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Molecular and Cellular Events in Early Thymocyte Development

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I. Introduction

It was recognized in the early 1980s that a minor population (~5% of the total number) of adult thymocytes, characterized by lack of expression of CD4 and CD8 [hence the designation "double-negative" (DN) thymocytes], contained progenitors for all thymocyte subsets. Upon intravenous or intrathymic transplantation of DN thymocytes into irradiated hosts, donor thymopoiesis was observed. However, in contrast to mice reconstituted with bone marrow-derived hematopoietic stem cells (HSC), thymus reconstitution from DN thymocyte progenitors was only transient, indicating that the thymus contains exhaustible rather than self-renewing "stem cells" (Fowlkes *et al.*, 1985). Well before then, it had been established that intrathymic progenitor cells are derived, like all other blood cells, from common HSC and that the thymus is dependent for as long as it performs its function on colonization from circulating ("blood-borne") progenitors (Moore and Owen, 1967a,b; Kadish and Bash, 1976; Le Douarin, 1984; Fowlkes *et al.*, 1985; Donskoy and Goldschneider, 1992). HSC populations, commonly characterized by multipotency, self-renewal capacity permitting long-term reconstitution following bone marrow engraftment and by their ability to protect lethally irradiated mice from bone marrow failure (for reviews and further references, see Uchida *et al.*, 1993; Zon, 1995; Morrison *et al.*, 1997), continuously give rise to lineage-restricted precursors. Lineage-restricted precursors refer to cell types that have lost the potential for some, but not for other, lineages. Their progeny is finally committed to a single lineage. In the case of thymocytes it has been a long-standing question whether the thymus is colonized by "true" HSC, by T-cell lineage-committed progenitors, or by both (reviewed by O'Neill, 1991; Ikuta *et al.*, 1992; Rodewald, 1995).

Technical advances such as multicolor flow cytometry, RNA and DNA analyses from minute numbers of, and even single cells by means of the polymerase chain reaction (PCR), and the availability of genetically modified mice expressing or lacking specific gene products made it possible to dissect the intrathymic pathways of differentiation in detail. Thus, the minor DN precursor population can now be resolved into multiple cellular subsets, each of which may be defined by cell surface

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phenotypes [based on expression of markers such as Thy-1, CD2, CD4, CD8, CD25 (also called Tac or IL-2 receptor (R) α chain), CD16/32 (FcγRIII/II), CD44, CD117 (c-kit), CD122 (IL-2 R β chain)], by precursor–product relationships (and the inclusion and exclusion of lineages among the progeny), by cell cycle status, and by rearrangements of their T-cell receptor (TCR) loci. In the future, analyses at the single cell level may provide information on the frequencies of cells within each subpopulation that bear a particular TCR rearrangement and an estimate how "synchronized" each cell within a given population is. Studies along this line have been reported from various stages of B-cell development (Ehlich *et al.*, 1994; ten Boekel *et al.*, 1995) but are not yet available for thymocytes.

This review focuses on early stages of thymocyte differentiation only. The stages covered here are defined as the events leading from HSC into lineage-restricted and finally T-cell lineage-committed precursors. Pro-T cells [TCR genes in germline or D(J)β rearranged] give rise to pre-T cells [TCR genes V(D)Jβ rearranged]. Subsequently, the TCR β chain forms, in association with the invariant pre-TCR α chain (pTα), the pre-TCR. Thymocytes are now ready to leave the DN stage and progress to the $CD4^+CD8^+$ [double-positive (DP)] stage, which is not discussed here. The field of thymopoiesis is addressed by a vast literature, thus, inevitably, the reference list cannot be complete. For additional reading, the reader may refer to reviews that have comprehensively covered overlapping topics (e.g., Kisielow and von Boehmer, 1995; Levelt and Eichmann, 1995; Anderson *et al.*, 1996; Malissen and Malissen, 1996; Shortman and Wu, 1996; Fehling and von Boehmer, 1997).

This review presents the following aspects: Available assays (Section II) to study early thymocyte development *in vivo* and *in vitro* are introduced. A brief survey of such assays, their strengths and weaknesses, may aid in the interpretation of data discussed later. Because pro-thymocytes originate from HSC, aspects of hematopoietic stem and extrathymic progenitor cell compartments (Section III) as they relate to pro-thymocyte development are presented. Precursor populations are described from the points of view of their phenotypes, developmental potential, and status of TCR gene rearrangements. The role of the pre-T-cell receptor complex in thymocyte development and allelic exclusion of functional TCR rearrangements is addressed in detail (Section IV). A discussion of the $\alpha\beta$ versus $\gamma\delta$ lineage commitment (isotypic exclusion) is beyond the scope of this review. New light has been shed onto essential functions of growth/differentiation factor receptors (Section V) in early thymocyte development. The implications of lack of growth factor receptors, particularly c-kit and cytokine receptors complexed

with the common cytokine receptor γ chain (γc), for thymocyte development and, in turn, for the fate of the thymic epithelium are discussed. Finally, early thymocyte development is considered in view of mutations in transcription factors (Section VI) (e.g., GATA-3, PU-1, Ikaros, TCF-1) and other molecules (Section VII) implicated in the process [e.g. adhesion molecules (α_4, or β_1 integrins, e-cadherin), CD44, CD81]. Although this review falls short of defining the molecular basis for T-cell commitment (for lack of data), it may outline critical molecular and cellular events accompanying the generation of early thymocytes.

II. Assays to Study Thymocyte Development *in Vivo* and *in Vitro*

A. *In Vitro* Systems

T-cell development is localized to and dependent on specialized environments provided primarily by the thymus. Here, progenitor T lymphocytes reside in close contact to the thymic "stroma." Thymic stromal cells are mostly of epithelial origin (for reviews, see van Ewijk, 1991; Boyd *et al.*, 1993; Anderson *et al.*, 1996), but hematopoietic elements (macrophages, dendritic cells) also contribute to the formation of a functional thymic environment. *In vivo,* thymocyte proliferation and differentiation are driven and controlled by precursor cell–stromal cell interactions (reviewed by Anderson *et al.*, 1996), and roles for soluble growth factors in thymocyte development have also been widely proposed.

Despite long-term efforts to study thymocyte development in suspension culture systems *in vitro,* a "structured" thymic stromal cell organization has proven indispensable in all systems supporting multiple stages of intrathymic T-cell development. Traditionally, fetal thymic lobes were depleted of endogenous thymocytes by treatment with 2′-deoxyguanosine to provide a functional thymic environment for HSC or T-cell-committed progenitors (reviewed by Jenkinson and Owen, 1990). However, such fetal thymic organ cultures (FTOC) are limited by the fact that the thymic stroma is not further accessible. Thus, insights into the requirements for each stromal cell compartment in various phases of thymocyte development cannot be obtained.

Precisely this can now be attempted with a more recently introduced modification of the FTOC method, the reaggregation fetal thymus organ culture (RFTOC) technique (Jenkinson *et al.*, 1992; Anderson *et al.*, 1993; Jenkinson and Anderson, 1994). This method involves the release of thymic stromal cell elements from freshly isolated or cultured fetal thymic lobes by means of enzymatic digestion of the lobes. Isolated thymic stromal cell populations, with or without addition of the appropriate progenitor cell populations, are centrifuged to form a densely

packed cellular pellet. This pellet, when placed as a standing drop onto a floating filter disk *in vitro*, can reaggregate to "reform" a functional thymic environment.

The RFTOC technique is now widely applied to examine interactions between stromal elements and developing thymocytes *in vivo*. For example, such experiments resulted in a report about stromal cell requirements for the emergence of T-cell receptor gene rearrangements in uncommitted progenitors (Oosterwegel *et al.*, 1997). Using RFTOC, an involvement of adhesion mediated by E-cadherin in thymus organogenesis (Müller *et al.*, 1997) was proposed. Finally, requirements guiding positive TCR repertoire selection were examined in RFTOC (reviewed by Fink and Bevan, 1995). It should be pointed out, however, that neither FTOC nor RFTOC methods allow analysis of T development from single progenitor cells. In the absence of a clonal assay, the frequency of T-cell progenitors among stem cell populations cannot be measured accurately.

A popular application in both FTOC and RFTOC systems is the usage of monoclonal antibodies (mAb) or antisense oligonucleotides to block stromal cell/thymocyte interactions. However, the number of "knock-out" mice lacking molecules on thymocytes or stromal cells is increasingly growing, and therefore many blocking studies can now be proven or disproven genetically. In this way, critical roles for expression of major histocompatibility complex (MHC) class I (Marusic-Galesic *et al.*, 1988), MHC class II (Mizuochi *et al.*, 1988), or the transcription factor GATA-3 (Hattori *et al.*, 1996a) were confirmed (Zijlstra *et al.*, 1990; Cosgrove *et al.*, 1991; Grusby *et al.*, 1991; Kontgen *et al.*, 1993; Ting *et al.*, 1996). In contrast, blocking studies suggesting roles for the IL-2 R α chain (Tentori *et al.*, 1988; Zuniga-Pflucker and Kruisbeek, 1990; Zuniga-Pflucker *et al.*, 1990) for CD81 (Boismenu *et al.*, 1996), for integrin $\alpha_6\beta_4$ (Imhof and Dunon, 1995; Ruiz *et al.*, 1995), for LFA-1 (Fine and Kruisbeek, 1991), or for CD44 (Wu *et al.*, 1993) are in discord with analyses of mutant mice [IL-2 R α (Willerford *et al.*, 1995), CD81 (Maecker and Levy, 1997; Miyazaki *et al.*, 1997), integrin α_6 (Georges-Labouesse *et al.*, 1996; D. Witherden and E. Georges-Labouesse, personal communication), integrin β_4 (van der Neut *et al.*, 1996), LFA-1 (Schmits *et al.*, 1996), and CD44 (Schmits *et al.*, 1997)]. Of course, some of these molecules may play redundant roles (Rajewsky, 1992) that may only be uncovered in mice lacking more than one gene product. Nevertheless, many mAb blocking studies suggested nonredundant roles that were not confirmed in the analyses of mutants. In conclusion, mAb-blocking experiments appear to be unreliable, and, where possible, mutants should be used to determine gene function even in thymus organ culture systems.

B. *In Vivo* Systems

1. Adoptive Transfers

Adoptive transfers of HSC or thymocyte subpopulations into recipient mice can recapitulate various aspects of thymocyte development *in vivo*. Following intravenous transfer, seeding of stem cell sites such as bone marrow by multipotent stem cells, or seeding ("homing") of the thymus by HSC or committed T-cell precursors, and the subsequent colonization of secondary lymphatic organs can be analyzed. In addition, direct intrathymic injection (Goldschneider *et al.*, 1986) of precursors is a very sensitive method to assess precursor–product relationships along the T-cell lineage. In both instances (intravenous and intrathymic transfers), host stem cells, committed progenitors, and mature lymphocytes are usually ablated using γ irradiation. It is a widely held view that hematopoietic stroma is radio resistant. However, a direct comparison of progenitor transfers into irradiated (600 rad) versus nonirradiated recipients revealed a substantial delay in developmental progression (~4 days) on intrathymic injection (Rodewald *et al.*, 1993). This delayed development in the irradiated thymic environment may be due to transient damage of thymic stromal cells or to the massive radiation-induced cell death causing depletion of endogenous thymocytes, which, in turn, disrupts the normal thymic architecture. Therefore, kinetic studies using intrathymic cell transfers into normal, nonirradiated recipients, albeit more laborious, are likely to reflect physiological development more closely (Petrie *et al.*, 1990; Rodewald *et al.*, 1993).

2. RAG-Deficient Blastocyst Complementation

Because many mutations are lethal at early stages of embryonic development, their potential effects on lymphocyte development and/or peripheral T- and B-cell functions cannot be studied directly. To overcome this problem, chimeric mice can be generated by injection of homozygous mutant embryonic stem cells (ES) into RAG-deficient blastocysts (reviewed by Chen, 1996). In RAG-deficient mice, lymphocyte development is arrested in early thymocyte and B-cell development (see later). Therefore, all progenitors and mature lymphocytes that develop beyond the "RAG block" are of ES cell (mutant) origin, which facilitates analyses of mutated genes selectively in lymphocytes.

3. Thymus Grafting

Transplantation of mutant fetal thymi into adult wild-type recipients can be used to assess the role of gene products produced within the thymic environment (e.g., stem cell factor; Asamoto and Mandel, 1981; Rodewald *et al.*, 1995). In this way, a gene defect can be restricted to a localized

environment and therefore separated from systemic effects caused by the mutation. However, this approach is only possible when homozygous mutant mice are viable until ~ day 15 of gestation.

III. Origin of Thymocytes: Hematopoietic Stem Cells and Early Thymocyte Progenitors

A. Thymus-Colonizing Progenitors: Committed or Not, a Long-Standing Question and No Answer

All blood cell lineages are ultimately derived from HSC. Depending on the life span of each lineage, HSC rapidly or slowly regenerate all lineages such that they are maintained at steady state throughout life. When artificially placed into the thymus, bone marrow HSC generate thymocytes. Notably, under these experimental conditions, HSC also generate other lineages, e.g., many donor-type granulocytes (Spangrude and Scollay, 1990), that are not present abundantly in the thymus under physiological conditions. The thymus contains spleen-colony-forming units (CFU-S) (myeloid–erythroid) progenitor activity at very low frequencies (~one CFU-S_{d12} per 2×10^5 $CD3^-CD4^-CD8^-$ thymocytes) (Papiernik *et al.*, 1988), whereas bone marrow HSC populations are highly enriched for CFU-S_{d12} activity (frequency estimates within the HSC compartment range from 1/10 (Spangrude and Weissman, 1988) to 1/60 (Morrison and Weissman, 1994). Does the thymus harbor any multipotent stem cells? If so, their self-renewal must be quite limited as thymus grafts discontinue to generate thymocytes of the "graft type" after ~4 weeks *in vivo* (Frey *et al.*, 1992); after this time, they produce host-type thymocytes exclusively. Bone marrow HSC and early thymic progenitors share some phenotypic markers (such as c-kit, CD44, Sca-1, the common cytokine receptor γ chain), whereas other markers (such as Sca-2 or the IL-7 R α chain) are expressed by early thymic progenitors only (see later). Thus, at least with regard to myeloid–erythroid potential, self-renewal, and phenotype, bone marrow HSC and intrathymic "stem cells" are clearly distinct. Are intrathymic "stem cells" T lineage committed? Not in all instances, as a potential to give rise, under appropriate conditions, to other cell types, notably natural killer (NK) cells, B cells, and some myeloid cells (dendritic cells), has been observed (see later). However, perhaps with the exception of the T/NK progenitor in the human thymus, clonality of the precursor–product relationship has not been demonstrated. Therefore, up to now, the possibility cannot be excluded that what are considered to be multi- or oligopotent progenitors in the thymus in fact represent mixtures of progenitors for each lineage. This caveat stated, the developmental potential of early intrathymic progenitor populations shall nevertheless be discussed (see later).

In the bone marrow, HSC undergoing lineage commitment into red blood cells, myeloid cells, platelets, or B lymphocytes presumably change their "lodge" from a "HSC stromal type" to a stromal cell type supporting their new lineage fate. In marked contrast, development of few lineages, notably T cells, mast cells, and dendritic cells is certainly not completed and may not even be initiated in the bone marrow. Instead, progenitors for these lineages have to migrate via the blood to peripheral mucosal and connective tissues (in the case of mast cells) or to the thymus (in the case of T cells) to continue their development. Thus, one should expect to find progenitors (which may or may not be committed) in the blood "en route" to their target tissues. Indeed, analysis of fetal blood (see later) led to the identification of committed progenitors for mast cells (Rodewald *et al.*, 1996), pro-thymocytes (Rodewald *et al.*, 1994), pro-B cells (Melchers and Abramczuk, 1980; Delassus and Cumano, 1996), and multipotent stem cells (Moore and Metcalf, 1970; Rodewald *et al.*, 1994, 1996; Delassus and Cumano, 1996; Marcos *et al.*, 1997). However, the finding of both committed and uncommitted progenitors in the fetal circulation does not resolve the question which cell types constitute the major pathways of thymus seeding.

Another level of complexity is introduced when data from analyzed mouse mutants are compiled. It is now apparent that differential molecular requirements exist for thymocyte development during fetal, early postnatal, adult, and late adult life (>6 month of age) (Fig. 1). For instance, mice lacking the C-terminal domain of the Ikaros gene were devoid of fetal, but not postnatal, thymocytes. In contrast, α_4 integrin$^{-/-}$ HSC generated normal numbers of thymocytes at fetal stages, but thymocyte numbers declined postnatally until they were undetectable by ~4 weeks of age. Likewise, in mice lacking TCF-1 it took ~6 month until thymopoiesis was blocked at the earliest stage. In contrast to these mutants revealing temporally modulated deficiencies, mice lacking the transcription factor GATA-3 or the growth factor receptors c-kit and the common cytokine receptor γ chain were completely devoid of thymocytes (all these mutants will be discussed in more detail later). The pattern shown in Fig. 1 is compatible with the idea that molecular requirements may differ over time. However, it is also conceivable that the thymus is seeded by different types of progenitors simultaneously or successively and that these cells may be committed or uncommitted at the time of thymus entry. A precedent for the ontogenetic regulation of the development of a particular lineage is Vγ3$^+$ dendritic epidermal T cells, which are only generated from a fetal precursor in a fetal thymus (for a review, see Ikuta *et al.*, 1992).[1] Section III,B summarizes available data on prethymic and intrathymic progenitor

[1] TCRδ nomenclature according to Garman *et al.*, 1986.

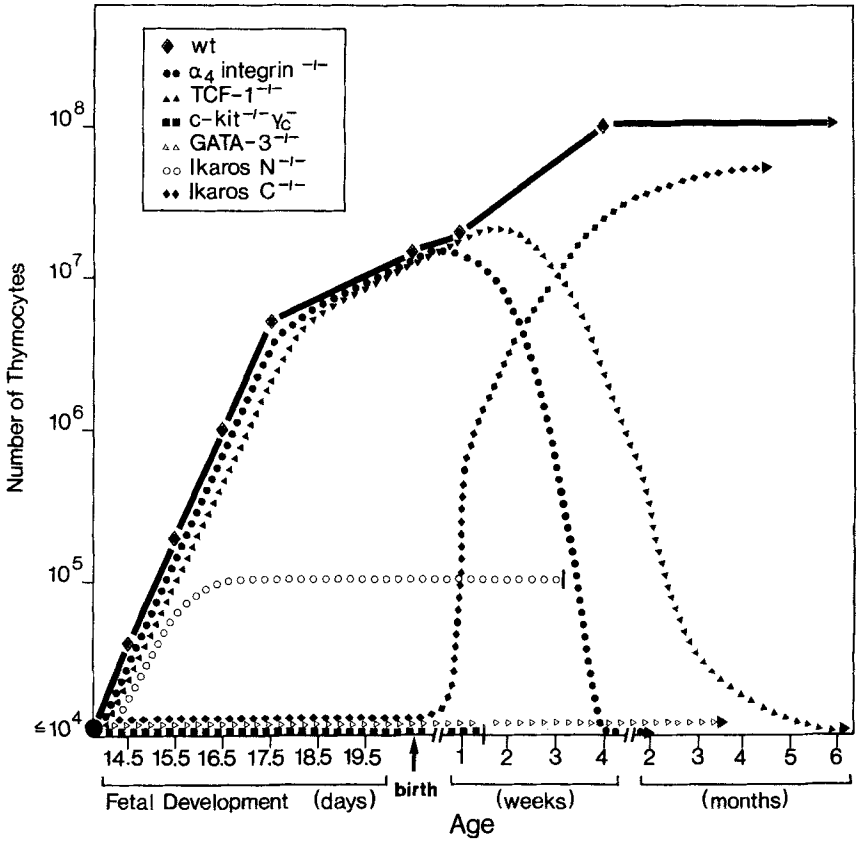


FIG. 1. Thymic cellularity as a function of age in wild-type and mutant mice. Total thymic cellularity is shown during both fetal and adult life. Cell numbers are only given as approximations based on data from the following mutant mice: Ikaros $N^{-/-}$ mice lack N-terminal sequences of Ikaros. This mutation appears to act in a domaint negative fashion (Georgopoulos *et al.*, 1994). Ikaros $C^{-/-}$ mice, considered as Ikaros null mutants, carry a deletion of the C terminus of Ikaros (J.-H. Wang *et al.*, 1996). For mutations with an early embryonic lethal phenotype, chimeric mice were generated by RAG complementation (see Section II). In such chimeric mice, the effects of lack of GATA-3 (Ting *et al.*, 1996) or α_4 integrin (Arroyo *et al.*, 1996) on T-cell development were determined. Mice lacking c-kit and γ_c^- (Rodewald *et al.*, 1997) live until ~10 days after birth, precluding analysis of adult thymocyte development. Disruption of TCF-1 in mice (Verbeek *et al.*, 1995) causes a block at the earliest stages in thymopoiesis in late adult, but not fetal or early postnatal life.

populations. The question of whether thymus-colonizing cells include T-cell lineage-committed progenitors remains controversial. In any case, bone marrow-derived HSC appear to be distinct from thymus-colonizing progenitors.

B. Thymocyte Progenitor Populations: Phenotypes, Developmental Potential, and Status of T-Cell Receptor Gene Rearrangements

1. Extrathymic Progenitor Populations

a. Fetal Liver and Bone Marrow. Fetal liver and bone marrow are the major sites of hematopoiesis during mid to late gestation and during postnatal life, respectively. Many laboratories have investigated thymic reconstitution kinetically and quantitatively from fetal liver or bone marrow cells following intravenous or intrathymic transfer into host mice or in FTOC *in vitro* (e.g., Kadish and Bash, 1976; Spangrude *et al.*, 1988); for further references, see, e.g., McKenna *et al.*, 1994).

Fetal liver cells generated thymocytes more rapidly compared to bone marrow cells (Kawamoto *et al.*, 1997), but this may reflect differences between HSC in fetal liver and bone marrow rather than provide evidence for the presence of pro-thymocytes in fetal liver. Based on fetal liver cell fractionation experiments, Sagara *et al.* (1997) proposed that T-cell progenitor activity was enriched in a population defined as Thy-1$^-$ c-kit$^+$ IL-7 R$^+$ CD3$^-$CD4$^{-/low}$ CD8$^-$ CD25$^-$ CD44$^+$ B220$^+$ FcγRII/III^{low}. These cells were potent progenitors, which gave rise to T or B lineage cells at frequencies of ~1/10 in the appropriate assays, but they also contained myeloid, particularly macrophage, potential (Sagara *et al.*, 1997). At first glance, expression of the B-cell marker B220 on progenitors that include pro-T potential may appear surprising, but, similarly, an early B220$^+$ bone marrow population that is included in "fraction A" according to Hardy *et al.* (1991) was shown to contain NK cell potential (Rolink *et al.*, 1996). B220$^+$ FcγRII/III^{low} cells from fetal liver contained pTα mRNA at low levels (Sagara *et al.*, 1997). pTα transcripts were undetectable in total liver between fetal days 11.5 and 14.5 (Bruno *et al.*, 1995). However, B220$^+$ FcγRII/III^{low} cells also expressed mRNA encoding Igα and VpreB (Sagara *et al.*, 1997), suggesting that lymphoid lineage-restricted transcripts are present at this stage, but may be derived from different progenitors in this population. In this and other reports (Rodewald, 1995), TCR β rearrangements [both DJ and V(D)J] were essentially undetectable in fetal liver and bone marrow (Soloff *et al.*, 1995), even by PCR analysis. Sterile transcripts derived from germline TCRβ and TCRγ loci were present in CD3-negative bone marrow cells (Soloff *et al.*, 1995; T. G. Wang *et al.*, 1996), but it was not analyzed whether sterile transcripts from the immunoglobulin heavy chain (IgH) loci were also present in the same samples. The significance of such germline TCR transcripts as an indication of lineage commitment is unknown.

In the bone marrow, Antica *et al.* (1994) searched for a progenitor population phenotypically reminiscent of the earliest intrathymic stem

cells in the adult mouse (see later). In contrast to "conventional" HSC, a population was found ("Sca-2$^+$ population") that shared phenotypic similarities with thymic "CD4^{low}" precursors (see later). Analysis of the developmental potential of bone marrow "Sca-2$^+$ cells" suggested that they represent an intermediate stage between Sca-2$^-$ HSC and immature thymocytes. Bone marrow "Sca-2$^+$ cells" differed, however, in their developmental potential from thymic "CD4^{low}", and it is not known whether the former represents the direct precursor of the latter (Antica *et al.*, 1994).

Fetal liver (McKenna *et al.*, 1994) or bone marrow (Chervenak *et al.*, 1992) cells could be propagated *in vitro* for ~7 to 10 days, while maintaining hematopoietic potency including pro-thymocyte activity. Suitable growth factors were IL-3 and SCF, but because these factors also act on HSC, it is possible that multipotent progenitors rather than pro-thymocytes were enduring the culture period.

Collectively, despite a long list of reports providing circumstantial evidence, neither pro-T cells nor the enigmatic "lymphoid stem cell" has been identified in fetal liver or bone marrow. (*Note:* By the time this review went to press, a candidate common lymphoid progenitor was reported in the bone marrow (Kondo *et al.*, 1997).) In fact, it is not even known whether T-cell commitment takes place in these sites.

b. Fetal Blood. Both T lineage-committed (Thy-1$^+$c-kit^{low}) (Rodewald *et al.*, 1994) and multipotent (Thy-1$^-$c-kit$^+$) (Rodewald *et al.*, 1994, 1996; Delassus and Cumano, 1996; Marcos *et al.*, 1997) progenitors have been identified in murine fetal blood. A fraction (~5%) of the fetal (day 15.5) blood-derived pro-thymocyte population carried TCR (D)J$_\beta$ rearrangements (Rodewald, 1995). Moreover, abundant levels of pTα mRNA were detected in Thy-1$^+$c-kit^{low}, whereas pTα mRNA was undetectable in multipotent Thy-1$^-$c-kit$^+$ progenitors in fetal blood (Bruno *et al.*, 1995). Upon transfer into the thymus, fetal blood pro-thymocytes generated a single wave of CD4$^+$CD8$^+$ thymocytes and subsequently mature TCR$\alpha\beta^+$ peripheral T cells. Whether these progenitors also have the potential to generate TCR$\gamma\delta^+$ T cells is not known. However, in the presence of growth factors (IL-2, IL-7, and SCF), both Thy-1$^+$c-kit^{low} and Thy-1$^-$c-kit$^+$ progenitors from fetal blood were capable of generating NK cells *in vitro* (see later). Nevertheless, fetal blood pro-thymocytes were clearly distinct from multipotent progenitors because they failed to reconstitute B lymphoid, myeloid, and erythroid lineages. The identification of pro-thymocytes in fetal blood led to the suggestion that T lineage commitment can precede thymus colonization (reviewed in Rodewald, 1995).

c. Intestine. In mice, a population with close phenotypic similarity to fetal blood-derived T-cell progenitors has been identified in the mucosal

layer in small and large intestine (Kanamori *et al.*, 1996). Tiny clusters consisting of ~1000 lymphoid cells each were identified microscopically in the cryptae of the lamina propria [termed "cryptopatches" (CP)]. Based on the estimation that the small intestine harbors ~1500 CP (the large intestine about 10-fold less CP), the total number of CP-associated cells was predicted to be in the order of 1.5×10^6 per mouse. Lymphoid cells located in CP were phenotypically heterogeneous, but most cells were Thy-1$^+$c-kit$^+$IL-7 R$^+$ and lacked mature T-cell (TCR$\alpha\beta$, TCR$\gamma\delta$, CD3) and B-cell (B220, Ig) markers. CP first appeared 2 weeks after birth; however, their phenotypic makeup raises the possibility that they are derived from one of the progenitors previously identified in fetal blood.

A multitude of lymphocytes lodging in the intestine are collectively referred to as intraepithelial lymphocytes (IEL) (for a review, see Klein, 1996). It is quite possible that IEL develop locally in the gut rather than descend from a mature, circulating T cell. CP-associated cells may represent such "local progenitor cells." The progenitor potential of CP-associated cells has not yet been determined. Given that CP-associated lymphocytes are rare and distributed throughout the gut, isolation of this population may be difficult.

2. *Intrathymic Progenitors at the Time of First Thymus Seeding*

Seeding of the thymus anlage begins around day 11 of fetal development (Auerbach, 1961; Owen and Ritter, 1969). Hattori *et al.* (1996b) determined the phenotypic heterogeneity and the developmental potential of cells retrieved from day 12 fetal thymus (FT). These ontogenetically earliest thymocytes are c-kit$^+$ CD3$^-$ CD4$^-$ CD8$^-$ CD25$^-$ CD44$^+$ and, in contrast to thymocytes on day 14 (and later), lack Thy-1 expression (Peault *et al.*, 1994; Hattori *et al.*, 1996b). This c-kit$^+$ population could be dissected further by expression of FcγRII/III (CD16/CD32) into FcγRII/III$^+$ (~30%) and FcγRII/III$^-$ (~70%) subsets. FcγRII/III receptors were shown previously to be expressed on the most immature thymocytes on fetal days 14–16 (Rodewald *et al.*, 1992, 1993; Falk *et al.*, 1993; reviewed by Leclercq and Plum, 1995; Sandor *et al.*, 1996; see later). Interestingly, on fetal day 12, c-kit$^+$ FcγRII/III$^+$, but not c-kit$^+$ FcγRII/III$^-$, thymocytes expressed two transcription factors crucially involved in thymocyte development: TCF-1 and GATA-3 (see later). In line with these T-cell lineage hallmarks, c-kit$^+$ FcγRII/III$^+$, but not c-kit$^+$ FcγRII/III$^-$, thymocytes also expressed mRNA for the T lineage-specific tyrosine kinase lck and for pTα. The latter finding is at variance with an earlier study in which pTα mRNA was not detected until FT 14.5 (Bruno *et al.*, 1995). C-kit$^+$ FcγRII/III$^+$ cells also expressed cytoplasmic CD3ε. However, cytoplasmic expression of CD3ε may not be T lineage specific, as, at least in the human,

fetal liver-derived NK cells also contained CD3ε (Phillips *et al.*, 1992). It is not known whether fetal day 12 c-kit$^+$ FcγRII/III$^+$ thymocytes carry any TCR rearrangements or express the RAG genes. On day 13, most fetal thymocytes were c-kit$^+$ FcγRII/III$^+$ and, subsequently, c-kit appeared to be downregulated before FcγRII/III (Hattori *et al.*, 1996b).

On fetal day 12, total thymocytes contained developmental potential for T- and non-T-cell (B cells, myeloid cells) lineages when analyzed in colony assays *in vitro* (Peault *et al.*, 1994; Hattori *et al.*, 1996b). This was taken as an indication, but was not proven, for the presence of multipotent progenitors in the thymus at this stage (Peault *et al.*, 1994). However, separation into c-kit$^+$ FcγRII/III$^+$ and c-kit$^+$ FcγRII/III$^-$ thymocytes revealed that the former but not the latter population was devoid of B-cell progenitor potential (and was very low in myeloid potential), suggesting that T-cell commitment can precede the onset of CD25 expression. C-kit$^+$ FcγRII/III$^-$ thymocytes may be multipotent cells or consist of mixtures of lineage-committed cell types.

Very immature thymocytes on FT day 14 were also found to express CD122 (IL-2 R β chain) together with FcγRII/III and CD44. A fraction of these cells expressed cytoplasmic CD3ε, but neither TCRβ nor CD25 (Falk *et al.*, 1993). Transfer experiments into FTOC revealed that CD122$^+$ thymocytes could generate both αβ and γδ progeny. CD122 expression in early thymocytes is most likely not functionally important, as shown in CD122-deficient mice (Suzuki *et al.*, 1997).

3. *Intrathymic Progenitors after First Thymus Seeding*

The available literature on the onset of TCR $D_\beta \rightarrow J_\beta$ and $V_\beta \rightarrow (D)J_\beta$ rearrangements, as well as the phenotypic markers that accompany rearrangements during development, is somewhat inconsistent. The available information is summarized for both fetal and adult thymocytes.

a. Fetal Development. Evidence for the earliest TCR_β rearrangements in ontogeny comes from the analysis of thymocytes on fetal day 13.5: Expression of intracellular TCR_β chains was detectable in few thymocytes (~100 per lobe) by confocal laser scanning microscopy (Falk *et al.*, 1996). On fetal days 14.5 and 15.5, both TCR DJ_β (Rodewald *et al.*, 1994; Falk *et al.*, 1996) and TCR $V(D)J_\beta$ (Rodewald *et al.*, 1993; Falk *et al.*, 1996) rearrangements were identified clearly by PCR-based analysis (van Meerwijk *et al.*, 1990; Anderson *et al.*, 1992; D'Adamio *et al.*, 1992). Another study found only TCR DJ_β but not TCR $V(D)J_\beta$ rearrangements in fetal day 15 thymus (Hozumi *et al.*, 1994a).

Combining the markers CD2 and FcγRII/III allowed further dissection of distinct developmental stages correlating with TCR_β rearrangements:

~50% of TCR_β loci from the earliest cells on day 15.5 ($CD2^-Fc\gamma RII/III^+$) were (on a population level and not estimated at the level of single cells) in germline and ~50% were DJ_β, but not $V(D)J_\beta$ rearranged. In a downstream population ($CD2^+Fc\gamma RII/III^-$), ~48 hr more advanced in development than $CD2^-Fc\gamma RII/III^+$ cells, TCR_β loci were entirely DJ_β, rearranged, and had abundant $V(D)J_\beta$ rearrangements (Rodewald *et al.*, 1993; Rodewald, 1995). Thus, as would be expected if DJ_β rearrangements precede $V(D)J_\beta$ rearrangements (Kronenberg *et al.*, 1986), fetal thymocytes can be separated by phenotype into a germline/DJ_β rearrangement stage and a $V(D)J_\beta$ rearrangement stage in fetal development. The lag time between both stages is ~2 days.

b. Adult Development. The adult thymus contains ~2% of thymocytes lacking TCR/CD3, CD4, and CD8 [triple negative (TN) thymocytes]. Based on expression of several cell surface markers, thymocytes at the TN stage can be dissected into subpopulations. The markers most commonly used are CD25 (IL-2 receptor α chain) (Ceredig *et al.*, 1985; Raulet, 1985; Shimonkevitz *et al.*, 1987; Pearse *et al.*, 1989), CD44 (Pgp-1) (Trowbridge *et al.*, 1985), and c-kit (Godfrey *et al.*, 1992; Matsuzaki *et al.*, 1993; Hozumi *et al.*, 1994a). In addition, Thy-1, CD2, HSA, and Sca-2 are useful markers at these stages. Based on the expression of CD25, CD44, and c-kit, thymocyte development follows the sequence $c\text{-}kit^+CD25^-CD44^+ \rightarrow c\text{-}kit^+CD25^+CD44^+ \rightarrow c\text{-}kit^{-/low}CD25^+ CD44^{-/low} \rightarrow c\text{-}kit^-CD25^-CD44^{-/low}$ (Pearse *et al.*, 1989; Godfrey *et al.*, 1993; for reviews, see, e.g., Levelt and Eichmann, 1995; Malissen and Malissen, 1996; Shortman and Wu, 1996; Fehling and von Boehmer, 1997).

The earliest thymocytes within this sequence are $c\text{-}kit^+CD44^+CD25^-$. In the adult thymus, these cells are $Thy\text{-}1^{low}$ and $CD4^{low}$ (Wu *et al.*, 1991b) (hence the designation "$CD4^{low}$" thymocytes) and express HSA and Sca-2 (Wu *et al.*, 1991a; for a review, see Shortman and Wu, 1996). Neither $(D)J_\beta$ nor $V(D)J_\beta$ rearrangements are detectable in $c\text{-}kit^+CD44^+CD25^-$ cells (Wu *et al.*, 1991b; Godfrey *et al.*, 1994; Petrie *et al.*, 1995; Ismaili *et al.*, 1996; Tourigny *et al.*, 1997), but rearrangement-associated enzymes (RAG, TdT) appear to be transcribed already at this stage (Ismaili *et al.*, 1996).

Upon downregulation of CD4 and upregulation of CD25, $c\text{-}kit^+CD25^-CD44^+$ cells give rise to $c\text{-}kit^+CD25^+CD44^+$ thymocytes. These cells still lack DJ_β or $V(D)J_\beta$ rearrangements (Godfrey *et al.*, 1994; Petrie *et al.*, 1995; Ismaili *et al.*, 1996; Tourigny *et al.*, 1997). Loss of CD44 and c-kit expression characterizes the next stage defined as $c\text{-}kit^{-/low}$ $CD25^+CD44^{-/low}$ (briefly: $CD25^+CD44^-$) thymocytes. Estimation by Southern blotting revealed that 70–90% of TCR_β loci are DJ_β rearranged in $CD25^+CD44^-$ thymocytes (Pearse *et al.*, 1989; Godfrey *et al.*, 1994) whereas $V(D)J_\beta$

rearrangements are still infrequent (<20%) (Tourigny *et al.*, 1997). The pre-TCR-mediated selection of thymocytes expressing functional TCR_β chains (β selection) occurs at the $CD25^+CD44^-$ stage (Fig. 2, see later).

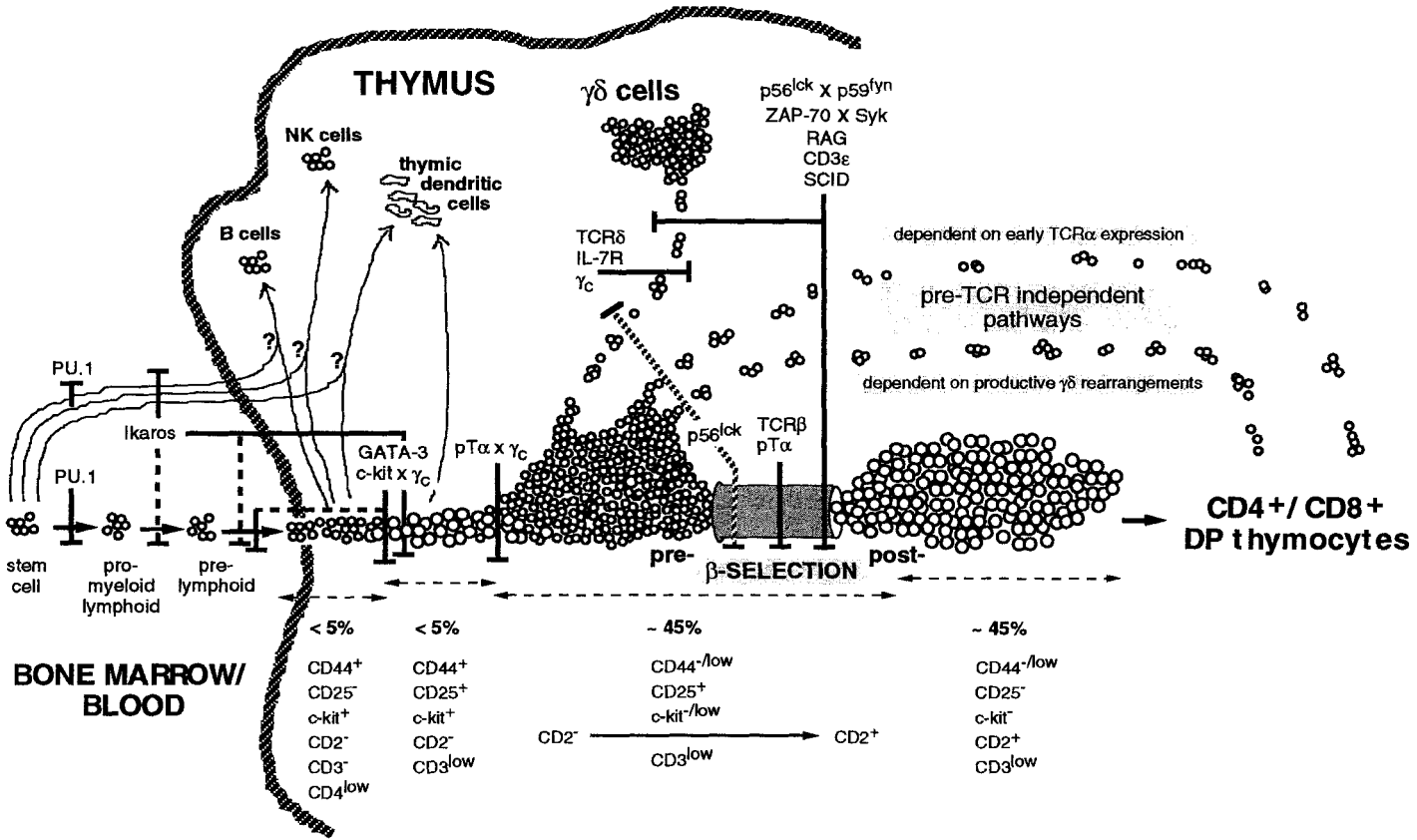
The final TN stage, according to these markers, is represented by a $CD44^{-/low}25^-$ phenotype (Pearse *et al.*, 1989). The vast majority of cells at this stage carry a single $V(D)J_\beta$ rearrangement (Pearse *et al.*, 1989; Mallick *et al.*, 1993; Dudley *et al.*, 1994), express a productive pre-TCR (Groettrup and von Boehmer, 1993b), and progress to the DP thymocyte compartment. This compartment is lacking in SCID (Bosma *et al.*, 1983), $RAG^{-/-}$ (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992), or $CD3\varepsilon^{-/-}$ (Malissen and Malissen, 1996) mice, or is strongly reduced in TCR $\beta^{-/-}$ (Mombaerts *et al.*, 1992b), $pT\alpha^{-/-}$ (Fehling *et al.*, 1995a) or $CD3\gamma^{-/-}$ (M. Haks, P. Krimpenfort, and A. Kruisbeek, personal communication) mice (see later).

C. Lineage Relationship of Thymocytes and Non-T-Cell Lineages

1. Thymocytes and Natural Killer Cells

T cells and natural killer cells share some phenotypic and functional properties, but only the former cell type uses genetic recombination to express a clonotypic antigen receptor. One effector function of NK cells, i.e., antibody-dependent cellular cytotoxicity (ADCC), is mediated by the low-affinity Fc receptor for IgG [CD16 (FcγRIII)]. CD16 forms a molecular complex with the common Fc receptor γ subunit (FcεRIγ) or CD3ζ

FIG. 2. Schematic illustration of early T-cell development. Dashed lines with arrows at both ends indicate the four, developmentally successive TN subsets defined by the differential expression of CD25, CD44, and c-kit surface markers. Percentages give the approximate proportion of each respective subset within the TN population. The three prethymic stages of T-cell development are defined only functionally, i.e., by their developmental potential. Thin arrows indicate minor pathways within the thymus that give rise to non-T lineage cells. It is not yet clear whether B, NK, and thymic dendritic cells are generated from extra- and/or intrathymic pathways. The dark bars symbolize points of complete developmental arrest due to null mutations in specific genes mentioned on top of the respective bar. In GATA-3-deficient and c-kit × γ_c double-deficient mice, T-cell development may actually be arrested before precursor entry into the thymus. This may also be true for some Ikaros-deficient mice as indicated by dashed bars (see text for details). The developmental block in $p56^{lck}$-deficient mice is partial (stippled bar). It has not been investigated to what relative extent the pre-TCR-dependent and the two minor pathways are inhibited by the absence of $p56^{lck}$. Other mutations that result in a partial block of thymopoiesis are not included in the scheme nor are mutations that cause a complete block only during fetal, but not adult thymopoiesis (see also Fig. 1). γ_c denotes the common cytokine receptor γ chain. The indicated block in thymopoiesis in $pT\alpha \times \gamma_c$ double-deficient mice has not yet been published (J. DiSanto, personal communication).


THYMUS
γδ cells
p56lck x p59fyn
ZAP-70 X Syk
RAG
CD3ε
SCID
NK cells
thymic dendritic cells
B cells
TCRδ
IL-7R
γc
dependent on early TCRα expression
pre-TCR independent pathways
dependent on productive γδ rearrangements
PU.1
Ikaros
PU.1
GATA-3
c-kit x γc
pTα x γc
p56lck
TCRβ
pTα
CD4+/ CD8+
DP thymocytes
stem cell
pro-myeloid lymphoid
pre-lymphoid
pre-
β-SELECTION
post-
BONE MARROW/ BLOOD
< 5%
CD44+
CD25-
c-kit+
CD2-
CD3-
CD4low
< 5%
CD44+
CD25+
c-kit+
CD2-
CD3low
~ 45%
CD44-/low
CD25+
c-kit-/low
CD2-
CD3low
CD2+
~ 45%
CD44-/low
CD25-
c-kit-
CD2+
CD3low

in which FcεRIγ or CD3ζ exists as either γ–γ or ζ–ζ homodimers or γ–ζ heterodimers (for further references, see Ravetch and Kinet, 1991; Flamand *et al.*, 1996).

Early in thymocyte ontogeny (days 12.5–16.5) most fetal thymocytes express FcγRII/III in association with FcεRIγ (Rodewald *et al.*, 1992, 1993; Flamand *et al.*, 1996; Hattori *et al.*, 1996b; Heiken *et al.*, 1996; reviewed by Leclercq and Plum, 1995; Sandor *et al.*, 1996). In thymocytes destined to become αβ T cells, FcγRII/III expression is shut down when TCR V(D)J_β chain rearrangements are completed (Rodewald *et al.*, 1993). In contrast, FcγRII/III expression is upregulated during maturation and activation of Vγ3 lineage cells in the thymus (Leclercq and Plum, 1994) and is maintained from immature to mature stages of B-cell development (Foy *et al.*, 1992).

Is there a role for the transient expression of FcγRII/III and FcεRIγ in early thymocyte development? It is evident from mutant mice that FcγRIII (CD16) (Hazenbos *et al.*, 1996), FcγRII (CD32) (Takai *et al.*, 1996), or the associated common Fc receptor γ subunit (FcεRIγ) (Takai *et al.*, 1994; Heiken *et al.*, 1996) is not essential for thymocyte or NK cell development. In contrast to mice lacking Fc receptor subunits, overexpression of FcεRIγ transgenes in the T-cell lineage markedly inhibited both thymocyte and NK cell development (Flamand *et al.*, 1996). In mice expressing the transgenic FcεRIγ chain at high levels, total thymic cellularity was reduced ~10 fold compared to wild-type mice, and proportions of thymic subsets were markedly distorted (DNs increased from 2 to 40%; DPs decreased from 80 to 40%). In addition, expression of FcγRII/III was maintained in adult DN thymocytes. Interestingly, both T and NK cell development were inhibited as shown by vastly reduced numbers of $NK1.1^+$ NK cells and lack of cytotoxic NK activity. Similarly, a block in both T and NK cell development was reported from mice expressing a human CD3ε transgene (Wang *et al.*, 1994). Because NK cell development is not thymus dependent, the inhibition of NK cell development may occur extrathymically and independently from the alteration in thymocyte development in these mice. Alternatively, a common T/NK precursor may be affected by expression of these transgenes.

The observed thymic phenotype was most likely not due to competition of FcεRIγ with CD3ζ or CD3η for assembly into the TCR/CD3 complex (Orloff *et al.*, 1990; Rodewald *et al.*, 1991; Ohno *et al.*, 1993) as Flamand *et al.* (1996) observed a similar phenotype in CD3ζ-deficient mice expressing transgenic FcεRIγ. However, another CD3ζ-deficient, FcεRIγ transgenic mouse showed normal numbers and proportions of DP and SP thymocytes and only moderately increased DN numbers (Liu *et al.*, 1997). Given the fact that FcεRIγ and FcγRII/III are dispensable for thymocyte develop-

ment (see earlier discussion), the findings in mice overexpressing FcεRIγ raise the possibility that FcγR/FcεRIγ complexes can provide an inhibitory signal in early thymocytes similar to FcγRII-mediated "negative" signaling in B cells and NK cells (for a review, see, e.g., Unkeless and Jin, 1997). The significance of this idea for normal thymocyte development remains to be determined.

The fact that FcεRIγ is expressed before CD3ζ or CD3η early in T-cell ontogeny is also relevant for the assembly of either FcεRIγ or CD3ζ into the TCR/CD3 complex. T cells that develop extrathymically, such as αβ and γδ IEL (Liu *et al.*, 1993; Guy-Grand *et al.*, 1994; Ohno *et al.*, 1994), or that develop from DN $CD3^+NK1.1^+Fc\gamma RIII^+$ thymocytes (Koyasu, 1994) express TCR isoforms containing the FcεRIγ subunit in homodimeric form or heterodimerized with CD3ζ. In contrast, CD3ζ, but not FcεRIγ, is expressed predominantly in thymus-dependent $CD4^+$ or $CD8^+$ αβ T cells. Thus, incorporation of CD3ζ and FcεRIγ appears to be diagnostic for thymic vs extrathymic origins of T cells, respectively.

Intrathymic expression of FcγRII/III marks cells that include the earliest fetal T lineage cells (Rodewald *et al.*, 1992; Falk *et al.*, 1993; Hattori *et al.*, 1996b). Transient expression of FcγRIII (CD16) on T-cell progenitors suggested that this population contained T and NK cell potential, either of which may be exercised dependent on the environment. Intrathymically, the developmental order is DN $CD3^-Fc\gamma RII/III^+ \rightarrow$ DP $CD3^{-/low}$ FcγRII/$III^- \rightarrow$ SP $CD3^+Fc\gamma RII/III^-$ thymocytes, whereas extrathymically, DN $CD3^-Fc\gamma RII/III^+$ give rise to DN $CD3^-Fc\gamma RII/III^+$ NK cells (Lanier *et al.*, 1992; Rodewald *et al.*, 1992). The finding that early thymic progenitors can give rise to NK cells *in vitro* and *in vivo* has been confirmed in several reports (Brooks *et al.*, 1993; Matsuzaki *et al.*, 1993; Moore and Zlotnik, 1995; Zuniga-Pflucker *et al.*, 1995). Although FcγRII/III expression is dispensable at this stage, it may reflect the relatedness of NK and T cells: NK cells may be an ancestral "T cell type" and developing thymocytes may reveal a NK-like phenotype before they, intrathymically, continue their development along the T-cell lineage.

In the presence of growth factors (IL-2, IL-7, and SCF), both multipotent ($Thy\text{-}1^-c\text{-}kit^+$) and pro-thymocyte ($Thy\text{-}1^+c\text{-}kit^{low}$) populations from fetal blood are capable of generating NK cells *in vitro* at frequencies of ~1/7 and 1/30, respectively. NK cell development *in vitro* is accompanied by the emergence of perforin mRNA expression (undetectable before culture) and acquisition of cytolytic activity toward NK-sensitive targets (H. R. Rodewald, unpublished). It would be interesting to study the expression of the recently identified killer inhibitory receptors (KIR) (Colonna, 1996; Lanier, 1997; Long and Wagtmann, 1997) during NK cell ontogeny.

In the mouse, evidence for a common progenitor for T cells and NK cells is based on population, but not single cell analysis. From human thymus, a clonogenic common T/NK precursor cell has been reported. A clonal assay for NK cell development was combined with FTOC reconstitution from pools of short-term cultured $CD34^{bright}$ precursors (Sánchez *et al.*, 1994). Because the potential of $CD34^{bright}$ intrathymic precursors for myeloid lineages was low or undetectable (Schmitt *et al.*, 1993; Sánchez *et al.*, 1994), these cells appeared to represent a common thymic T/NK precursor downstream from the myeloid branch.

2. *Thymocytes and B Lymphocytes*

Several laboratories reported that progenitors isolated from thymus have the potential to generate B lineage cells *in vitro* (on stromal cells and in the presence of IL-7) or *in vivo* on transfer into recipient mice. In the fetus, B-cell progenitor activity was noted in FT day 12 (Peault *et al.*, 1994; Hattori *et al.*, 1996b) and among $c\text{-}kit^+CD25^-$, but not $c\text{-}kit^+CD25^+$ progenitors (Zuniga-Pflucker *et al.*, 1995). However, in the latter study, lineage assignment was based solely on B220 expression, which is not restricted to B cells (Rolink *et al.*, 1996). In the adult thymus, $c\text{-}kit^+$ (Matsuzaki *et al.*, 1993) or $c\text{-}kit^+CD4^{low}CD25^-CD44^+$ cells (L. Wu *et al.*, 1991a, 1996; Moore and Zlotnik, 1995) contained B-cell progenitor potential following iv transfer. This potential was lost when a downstream population (defined as $CD25^+CD44^+$) was analyzed (Moore and Zlotnik, 1995; Zuniga-Pflucker *et al.*, 1995; L. Wu *et al.*, 1996).

These results, together with data showing the potential of thymic progenitors for NK cells (see earlier) and myeloid cells (see later), suggest that intrathymic progenitors are not committed to the T-cell lineage until the onset of CD25 expression. Alternatively, T- and B-cell progenitor activity may stem from distinct clonal progenitors that could not be separated on the basis of the cell surface markers used in these experiments.

3. *Thymocytes and Myeloid Lineages*

a. CFU-S, Macrophages, and Granulocytes. Fetal day 11/12 thymic rudiments contained developmental potential for myeloid cells, including macrophages and granulocytes (Peault *et al.*, 1994; Hattori *et al.*, 1996b). Frequencies of clonable macrophage precursors declined over time and were undetectable by day 14 of gestation (Peault *et al.*, 1994). In the adult thymus, as stated earlier, myeloid–erythroid progenitor activity occurred at very low frequencies (~one $CFU\text{-}S_{d12}$ per 2×10^5 $CD3^-CD4^-CD8^-$ thymocytes) (Papiernik *et al.*, 1988), a feature clearly distinct from bone marrow HSC populations. In accord with this early work, $c\text{-}kit^+CD4^{low}CD44^+CD25^-$ were essentially free of $CFU\text{-}S_{d12}$ activity

and lacked myeloid progeny (Mac-1 or Gr-1 positive cells) following iv transfer (L. Wu *et al.*, 1991a, 1996; Moore and Zlotnik, 1995). However, using *in vitro* colony assays, two studies showed that the "$CD4^{low}$" population contained colony forming cells (CFC) at frequencies ranging from ~1/200 (mostly macrophages) (Wu *et al.*, 1991a) to ~1/2500 (Matsuzaki *et al.*, 1993). Thus, although myeloid progenitor potential, including CFU-S activity, is very low, the thymus is not completely devoid of such hematopoietic potential. This notion would be compatible with the presence of a steady, but very low number of multipotent or even "true" HSC in the thymus, and it cannot be excluded that such rare cells are responsible for the generation of non-T lineages in cell transfer experiments from thymus-derived progenitor populations.

b. Thymic Dendritic Cells. Another cell type traditionally assigned to the myeloid lineage, bone marrow-derived dendritic cells (DC), has been linked to thymopoiesis. This "linkage" is based on two findings: (1) intrathymic and intravenous transfer of "$CD4^{low}$" cells resulted in the appearance of donor-type thymic DC and (2) most thymic and a subset of splenic DC expressed CD8α (Vremec *et al.*, 1992). These data led to the proposal that thymocytes and thymic DC share a common intrathymic progenitor (Ardavin *et al.*, 1993). Even a later stage progenitor population ($CD25^+CD44^+$) was shown to express potential for thymic and splenic DC (L. Wu *et al.*, 1996). At the $CD25^+CD44^-$ stage, DC potential was essentially lost. Because the "$CD4^{low}$" but not the $CD44^+CD25^+$ population also harbors B-cell progenitor potential (see earlier), this was taken as evidence that thymocytes and DC share a "late" common progenitor downstream from the putative T/B branch in development (L. Wu *et al.*, 1996).

Transfer studies showed that numbers of T cell vs dendritic cell progeny differed by a factor of ~2000 (i.e., intrathymic transfer of 10^4 "$CD4^{low}$" cells yielded 5×10^6 thymocytes, but only 4×10^3 dendritic cells of donor type). Thus, more than two "$CD4^{low}$" precursors were required to generate one thymic DC. These numbers do not rule out the possibility that the DC progeny observed in adoptive transfers was derived from a contaminating multipotent or DC-restricted progenitor. However, the finding that all thymus-derived DC progeny (both thymic and splenic) expressed CD8α, whereas bone marrow-derived DC generated both $CD8\alpha^+$ and $CD8\alpha^-$ subsets, supports the idea of separate DC lineages (L. Wu *et al.*, 1996).

In this context, the authors would like to point out that development of thymic DC is apparently not compromised in mice lacking pro-thymocytes due to mutations in both c-kit and γ_c (H. R. Rodewald, unpublished). Although pro-thymocytes may be able to serve as progenitors for thymic DC on adoptive transfer, the presence of thymic DC in the absence of

pro-thymocytes suggests that pro-thymocytes may not be the obligatory precursors of thymic DC (see Section V,C,4,c).

IV. Role of the Pre-T-Cell Receptor Complex in Thymocyte Development and Allelic Exclusion of the TCR β Chain

A. Identification of the Pre-TCR

The severe-combined immunodeficient (SCID) mouse (Bosma *et al.*, 1983) cannot efficiently rearrange its TCR and immunoglobulin loci due to a naturally occurring mutation in a gene encoding a DNA-dependent protein kinase that participates in some unknown way in receptor gene recombination (Blunt *et al.*, 1995; Kirchgessner *et al.*, 1995). Fluorocytometric (FACS) analysis of SCID thymocytes has revealed a severe block in early thymopoiesis that affects the transition of $CD25^+CD44^{-/low}$ TN thymocytes to the $CD25^-CD44^{-/low}$ TN stage, resulting in a virtually complete absence of $CD4^+8^+$ thymocytes and subsequent maturational stages (Habu *et al.*, 1987; Shores *et al.*, 1990). The fact that this developmental block can be overcome by breeding functionally rearranged TCR$\alpha\beta$ transgenes onto the SCID background has demonstrated that productive TCR rearrangements can promote the TN → DP transition of developing thymocytes (Scott *et al.*, 1989) and hence may be required at a much earlier developmental stage than previously thought. Interestingly, introduction of a transgenic TCRβ-chain alone also resulted in the generation of DP thymocytes in SCID mice, despite the absence of TCRα chains, clearly indicating a specific role of TCRβ in early thymopoiesis independent of its function in the mature TCR (von Boehmer, 1990; Kishi *et al.*, 1991). However, the absolute number of DP thymocytes in TCRβ transgenic SCID mice did not exceed 10% of the number found in nontransgenic wild-type mice, which left the possibility that TCRβ alone might not be sufficient to mediate the same degree of expansion normally associated with the TN → DP transition. Subsequent analysis of several different gene-targeted and TCR transgenic mouse strains demonstrated that this was not the case and that the failure of the transgenic TCRβ chain to fully reconstitute the DP compartment was a SCID-specific defect, most likely due to the inability of SCID thymocytes to properly process DNA double strand breaks accompanying the normal rearrangement process. For instance, introduction of a transgenic TCRβ chain alone into $RAG^{-/-}$ mice, which contain exclusively TN thymocytes due to a complete arrest of thymopoiesis at the same developmental stage as in SCID mice (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992), could again overcome this early block in thymopoiesis, this time, however, giving rise to normal numbers of DP thymocytes (Shinkai *et al.*, 1993). In contrast, functionally rearranged

TCRα transgenes were completely unable to promote T-cell development in RAG$^{-/-}$ mice (Shinkai *et al.*, 1993). These findings were substantiated in TCRα and TCRβ knockout mice, which were found to exhibit developmental defects at very different stages of T-cell development. TCRα-deficient mice showed no significant reduction in total thymocyte number, as T-cell development could proceed normally up to the DP stage (Philpott *et al.*, 1992; Mombaerts *et al.*, 1992b). Only the transition from the DP to the SP stage was blocked, reflecting the requirement for a functional TCRα chain in the process of positive selection, which is obligatory for the generation of mature $\alpha\beta$ thymocytes. T-cell development in TCRβ-deficient mice, however, was severely impaired much earlier, at exactly the same developmental stage where the SCID and RAG mutations revealed their effects (Mombaerts *et al.*, 1992b; Godfrey *et al.*, 1994). The analysis of these mouse mutants led inevitably to the conclusion that the presence of a TCRβ, but not of a TCRα chain, was important for the progression of thymic development beyond the CD25^{+}CD44$^{-/low}$ TN stage. This notion was strongly supported by the finding that TCRβ rearrangements in DP thymocytes of TCR$\alpha^{-/-}$ mice as well as in late (CD25^{-}CD44$^{-/low}$) TN thymocytes from normal mice were predominantly productive, in contrast to the statistical prediction for a random rearrangement process (Mallick *et al.*, 1993). Because the analysis of V(D)Jβ joints in the preceding developmental stage (CD25^{+}CD44$^{-/low}$) revealed the expected preponderance of out-of-frame joints, indicating random, non-selected rearrangements, the experiments suggested that the maturation of TN thymocytes beyond the CD25^{+}CD44$^{-/low}$ stage was accompanied by selection of cells with productive TCRβ rearrangements. Taken together, the results of all these studies clearly indicated an important role of the TCRβ chain, independent of TCRα, in mediating the transition of CD25^{+}CD44$^{-/low}$ TN thymocytes to the DP stage via the CD25^{-}CD44$^{-/low}$ TN stage. These data also provided a good rationale for the observation made much earlier that the TCRβ locus is expressed well before the TCRα locus, both in ontogeny and during thymopoiesis in adult mice (Raulet *et al.*, 1985; Samelson *et al.*, 1985; Snodgrass *et al.*, 1985a,b).

How can a TCRβ chain promote T-cell development in the absence of a functional TCRα chain? The cloning and characterization of certain pre-B-cell-specific genes provided an important clue. These nonrearranging genes, termed λ5 and VpreB, were shown to encode novel immunoglobulin-like molecules that could associate with Ig heavy chains, in the absence of Ig light chains, to form a previously unrecognized type of receptor, the pre-B-cell receptor (pre-BCR) (reviewed in Melchers *et al.*, 1993). Targeted disruption of the λ5 gene gave rise to mutant mice in which B-cell development was severely (although not completely)

blocked at the same developmental stage as in $RAG^{-/-}$ mice, demonstrating a critical role of the pre-BCR in early B-cell development (Kitamura *et al.*, 1992). If T- and B-cell development proceeded in an analogous fashion, the TCRβ chain would be expected to associate with nonrearranging gene products that could substitute for the TCRα chain, giving rise to a pre-TCR, much like the Ig heavy chain was shown to associate with the surrogate light chains $\lambda 5$ and VpreB to form a signaling-competent pre-BCR. Such a scenario would neatly explain the perported effects of TCR transgenes and gene deficiencies on T-cell development described earlier.

Initially, the analysis of TCRβ transgenic mice seemed to provide direct evidence for the existance of a pre-TCR complex, as it was found that isolated $CD4^-8^-$ immature thymocytes of non-SCID origin and total thymocytes of SCID origin were able to express the transgenic TCRβ chain on the cell surface in the absence of any other TCR chains, including TCRα (Kishi *et al.*, 1991). However, the biochemical characterization of such surface-expressed, transgene-encoded TCRβ chains did not reveal any pre-BCR-like structure. Rather, it was shown that the vast majority of transgenic TCRβ chains were expressed in a perplexing variety of different structures that lacked CD3 components and partly consisted of GPI-linked TCRβ monomers (Kishi *et al.*, 1991; Groettrup *et al.*, 1992). However, it soon became clear that all these TCRβ configurations, including GPI-linked monomers, were transgene-specific peculiarities with little or no physiological significance, as they were found neither in normal, non-transgenic mice nor on a cell line derived from immature SCID thymocytes (see later) that had been transfected with the same functionally rearranged TCRβ gene used before to generate TCRβ transgenic mice (Groettrup and von Boehmer, 1993a,b). Collectively, these studies clearly showed that TCRβ-transgenic mice were not, after all, the appropriate system to search for a putative pre-TCR.

The ideal tool to investigate the biochemical basis of how a TCRβ chain could mediate developmental effects in the absence of other rearrangement-dependent proteins was finally found in a newly established pre-T cell line (termed Sci/ET27F) that had been derived from immature SCID thymocytes and was shown to be devoid of any functional α, β, γ, and δ T-cell receptor chains (Groettrup *et al.*, 1992). When this pre-T-cell line was transfected stably with functionally rearranged TCRβ genes, a TCRβ/CD3 complex could be detected on the cell surface, whereas there was no such cell surface expression upon transfection of mature T-cell lines. An initial biochemical characterization of this apparently pre-T-cell-specific complex revealed that the TCRβ chain was disulfide linked to a second protein with a roughly similar molecular mass as TCRβ itself, suggesting that the complex may contain a TCRβ homodimer (Groettrup

et al., 1992). Subsequent experiments, however, rendered this possibility increasingly unlikely. The use of two-dimensional gel electrophoresis in combination with a different labeling technique led eventually to the detection of a novel, heavily glycosylated and sialylated protein that had a molecular mass of approximately 33 kDa (Groettrup *et al.*, 1993). This glycoprotein, designated provisionally "gp33," was shown to be the disulfide-linked partner chain of TCRβ in the receptor complex found on the transfected SCID cell line. Most important, the same protein was also associated with TCRβ chains on the surface of large thymocytes from TCRα-deficient mice, demonstrating that the gp33–TCRβ-containing complex was not a transgenic or cell line artifact (Groettrup *et al.*, 1993).

The possibility to grow large amounts of TCRβ-transfected cells of the SCID cell line and to precipitate gp33 with TCRβ-specific antibodies, thanks to its disulfide linkage with the β chain, permitted the purification of sufficient amounts of gp33 to obtain a partial, N-terminal amino acid sequence. This then led to the isolation of full-length gp33 cDNA (Saint Ruf *et al.*, 1994). The cloning of gp33 and the subsequent analysis of its structural features and expression patterns firmly established the existance of a pre-TCR on maturing thymocytes, in analogy to the pre-BCR on early B cells. Because gp33 supplies the partner chain for TCRβ in pre-T-cells, before TCRα is available, gp33 was renamed the pre-TCR α (pTα) chain. The identification of certain structural similarities between pTα and TCRα chains and the demonstration of a comparable genomic organization of the corresponding genes confirmed that this new name was not chosen inappropriately.

B. Structural and Biological Features of pTα and the Pre-TCR

1. Structure of pTα and Organization of the pTα Gene

The mature murine pTα chain is an invariant transmembrane protein that consists of a single extracellular immunoglobulin-like domain, a hydrophobic transmembrane region, and a cytoplasmic tail of approximately 30 amino acids (Saint Ruf *et al.*, 1994; reviewed by von Boehmer and Fehling, 1997). The identification of a genuine cytoplasmic tail in pTα was unexpected, as all other known TCR and Ig molecules are devoid of such a structure. Inspection of the amino acid sequence of the cytoplasmic tail revealed potential serine/threonine phosphorylation sites for protein kinase C and motifs that could potentially serve as attachment sites for molecules with SH3 domains, resembling known functional sequence elements in the cytoplasmic tail of CD2. These findings suggested initially that the cytoplasmic portion of pTα might be involved directly in the transduction

of pre-TCR-mediated signals (Saint Ruf *et al.*, 1994). However, cloning of human pTα cDNA and comparison with its mouse homolog revealed that the cytoplasmic portion in both species did not share any sequence similarities, whereas all other parts of both molecules were highly conserved (Del Porto *et al.*, 1995). Although this finding did not formally exclude an essential role of the cytoplasmic tail in signal transduction, it rendered such a possibility much less likely. Experiments with transgenic mice expressing a truncated form of pTα have strengthened the view that the cytoplasmic portion of pTα is not essential for the proper functioning of the pre-TCR (see later).

Although pTα clearly belongs to the immunoglobulin superfamily, it exhibits no significant sequence similarity to any particular member, including the T-cell receptor chains. However, its transmembrane region contains two basic residues that are separated from each other by four hydrophobic amino acids (Saint Ruf *et al.*, 1994). The same two polar residues with exactly the same spacing are found in the transmembrane regions of the TCRα and δ chains, reflecting similar structural and/or functional constraints in these three molecules. Analysis of the pTα gene has in fact provided strong evidence for an evolutionary relationship among pTα, TCRα, and TCRδ, as the exon/intron organization of the pTα gene resembles the structure of the TCRα, and also TCRδ, constant region genes to a surprising degree (Fehling *et al.*, 1995b). However, the presence of a full-length pTα message in RAG-deficient mice (Saint Ruf *et al.*, 1994) and analysis of the genomic sequence of the pTα locus (Fehling *et al.*, 1995b) have proven that expression of a functional pTα polypeptide does not depend on RAG-mediated gene rearrangements, in contrast to TCRα and TCRδ chains.

2. *Expression Pattern of pTα*

The expression pattern of pTα has been analyzed in detail by RT-PCR (Saint Ruf *et al.*, 1994; Bruno *et al.*, 1995). In the thymus, pTα seems to be expressed in all immature TN stages with the possible exception of the c-kit$^+$CD25$^-$CD44$^+$ subpopulation. For unknown reasons, the pTα message has been detected in this earliest thymic subpopulation only in RAG$^{-/-}$ but not in normal mice. pTα transcripts are clearly present in DP thymocytes, but not in SP thymocytes. The fact that the pTα and TCRα message can be found together in certain DP thymomas (Jacobs *et al.*, 1996a) suggests that both genes can be coexpressed at the DP stage in the same cell. Mature T cells of both the $\alpha\beta$ and $\gamma\delta$ lineage do not express pTα. Accordingly, spleen and lymph node lack pTa mRNA, like most nonlymphoid organs. However, the pTα message can be detected in gut and liver, which are potential sites of extrathymic T-cell development.

Together, these findings strongly suggest that pTα expression is confined strictly to immature cells of the T-cell lineage. Interestingly, the pTα message has been detected in the bone marrow of normal and RAG$^{-/-}$ mice, although pre-B cells, B cells, and other hematopoietic lineages do not express pTα (Bruno *et al.*, 1995), suggesting that the bone marrow contains T-committed precursors. If so, it may be possible to use pTα as a marker to identify and purify these interesting cells, which may represent a stage in development close to the proposed but elusive common lymphoid stem cell. The observation that pTα is also expressed in a cell population from mouse fetal blood that has been shown to have T precursor activity and to be restricted to the T lineage (Bruno *et al.*, 1995; Rodewald, 1995) underlines the potential diagnostic value of pTα as a marker for early T-cell precursors.

3. *Biochemical Composition of the Pre-TCR*

In the pre-T cell receptor, pTα is disulfide linked with TCRβ and is associated with certain CD3 components forming a signaling-competent receptor complex that is expressed on the cell surface of pre-T cells at very low levels. Because of these low expression levels, it has been technically difficult to identify the complex with cytofluorometric means in normal or TCRα-deficient mice, although the pre-TCR can be detected readily with biochemical methods. For unknown reasons, immortal pre-T-cell lines in general express much higher levels of the pre-TCR complex than normal thymocytes on the cell surface (Punt *et al.*, 1991; Groettrup *et al.*, 1992; Mombaerts *et al.*, 1995). This situation has led to some confusion about the precise physiological composition of the pre-TCR with regard to the CD3 components. At present, biochemical data suggest that CD3ε and CD3γ are core components (reviewed in Borst *et al.*, 1996). CD3ζ appears to participate as well, as stimulation of the pre-TCR complex in a pre-T-cell line leads to tyrosine phosphorylation of ζ and concomitant binding of the tyrosine kinase ZAP-70 (van Oers *et al.*, 1995), although earlier studies in the same cell line have shown that CD3ζ association with the pre-TCR is much weaker than with the mature TCR/CD3 complex (Groettrup *et al.*, 1992). Variable results have been obtained with regard to the presence of CD3δ in the pre-TCR complex. Although three different groups reported the presence of CD3δ in the pre-TCR of three distinct pre-T-cell lines (Punt *et al.*, 1991; Groettrup *et al.*, 1992; Mombaerts *et al.*, 1995), a fourth group could not detect CD3δ in the pre-TCR complex of an additional pre-T-cell line (Jacobs *et al.*, 1994), in the pre-TCR of thymocytes from TCRα-deficient mice (Jacobs *et al.*, 1994), or in RAG-deficient mice that were reconstituted with a transgenic TCRβ chain (Jacobs *et al.*, 1996a).

Whether CD3δ and CD3ζ are required for the proper functioning of the pre-TCR has become most obvious in appropriate gene knockout mice. In mouse mutants lacking CD3ζ, TCR surface expression is very low and the generation of DP thymocytes is very inefficient, suggesting that ζ contributes to the structural integrity of the pre-TCR complex and/or to pre-TCR signaling (reviewed in Shores and Love, 1997). To distinguish between these two possibilities, Shores and colleagues introduced a transgene encoding a truncated CD3ζ chain without a cytoplasmic portion into CD3ζ-deficient mice. The cytoplasmic tail of CD3ζ is known to be essential for the signal transduction function of ζ, but not for its structural role in promoting surface expression of the TCR complex. Efficient restoration of thymopoiesis in such reconstituted mice demonstrated that CD3ζ was required mainly for cell surface expression of the pre-TCR, but not for pre-TCR signaling. However, CD3ζ might contribute to efficient signal transduction as well, as the number of thymocytes in the reconstituted mice was somewhat reduced compared to normal controls, despite completely restored levels of TCR/CD3 surface expression (Shores *et al.*, 1994). Analysis of CD3δ-deficient mice, however, demonstrated that δ is dispensable for a proper function of the pre-TCR, as early (but not late) T-cell development in δ-deficient mice was shown to be completely normal (Dave *et al.*, 1997). Interestingly, CD3δ was also not required for the development of $\gamma\delta$ T cells (Dave *et al.*, 1997). Taken together, these data suggest important structural differences between the $\alpha\beta$TCR and the pre-TCR. The former is thought to have the following composition: TCRα–β, CD3$\gamma\varepsilon$, CD3$\delta\varepsilon$, and ζ–ζ/η. In the pre-TCR, TCRα is replaced by pTα, and CD3$\delta\varepsilon$ most likely by a second CD3$\gamma\varepsilon$ module. These presumed alterations would give rise to the following structure for the pre-TCR: pTα–TCRβ, $(CD3\gamma\varepsilon)_2$, ζ–ζ/η. Whether these receptor-specific compositions alter the mode of how a cell perceives CD3-mediated signals remains an important issue to be investigated. It is also not yet clear whether this basic pre-TCR complex assembles into structures of higher order *in vivo*, potentially adding another degree of complexity, as suggested by Jacobs (1997).

The present view of the pre-TCR depicts a rather asymmetric structure that lacks a partner chain for the TCRβ V-region domain. It is still a matter of speculation whether a separate as yet unidentified "VpreT" subunit exists. Cotransfection of TCRβ and pTα into a mature TCR-negative hybridoma does not lead to efficient cell surface expression of a pre-TCR complex (Saint Ruf *et al.*, 1994), an observation that can be interpreted as indication that an additional component, not present in mature cell lines, is mandatory. However, it is equally possible that mature T cells actively retain pre-TCR complexes. Interestingly, a defective TCRβ transgene that lacks a V-region domain can induce the generation of DP thymo-

cytes in RAG$^{-/-}$ mice (Jacobs *et al.*, 1996a), suggesting that neither a Vβ nor a hypothetical Vpre-T domain is absolutely required for pre-TCR function. However, although the number of thymocytes is strongly increased in these mice compared to RAG$^{-/-}$ controls, only about 30% of wild-type levels are reached (Jacobs *et al.*, 1996a). Although certain similarities of the pre-TCR and pre-BCR (reviewed in Borst *et al.*, 1996) provide an appealing argument in favor of a VpreT subunit, the difference between TCRβ and IgH chains in terms of folding, as well as structural differences between pTα and λ5 chains (reviewed in von Boehmer and Fehling, 1997), should also be considered.

C. Pre-TCR Function in Early Thymocyte Development

1. Physiological Role of the Pre-TCR

In wild-type mice, the developmental transition from the TN CD25^{+} pre-T-cell stage to the DP stage is accompanied by a series of differentiation events, including downregulation of CD25, upregulation of CD2, allelic exclusion of the TCRβ locus, initiation of germline transcription at the TCRα locus, surface expression of a TCRβ/CD3 complex, and a 20- to 50-fold cellular expansion that is responsible for more than 95% of thymic cellularity (reviewed in Levelt and Eichmann, 1995; Shortman and Wu, 1996; Fehling and von Boehmer, 1997). The crucial role of the pTα chain, and thus the pre-TCR, in this process has become evident in pTα-deficient mice (Fehling *et al.*, 1995a). Such animals were generated via gene targeting by deleting the two last exons of the pTα gene encoding the transmembrane region, the cytoplasmic tail, and the cysteine involved in heterodimer formation. The lack of functional pTα chains in homozygous pTα knockout mice severely hampered the development of thymocytes along the $\alpha\beta$ pathway. In adult pT$\alpha^{-/-}$ mice, the total number of thymocytes was on average between 5 and 10×10^6 compared to about 100×10^6 in littermate controls. Staining with CD4- and CD8-specific antibodies revealed that this strong reduction in total thymic cellularity was due mainly to a decrease of DP thymocytes to less than 5% the number found in wild-type controls. TCR$\alpha\beta$-expressing SP thymocytes were also present in pTα-deficient mice, although their absolute number was reduced 10- to 20-fold as well, i.e., to a roughly similar degree as DP thymocytes. The large parallel reduction in the number of DP and SP thymocytes suggested that the relative decrease of SP thymocytes in pT$\alpha^{-/-}$ mice was simply due to an insufficient supply of DP precursors and that pTα was not required for the DP to SP transition itself.

In sharp contrast to the development of $\alpha\beta$ T cells, the generation of $\gamma\delta$-expressing cells was not at all impaired in pTα-deficient thymi, establish-

ing that pTα and the pre-TCR are not required for normal development of $\gamma\delta$ lineage cells (Fehling *et al.*, 1995a). In fact, the absolute number of $\gamma\delta$ thymocytes was consistently about 3- to 10-fold higher in pT$\alpha^{-/-}$ mice compared to littermate controls. This significant increase in $\gamma\delta$ thymocytes, which is seen to a similar extent in TCR$\beta^{-/-}$ mice (Mombaerts *et al.*, 1992b; H. J. Fehling, unpublished observation), could indicate a direct role of pTα and the pre-TCR in the $\alpha\beta/\gamma\delta$ lineage decision. Alternatively, it may simply reflect an imbalance in thymic homeostasis due to the availability of additional space in the pTα-deficient thymus lacking more than 90% of its contingent of $\alpha\beta$ lineage cells.

To localize the defect in pT$\alpha^{-/-}$ mice more precisely, the subset composition of immature TN thymocytes was analyzed with antibodies specific for CD25, CD44, and c-kit (Fehling *et al.*, 1995a, 1997). The total number of immature TN thymocytes was not significantly different in mutant and wild-type thymi. However, the number of cells in the CD25$^+$CD44$^{-/low}$ subset was approximately twofold augmented, whereas the subset of cells with gradually decreasing levels of CD25, giving rise to and including CD25$^-$CD44$^{-/low}$ cells, was severely depleted. The two earlier TN subsets (CD25$^-$CD44$^+$c-kit$^+$ and CD25$^+$CD44$^+$c-kit$^+$), however, were present in normal numbers and proportions in pTα-deficient mice. Collectively, the data demonstrated that the impaired development of $\alpha\beta$ lineage cells in pT$\alpha^{-/-}$ mice was due to a block in the transition of CD25$^+$CD44$^{-/low}$ thymocytes to the CD25$^-$CD44$^{-/low}$ stage, exactly as in SCID, RAG$^{-/-}$, CD3$\varepsilon^{-/-}$, CD3$\gamma^{-/-}$, and TCR$\beta^{-/-}$ mice.

The identification of pTα as a partner chain for TCRβ in immature thymocytes and the demonstration of the crucial role of pTα in $\alpha\beta$ thymopoiesis have provided a satisfactory explanation for the initially surprising observation that a functional TCRβ chain can promote T-cell development despite the absence of TCRα. It is now clear that TCRβ exerts its effect in early T-cell development predominantly, if not exclusively, by associating with pTα and CD3 to form a signaling-competent pre-TCR complex. What is the biological purpose of the pre-TCR? The identification of a common point of developmental arrest in SCID, RAG$^{-/-}$, CD3$\varepsilon^{-/-}$, CD3$\gamma^{-/-}$, TCR$\beta^{-/-}$, and pT$\alpha^{-/-}$ mice shows that TN thymocytes have to pass a checkpoint at the CD25$^+$CD44$^{-/low}$ stage, at which further development stalls, unless certain developmental signals are provided. The pre-TCR acts as a molecular sensor that informs a cell that it has generated a functional TCRβ chain. The cell responds with intense proliferation, differentiation, and rapid progression to the DP stage, where TCRα rearrangements are predominantly taking place. In contrast, cells that fail to produce a functional TCRβ chain cannot form a pre-TCR and are therefore not allowed to progress along the $\alpha\beta$ developmental pathway (with a few

exceptions that are discussed later). Unless they have the ability to develop along the $\gamma\delta$ lineage, they are doomed to die. The biological function of the pre-TCR is therefore to selectively expand those T-cell precursors that have a productive TCRβ chain and hence the potential to form an $\alpha\beta$ TCR after successful TCRα rearrangement. In this way, useless T-cell precursors without functional TCRβ chains are effectively excluded from further development at the earliest possible stage. The process in which cells with a functional TCRβ rearrangement are preferentially allowed to progress in their development is generally referred to as "β selection" (Mallick *et al.*, 1993; Dudley *et al.*, 1994).

2. *Potential Modes of Action*

Although the developmental consequences of pre-TCR signaling are well understood, it is not yet clear which of these effects are a direct consequence of pre-TCR activity and which are controlled cell autonomously or influenced by other signals. Like many developmental processes that are regulated by receptors, the main function of the pre-TCR could be to control cell survival, proliferation, differentiation, or certain combinations thereof. For instance, it is conceivable that the only function of the pre-TCR might be to extend the life span of $CD25^+$ pre-T cells, allowing these cells to unfold a cell-autonomous program that includes differentiation and proliferation and depends only on the presence of certain cytokines, e.g., IL-7, rather than being triggered directly by pre-TCR-derived signals. The idea that the pre-TCR is indeed not required for differentiation is supported by the finding that $CD25^+$ pre-T cells can be induced to mature into DP thymocytes by a number of alternative stimuli that are very different from pre-TCR-mediated signals, including some that seem to do little more than inhibiting programmed cell death (see later). However, because there is no evidence so far that any of these alternative, CD3-independent stimuli can lead to a similar proliferative expansion as the one that is associated with normal pre-TCR function, these findings are still consistent with a direct role of the pre-TCR in mediating cell division.

Three studies have impinged on the relationship between pre-TCR function and cellular proliferation. Hoffman and colleagues (1996) investigated the cell cycle status of $CD25^+CD44^{-/low}$ TN thymocytes in normal and pre-TCR-deficient ($TCR\beta^{-/-}$, $RAG^{-/-}$) mice. It was found that about 8–10% of these cells in normal mice were of large size with a DNA content of greater than 2*N*, characteristic of the S/G2 phases of cell cycle. This population (denoted L for "large size") was found to exhibit additional hallmarks of rapidly dividing cells, such as augmented expression of cyclins A and B, elevated amounts of the active form of cdc-2 (cell division cycle-2) protein, increased activity of the cyclin-dependent kinase cdk-2, reduced

expression of p27, a potent inhibitor of several cyclin-dependent kinases, and hypophosphorylation of the retinoblastoma (Rb) gene product. In contrast, none of these features were found in the population containing apparently nondividing, small cells (denoted E for "expected size"). Most important, the analysis of V(D)Jβ rearrangements by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) revealed that there was no enrichment of in-frame joints in population E. In contrast, cells of subset L had predominantly in-frame rearrangements. Interestingly, $CD25^+CD44^{-/low}$ thymocytes from mutant mice unable to form a functional pre-TCR were reported to be almost exclusively of type E. Taken together, these results strongly suggested that the $CD25^+CD44^{-/low}$ subpopulation can be sorted into an earlier, preselection (population E) and a later, postselection subset based on cell cycle status (cell size, DNA content). This interpretation intimated that the generation of a productive TCRβ chain and formation of a functional pre-TCR were obligatory for the proliferation of $CD25^+CD44^{-/low}$ precursors.

These findings have been challenged by a separate study. Tourigny and co-workers (1997) set out to determine the extent of DJβ and V(D)Jβ rearrangements in each of the four immature TN thymic subsets by quantitative Southern blotting. Consistent with previous data (Godfrey *et al.*, 1994; Petrie *et al.*, 1995), no TCRβ rearrangements were detected in the first two subsets ($CD25^-CD44^+$ and $CD25^+CD44^+$ TN thymocytes). In contrast, almost 90% of all TCRβ alleles were shown to exhibit DJβ rearrangements at the subsequent stage ($CD25^+CD44^{-/low}$ TN thymocytes). However, only about 10% of all alleles at this very stage exhibited a complete V(D)J rearrangement, whereas in the subsequent $CD25^-CD44^{-/low}$ subset, 60% of all TCRβ alleles had V(D)J rearrangements. These findings suggested that D $\rightarrow$ Jβ recombination occurred immediately before the acquisition of the $CD25^+CD44^{-/low}$ phenotype, whereas V $\rightarrow$ (D)Jβ rearrangements occurred substantially later, during the 2- to 3-day period of residence at the $CD25^+CD44^{-/low}$ stage (Shortman *et al.*, 1990), but before transition to the $CD25^-CD44^{-/low}$ stage. Tourigny and colleagues (1997) also assessed the rearrangement status of the TCRβ locus in those $CD25^+CD44^{-/low}$ cells that were actively cycling. These cells were identified based on their larger than 2*N* DNA content. Surprisingly, the experiment showed that the extent of V(D)Jβ rearrangements among $CD25^+CD44^{-/low}$ dividing cells was not significantly different from that found in noncycling $CD25^+CD44^{-/low}$ cells (2N DNA) or in the total $CD25^+CD44^{-/low}$ population. If proliferation were an exclusive feature of cells that have passed β selection, as suggested by the studies of Hoffman *et al.* (1996), one would have expected a much higher percentage of complete V $\rightarrow$ (D)Jβ rearrangements in cycling $CD25^+CD44^{-/low}$ cells, similar to what was found at the

$CD25^-CD44^{-/low}$ postselection stage (~60%). Moreover, Tourigny and colleagues (1997) found substantial levels of cycling $CD25^+CD44^{-/low}$ cells also in $RAG^{-/-}$, $TCR\beta^{-/-}$, and SCID mice. Collectively, these data were interpreted to indicate that differentiation and proliferation were not inherently linked to TCRβ recombination or the expression of a functional TCRβ chain. Rather, TCRβ rearrangements appeared to occur in parallel with these events as part of an intrinsic developmental program. The authors therefore proposed that the outcome of TCRβ gene recombination would not influence the developmental progression, including proliferation per se, but would serve instead simply to promote the survival of those developing cells that have managed to express a functional TCRβ chain.

In fetal mouse thymocytes, the proliferation kinetics associated with β selection have been studied in detail by Falk and co-workers (1996). Using a combination of cytoplasmic TCRβ staining, propidium iodide staining, bromodeoxyuridine (BrdU) labeling, and confocal laser scanning microscopy, the proliferation dynamics of $TCR\beta^+$ and $TCR\beta^-$ thymocytes were compared between days of gestation 14.5 and 18.5 both in FTOC and in fetal thymi freshly recovered *ex vivo*. Based on the DNA content, all $TCR\beta^+$ cells were reported to be in cell cycle during the entire 4-day observation period. In contrast, the percentage of cells in S/G2 among $TCR\beta^-$ cells was considerably lower and decreased continuously from day 14.5 to day 18.5. Moreover, from day 17.5 onward, $TCR\beta^-$, but not $TCR\beta^+$, cells were shown to contain a proportion of hypodiploid (DNA content $<2N$) cells, suggesting ongoing cell death by apoptosis in this population. BrdU labeling and cell counting by flow cytometry and confocal microscopy with *ex vivo*-isolated thymocytes indicated that $TCR\beta^+$ cells experienced an approximately 500-fold expansion between days 14.5 and 18.5, whereas the number of $TCR\beta^-$ cells increased only about 8- to 14-fold. Combined with other data in this study (Falk *et al.*, 1996), the findings suggested that the major consequence of pre-TCR-mediated β selection of fetal thymocytes consisted of a burst of up to nine rapid cell divisions that lasted for about 4 days. In contrast, lack of selection of TCRβ-negative cells seemed to be reflected by a limited maintenance of slow proliferation, then cessation of proliferation, and finally cell death by apoptosis.

Assuming that β selection during fetal development is not significantly different from β selection in the adult thymus, these results support the conclusion of Hoffman and co-workers (1996) that expression of a functional TCRβ chain is closely linked to proliferation. However, using confocal laser scanning microscopy to identify individual $TCR\beta^+$ cells in freshly isolated thymic lobes, Falk and colleagues (1996) also showed that little cell division occurred for at least 1 day after thymocytes first expressed a

TCRβ chain, despite the fact that most of these cells were hyperdiploid (DNA content $>2N$). This interesting observation suggested that TCRβ expression was followed by a delay period, in which most cells outlasted in the G2 phase of the cell cycle, before undergoing a burst of cell divisions. The biological role of this apparent delay remained obscure, but it was hypothesized that the transient arrest in G2 could indicate the requirement for a secondary, mitogenic signal, e.g., through cytokine–cytokine receptor interactions (Falk *et al.*, 1996). Taken together, available data do not yet provide a conclusive answer to the question whether pre-TCR-derived signals directly trigger a transition in the cell cycle or whether cell division is secondary to pre-TCR activity.

D. Pre-TCR-Independent Pathways to the DP Stage

1. Pathways Promoted by the Presence of $\gamma\delta$ Thymocytes or the Formation of a $\gamma\delta$ TCR

If the pre-TCR were absolutely required for the developmental progression of $CD25^+CD44^{-/low}$ pre-T cells, then mutant mice in which a functional pre-TCR cannot be formed should be completely devoid of DP thymocytes. This is indeed the case in $RAG^{-/-}$ (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992) and $CD3\varepsilon^{-/-}$ (Malissen *et al.*, 1995) mice, but not in TCRβ- (Mombaerts *et al.*, 1992b) or pTα-deficient mice (Fehling *et al.*, 1995a), suggesting that there are other pre-TCR-independent mechanisms that can foster the generation of DP thymocytes, albeit inefficiently. One pre-TCR-independent mechanism to generate DP from DN thymocytes is induced by TCR-expressing cells. This was first suggested by the analysis of "leaky" SCID mice, which revealed a striking correlation between the presence of SP TCR-bearing cells in some SCID thymi and the appearance of TCR-negative $CD4^+CD8^+$ thymocytes (Shores *et al.*, 1990). Additional experiments demonstrated that intravenous transfer of normal bone marrow cells, including multipotential precursors, into SCID mice would not only give rise to donor-derived TCR-positive thymocytes, but also promote the differentiation of SCID thymocytes from TN into $CD4^+CD8^+$ DP thymocytes, although the SCID-derived cells remained TCR negative (Shores *et al.*, 1990). A second study found that intravenous injection of *in vitro* expanded populations of $\gamma\delta$ T cells or cloned $\gamma\delta$ T cell lines into SCID mice resulted in the generation of small but significant numbers of SCID-derived DP thymocytes. Thus, $\gamma\delta$ T cells provide signals, which can promote *in trans* the differentiation of thymocytes in the absence of a pre-TCR (Lynch and Shevach, 1993). Whether the effect of $\gamma\delta$-expressing cells is brought about by direct thymocyte–

thymocyte contacts, by secretion of cytokines that act on pre-T cells, or indirectly by activation of thymic stromal cells, which, in turn, stimulate the entry of $CD4^-CD8^-$ thymocytes into the CD4/CD8 differentiation pathway, has remained obscure. Moreover, it is not clear whether DP thymocytes generated in this way are "genuine" DP thymocytes with the potential to mature into functional T cells (if they could express a functional TCR) or whether they are some aberrant, dead-end cells that just happen to express CD4 and CD8 coreceptors.

A very interesting finding has been reported by Passoni and colleagues (1997). These researchers analyzed the reading frame of TCRδ rearrangements in the residual DP thymocyte population of TCRβ-deficient mice. Using the PCR-RFLP technique and primers specific for Vδ4, Vδ5, Vδ6, and Jδ1, it was found that more than 70% of the corresponding TCRδ rearrangements in DP thymocytes of TCR$\beta^{-/-}$ mice were in frame. A comparable value (66%) was found for Vγ2 $\rightarrow$ Jγ1 rearrangements. A random rearrangement process is expected to generate in-frame joints in only one out of three rearrangements, which was actually found when the preceding $CD25^+CD44^{-/low}$ DN population was analyzed with the same sets of primers. The data therefore indicated that at least some DP thymocytes in TCRβ-deficient mice had been selected for productive TCRδ and TCRγ rearrangements. This conclusion obviously suggests that expression of a $\gamma\delta$ TCR can promote the generation of DP thymocytes not only *in trans*, but also *in cis*. In other words, the generation of some DP thymocytes in TCR$\beta^{-/-}$, and by inferrence in pT$\alpha^{-/-}$ mice, appears to be contingent on the formation of productive TCRδ and TCRγ rearrangements. Unfortunately, the type of analysis peformed by Passoni *et al.* (1997) does not allow to estimate the percentage of DP thymocytes generated by this *cis* pathway.

2. *A Pathway Dependent on Early Expression of Functional TCRα Chains*

If the development of DP thymocytes in TCRβ- and pTα-deficient mice was solely due to the presence of $\gamma\delta$-expressing cells or in-frame γ/δ rearrangements, one would expect a complete arrest of thymopoiesis at the $CD25^+CD44^{-/low}$ pre-T cell stage in doubly deficient mice lacking, apart from pTα or TCRβ, also functional TCRδ chains. This prediction was essentially fulfilled in TCR$\beta^{-/-}$ TCR$\delta^{-/-}$ mutant mice, in which the frequency of DP thymocytes dropped to around or less than 1% (Mombaerts *et al.*, 1992b). In contrast, the percentage and absolute number of DP thymocytes was not reduced in pT$\alpha^{-/-}$ TCR$\delta^{-/-}$ doubly deficient mice compared to pT$\alpha^{-/-}$ singly deficient mice (Buer *et al.*, 1997), suggesting a third pathway to the DP stage. This pathway seemed to involve the

TCRβ chain, as it was blocked in TCR$\beta^{-/-}$ TCR$\delta^{-/-}$ mice (Mombaerts *et al.*, 1992b). The analysis of TCRβ expression in DP thymocytes of various mutant and wild-type mice provided a clue about the molecular mechanism of this putative third pathway. Approximately 50–60% of DP thymocytes in normal mice express intermediate levels of TCRβ on the cell surface. This population was shown to be significantly reduced in pTα-deficient mice, most likely reflecting the fact that many of these cells lacked a TCRβ chain because they had developed in the absence of pre-TCR-mediated "β selection" (Fehling *et al.*, 1997). This was in fact confirmed by cytoplasmic stainings with TCRβ-specific antibodies, which revealed that only about 40% of DP cells on a pT$\alpha^{-/-}$ background expressed a functional TCRβ chain, whereas essentially all DP cells were cytoplasmic TCRβ positive in the thymus of normal mice (Buer *et al.*, 1997). Surprisingly, almost 100% of DP thymocytes in pT$\alpha^{-/-}$ × TCR$\delta^{-/-}$ doubly deficient mice were found to be positive for cytoplasmic TCRβ expression. This indicated that all DP thymocytes formed in the absence of both pTα molecules and $\gamma\delta$-expressing cells had been subjected to some sort of "β-selection" (Buer *et al.*, 1997). Two types of observation finally suggested that the DP thymocytes in pT$\alpha^{-/-}$ TCR$\delta^{-/-}$ mice were most likely derived from pre-T cells with productive TCRβ joints, in which "premature" TCRα rearrangements had taken place and given rise to functional TCRα chains, which then could form a normal $\alpha\beta$ TCR in lieu of the pre-TCR. First, although the number of DP thymocytes in pT$\alpha^{-/-}$ and pT$\alpha^{-/-}$ TCR$\alpha^{-/-}$ mice was found to be rather similar, cytoplasmic staining revealed that only about 15% of DP thymocytes from the doubly deficient background were TCRβ positive versus 40% in pT$\alpha^{-/-}$ single-deficient mice, implying that a significant fraction of DP thymocytes in pT$\alpha^{-/-}$ mice had been generated in a TCRα-dependent fashion (Buer *et al.*, 1997). Second, introduction of TCR$\alpha\beta$ transgenes into pTα-deficient mice restored the development of CD4$^+$CD8$^+$ thymocytes very efficiently, indicating that early expression of TCRα could functionally substitute for pTα expression (Bruno *et al.*, 1996; Buer *et al.*, 1997). In fact, in TCR$\alpha\beta$ transgenic, pT$\alpha^{-/-}$ mice the number of thymocytes was reduced by only about 50% compared to TCR$\alpha\beta$ transgenic, pTα^+ mice, although the transgenic TCRα chain was expressed in only 9% of CD25$^+$ pre-T cells, demonstrating that an $\alpha\beta$ TCR could mediate expansion of pre-T cells at least as efficient as a pre-TCR.

Taken together, these data show that DP thymocytes can be generated by at least three distinct pathways, two of which are noticeable solely in mice in which the main route is experimentally blocked. The first pathway involves the formation of a pre-TCR, accounts for at least 95% of all DP thymocytes in normal mice, and therefore represents by far the physiologi-

cally most relevant route. The second pathway depends either on successful generation of a $\gamma\delta$ TCR in apparently $\alpha\beta$ lineage cells or on the presence of $\gamma\delta$-expressing cells, which somehow create a thymic environment that promotes the TN to DP transition. Finally, the third pathway involves premature formation of an $\alpha\beta$ TCR due to TCRα rearrangements apparently occurring at low frequency in $CD25^{+}CD44^{-/low}$ cells. This pathway is expected to be completely irrelevant in normal mice because, in the presence of pTα, formation of a functional TCRβ chain alone is sufficient to induce proliferation and transition to the DP stage. The assumption that the latter two pathways do not play any significant role in normal $\alpha\beta$ T-cell development is supported by the finding that the number of DP thymocytes is not reduced in TCRα- or TCRδ-deficient mice when compared with wild-type littermates. Irrespective of the physiological significance, the identification of three potential pathways to the DP stage finally provides a satisfactory explanation for the observed differences in the phenotypes of $pT\alpha^{-/-}$ and $TCR\beta^{-/-}$ mice on the one hand and $RAG^{-/-}$ and $CD3^{-/-}$ mice on the other hand, whereas in RAG- and CD3-deficient mice all three pathways are obstructed, resulting in a complete arrest of thymopoiesis at the TN to DP transition. TCRβ-deficient mice are left with pathway 2 and pTα-deficient mice with pathways 2 and 3, accounting for the apparent "leakiness" of these latter two mouse mutants (see also Fig. 2).

3. *Experimental Manipulations Generating DP Thymocytes in the Absence of a Pre-TCR*

Another line of investigation has revealed a number of artificial ways to induce the differentiation of $CD4^{-}CD8^{-}$ TN thymocytes into DP cells. Although of little or no physiological importance, these pathways do provide some hints about the possible mode of action of the pre-TCR in the normal situation. In an effort to use RAG-2-deficient mice as hosts for thymic reconstitution, Zúniga-Pflücker and colleagues (1994) found that sublethal doses of γ radiation allowed immature $CD25^{+}CD44^{-/low}$ TN cells to differentiate into DP thymocytes in the absence of any detectable TCRβ gene rearrangements. This intriguing observation was also reported by other investigators (Guidos *et al.*, 1995). Two possible mechanisms were proposed: a radiation-induced change in the thymic microenvironment, which would then somehow stimulate maturational processes (Guidos *et al.*, 1995), or induction of somatic mutations in genes that normally suppress the TN to DP transition (Zuniga-Pflucker *et al.*, 1994; Guidos *et al.*, 1995). A follow-up study revealed that DP thymocytes from irradiated RAG-deficient mice had indeed accumulated somatic mutations, interestingly, in the tumour suppressor gene p53, a key regulator of apoptosis (Jiang *et*

al., 1996). To assess the role of p53 in the generation of DP thymocytes directly, $RAG^{-/-}$ mice were bred with p53-deficient mice, in which T-cell development had been shown previously to be essentially normal (Donehower *et al.*, 1992). Analysis of thymocytes from $p53^{-/-}$ $RAG^{-/-}$ mice revealed the presence of $CD4^+CD8^+$ thymocytes (Jiang *et al.*, 1996; Mombaerts *et al.*, 1995), demonstrating that lack of p53 function is sufficient to allow for the differentiation of $CD4^-CD8^-$ into $CD4^+CD8^+$ thymocytes. However, both γ irradiation and disruption of the p53 tumor suppressor gene in $RAG^{-/-}$ mice did not restore more than 10% of normal thymic cellularity, possibly indicating that the transition to the DP stage was not accompanied by a similar proliferative burst as observed in normal mice or $RAG^{-/-}$ mice reconstituted with a functional TCRβ transgene (but see later for another interpretation). Interestingly, a further decrease in the number of DP thymocytes was seen in γ-irradiated $RAG^{-/-}$ mice lacking the src kinase $p56^{lck}$ (G. Wu *et al.*, 1996). Moreover, the few DP thymocytes that were found after irradiation of $RAG^{-/-}$ $lck^{-/-}$ mice had a more immature phenotype than the majority of DP thymocytes in normal or γ-irradiated $RAG^{-/-}$ singly deficient mice. These findings suggest that radiation-induced generation of DP thymocytes in $RAG^{-/-}$ mice is dependent on the presence of src kinases, such as $p56^{lck}$ (the role of src kinases in early T-cell development is discussed in Section IV,F,3).

γ irradiation results in the formation of DP thymocytes also in SCID mice. However, in these mouse mutants a pre-TCR-dependent mechanism appears to be responsible for most of the observed effects of γ irradiation, which is in contrast to similarly treated $RAG^{-/-}$ mice. This is suggested by several observations: Analysis of thymus DNA in γ-irradiated SCID mice revealed diverse TCRβ rearrangements detectable concomitant with the appearance of DP thymocytes (Danska *et al.*, 1994). Cytoplasmic staining showed that on average 70% of radiation-induced DP thymocytes expressed a TCRβ chain, and sequence analysis of PCR-amplified V(D)Jβ joints revealed a high degree of diversity, indicating that this DP thymocyte population was polyclonal. Moreover, 90% of the V(D)Jβ rearrangements were in frame, suggesting that DP thymocytes in irradiated SCID mice had undergone "β selection." Notably, these rearrangements completely lacked the structural anomalies associated with aberrant rearrangements found occasionally in nonirradiated SCID mice. Other studies revealed normal gene rearrangements also at the TCRγ and TCRδ loci of irradiated SCID mice (Livak *et al.*, 1996). However, complete Vα $\rightarrow$ Jα rearrangements could not be detected, although rearrangements at the TCRα loci were shown to be initiated after radiation-induced formation of DP thymocytes. Because TCRα rearrangements occur significantly after TCRβ, δ and γ rearrange-

ments, radiation-induced restoration of V(D)J recombination appears to be temporally restricted. Taken together, these findings strongly suggest that γ irradiation of SCID mice induces a DNA repair pathway that can transiently compensate for the SCID defect in V(D)J recombination, leading to normal TCRβ expression, formation of a pre-TCR, and generation of DP thymocytes in a pre-TCR-dependent fashion. In contrast, the mechanism by which γ irradiation generates DP thymocytes in $RAG^{-/-}$ mice is independent of a functional TCRβ protein, as these mice are inherently unable to perform gene rearrangement, even after irradiation (Zuniga-Pflucker *et al.*, 1994; Guidos *et al.*, 1995).

Most interesting, radiation-induced rescue of V(D)J rearrangements in SCID mice is absolutely dependent on the presence of p53, as this pathway is completely blocked in p53-deficient SCID mice, which fail to rearrange their TCR loci even after irradiation (Bogue *et al.*, 1996). However, and somewhat confusing, thymocytes in SCID mice lacking p53 exhibt limited developmental progression to the DP stage even in the absence of irradiation (Bogue *et al.*, 1996), much like mice deficient in both p53 and RAG, as described previously. Thus, in SCID mice two mechanisms might be responsible for irradiation-induced development of DP thymocytes, and p53 appears to be involved in both mechanisms, albeit in opposing fashions. The first pathway most likely represents a DNA repair response, requires the presence of p53, and results in the restoration of functional TCR rearrangements and formation of a conventional pre-TCR. This pathway is expected to dominate in irradiated SCID mice, as functional TCRβ rearrangements should confer a significant advantage for cellular expansion, an assumption that is supported by the fact that about 90% of TCRβ rearrangements found in thymi of irradiated SCID mice are productive. The second pathway is rearrangement independent and requires irradiation, not to induce a DNA damage response, but to mutationally inactivate p53 alleles in developmentally arrested thymocytes. For unknown reasons, inactivation of p53 then results in limited developmental progression to the DP stage.

Although mutations in p53 are known to suppress apoptosis, it seems unlikely that such mutations can bring about differentiative signals. Therefore, the promoting effects of γ irradiation or p53 deficiency on the differentiation of $CD25^{+}CD44^{-/low}$ TN cells in $RAG^{-/-}$ mice could merely be a consequence of prolonged survival. Consistent with this interpretation, induction of DP thymocytes, again in the absence of a significant increase in thymic cellularity, was also observed in RAG-deficient mice expressing transgenic bcl-2 under the control of the lck proximal promoter (Linette *et al.*, 1994). Bcl-2 is a potent antiapoptotic protein that can protect developing and mature T cells against a variety of apoptotic signals (Cory, 1995).

However, this promoting effect of transgenic bcl-2 on thymopoiesis in $RAG^{-/-}$ mice seems to depend critically on some additional, unknown factors, as the generation of DP thymocytes was not observed in a separate study using a different line of bcl-2-transgenic $RAG^{-/-}$ mice (Maraskovsky *et al.*, 1997).

It is also possible that the generation of DP cells in p53-deficient, in γ-irradiated, or in some Bcl-2-transgenic $RAG^{-/-}$ mice is the result of secondary mutations in a few $CD25^+$ pre-T cells, which then differentiate and proliferate like normal $CD25^+CD44^{-/low}$ precursors that express a pre-TCR, rather than reflecting extensive differentiation without proliferation. To distinguish between these two possibilities, one would need to assess the cell cycle status of transitory $CD25^-CD44^{-/low}$ thymocytes in $p53^{-/-}$ $RAG^{-/-}$ mice and/or the clonality of the resulting DP cells. If the DP thymocytes in $p53^{-/-}$ $RAG^{-/-}$ mice were derived from a limited number of rapidly cycling precursors with secondary mutations, they should be highly oligoclonal and the intermediate $CD25^-CD44^{-/low}$ subset should contain a relatively large proportion of cells with a high ($>2n$) DNA content. The clonal composition of DP thymocytes may be directly measurable in $p53^{-/-}$ $CD3^{-/-}$ mice by studying the TCRβ repertoire of the DP population, provided that introduction of the p53 mutation in CD3-deficient mice also results in pre-TCR-independent generation of DP thymocytes, as one might predict.

The analysis of TCRβ-independent pathways to the $CD4^+CD8^+$ stage suggests an important general conclusion, namely that the pre-TCR is not directly required for differentiation. Rather, the main function of the pre-TCR seems to be to directly promote cell survival and directly or indirectly also proliferation. This interpretation implies that $CD25^+CD44^{-/low}$ TN cells are already endowed with a developmental program that they unfold in the absence of any specific differentiative signals, simply on extension of their life span. This program may include extensive proliferation, which, in the latter case, would depend on specific mitotic signals provided by the pre-TCR and/or other receptor systems. Whether p53 and/or Bcl-2 is physiologically involved at this stage of differentiation is not yet clear.

E. Role of the Pre-TCR in Allelic Exclusion

Although individual T lymphocytes have the potential to generate two distinct T-cell receptor β chains, they usually express only one allele, a phenomenon termed allelic exclusion. Analysis of T-cell clones and hybridomas has revealed that allelic exclusion at the TCRβ locus is largely due to the fact that $\alpha\beta$ T cells carry in general only one productive TCRβ rearrangement, whereas the rearrangement on the other allele is either

incomplete (DJβ) or out of frame (reviewed in Malissen *et al.*, 1992). These findings are in line with the view that a productive TCRβ rearrangement can somehow prevent further rearrangements at the TCRβ locus. Strong support for this hypothesis has been obtained in mice expressing productively rearranged TCRβ transgenes (Uematsu *et al.*, 1988; Krimpenfort *et al.*, 1989), which enforce almost complete inhibition of endogenous Vβ → (D)Jβ rearrangements, whereas Dβ → Jβ rearrangements are essentially unimpaired. In contrast, mice expressing a nonproductive TCRβ transgene exhibit no inhibition of endogenous TCRβ rearrangements (Krimpenfort *et al.*, 1989).

In mature T cells, the rearrangement status of the TCRα locus differs from that of the TCRβ locus in that usually both alleles carry Vα → Jα rearrangements and cells with two functional TCRα alleles are found in approximately 30% of all T cells (Casanova *et al.*, 1991; Malissen *et al.*, 1992; Padovan *et al.*, 1993). In fact, in TCRα transgenic mice there is no or only very inefficient inhibition of endogenous Vα → Jα rearrangements (Borgulya *et al.*, 1992; Hardardottir *et al.*, 1995; Heath *et al.*, 1995). Thus, it appears that rearrangements at the TCRα locus continue on both alleles until a receptor is formed that can bind to thymic MHC molecules. Positive selection will then lead to downregulation of RAG expression and complete termination of all TCR rearrangements (Turka *et al.*, 1991; Borgulya *et al.*, 1992; Brändle *et al.*, 1992; Petrie *et al.*, 1993; Kouskoff *et al.*, 1995). Although, in general, TCRα rearrangements occur relatively late during thymocyte development, primarily at the transition from the TN to DP stage and during the DP stage itself (Petrie *et al.*, 1995; Wilson *et al.*, 1996), TCRβ rearrangements are initiated and completed much earlier, namely at the $CD25^{+}CD44^{-/low}$ TN stage (Godfrey *et al.*, 1994; Petrie *et al.*, 1995; Tourigny *et al.*, 1997) (see Section III,B). Therefore, functional TCRβ chains relay a negative feedback to the second allele in the absence of TCRα, strongly suggesting a role of the pre-TCR in mediating allelic exclusion.

If the pre-TCR were implicated in the arrest of further TCRβ rearrangements, pTα-deficient mice should show signs of inefficient allelic exclusion, a hypothesis that has been tested in three experimental approaches. Xu and co-workers (1996) generated ES cell lines in which either one or both of the pTα alleles were inactivated by targeted gene disruption. $pT\alpha^{+/-}$ or $pT\alpha^{-/-}$ ES cell clones were then transfected separately with a construct encoding a functionally rearranged TCRβ chain to establish $TCR\beta^{+}pT\alpha^{+/-}$ and $TCR\beta^{+}pT\alpha^{-/-}$ ES cell clones. The ability of the TCRβ transgene to mediate allelic exclusion in the absence or presence of pTα was determined in the "RAG complementation assay" (see Section II,B,2). Essentially all CD3-expressing thymocytes

in the chimeric mice (TCRβ^+pT$\alpha^{+/-}$ or TCRβ^+pT$\alpha^{-/-}$) were positive for the transgenic TCRβ chain. Interestingly, no evidence for the expression of endogenously encoded TCRβ chains was found either in chimeras with a pT$\alpha^{+/-}$ or a pT$\alpha^{-/-}$ background, as determined by FACS analysis of thymocytes using an antibody specific for a particular endogenous Vβ element (Xu *et al.*, 1996). This result suggested that allelic exclusion was operating even in the absence of pTα, at least at the level of cell surface expression. Xu and colleagues (1996) also tested the effect of TCRβ transgene expression at the level of DNA rearrangements. This was done by analyzing genomic DNA from thymocytes of TCRβ^+pT$\alpha^{+/-}$ and TCRβ^+pT$\alpha^{-/-}$ chimeric mice with Vβ6/Vβ7- and Jβ2-specific primers in a quantitative PCR assay as originally described by van Meerwijk *et al.* (1990) and in a modified form by Anderson *et al.* (1992) and D'Adamio and colleagues (1992). It was found that expression of the transgenic TCRβ chain inhibited Vβ7/Vβ6 to Jβ2 rearrangements by approximately 90% in the presence of pTα. Most important, the same degree of inhibition was seen in pTα-deficient thymocytes. Assuming that Vβ6/Vβ7 $\rightarrow$ Jβ2 rearrangements were representative for the overall status of the endogenous TCRβ alleles, these data appeared to indicate that pTα was not required for the inhibition of β rearrangements by a transgenic TCRβ chain. A potential problem of this experimental approach was the fact that pTα^+ and pT$\alpha^{-/-}$ chimeric mice were not of the same transgenic background, as they were generated from pT$\alpha^{+/-}$ and pT$\alpha^{-/-}$ ES cell clones that had been transfected individually with the respective TCRβ transgene, almost certainly giving rise to animals with distinct transgene copy numbers and/or insertion sites. Nevertheless, the authors concluded that the pre-TCR may not be involved in the signaling of allelic exclusion at the TCRβ locus (Xu *et al.*, 1996).

The opposite conclusion was reached in a subsequent study using stable lines of pT$\alpha^{-/-}$ and TCRβ-transgenic, pT$\alpha^{-/-}$ mice rather than chimeric animals, which permitted a more rigorous analysis. The efficiency of allelic exclusion in the absence of pTα was determined by measuring the frequency of mature SP thymocytes and peripheral LN T cells with two distinct TCRβ chains on the cell surface in both pT$\alpha^{-/-}$ and pTα^+ mice. To calculate the maximum number of cells expressing two distinct TCRβ chains in the complete absence of allelic exclusion, the following assumptions were made: (1) Sufficient time for rearrangements on both alleles, (2) absolutely no feedback inhibition of the functional β chain on the other allele, and (3) no selective disadvantage of cells with two functional β chains. Under these conditions (complete absence of allelic exclusion), it

was calculated (see Fig. 3) that 20% of TCRβ positive cells would express two distinct β chains. Any, even partial, violation of these assumptions would reduce the percentage of double expressing cells. Given the low frequency of cells that express a particular Vβ element in normal, nontransgenic mice, it was clear that evidence for incomplete allelic exclusion would not be easy to obtain by cell surface staining with antibodies specific for just two different Vβ elements. To alleviate this problem, a combination of antibodies directed against Vβ6 or Vβ8.1/8.2 elements on one hand and a pool of antibodies recognizing TCRs containing most of the remaining Vβ elements on the other hand was used. The sensitivity of this analysis was further increased by gating on CD3-positive thymocytes/lymphocytes, thus excluding all other cells that did not express a TCR or only at low levels. This analysis revealed that the frequency of $CD3^+$ thymocytes and lymph node cells scoring positive for both Vβ8.1/8.2 or Vβ6 and one of the Vβs represented in the antibody pool was extremely low (~0.1–0.2%) in normal pTα^+ mice (Krotkova *et al.*, 1997). This value might have reflected the low proportion of T cells that genuinely expressed two β chains, violating allelic exclusion, as suggested by studies of human T lymphocytes (Davodeau *et al.*, 1995; Padovan *et al.*, 1995). Alternatively, it might have represented the level of nonspecific background staining in the experiment. Most important, however, no significant increase in the percentage of potentially double-expressing cells could be detected in pT$\alpha^{-/-}$ mice (Krotkova *et al.*, 1997). These data clearly demonstrated that T cells and thymocytes expressing intermediate to high levels of the $\alpha\beta$ TCR were indeed allelically excluded at the level of cell surface expression, even in pTα-deficient mice, extending the FACS results of Xu *et al.* (1996) to normal, nontransgenic mice.

This analysis was also performed with TCRβ transgenic mice expressing or lacking pTα (Krotkova *et al.*, 1997). Cells expressing endogenous TCRβ chains were equally infrequent in pTα^+ and pT$\alpha^{-/-}$ mice, although the presence of the transgenic β chain on essentially all $CD3^+$ cells should have allowed the detection of even very small differences in the number of TCRβ double-expressing cells. These findings confirmed that pTα was not required to establish allelic exclusion at the level of cell surface expression. However, evidence for a role of pTα in allelic exclusion was found at the level of DNA rearrangements. Using the same DNA-PCR assay employed by Xu and colleagues (1996), it was shown that TCRβ transgenic thymocytes of pTα^+ mice contained nearly undetectable levels of endogenous V$\beta \rightarrow$ (D)Jβ rearrangements due to the negative feedback inhibition mediated by the transgenic TCRβ chain, as had been reported previously (Uematsu *et al.*, 1988; van Meerwijk *et al.*, 1990; Anderson *et al.*, 1992). In contrast, although the transgenic TCRβ chain was still able to inhibit

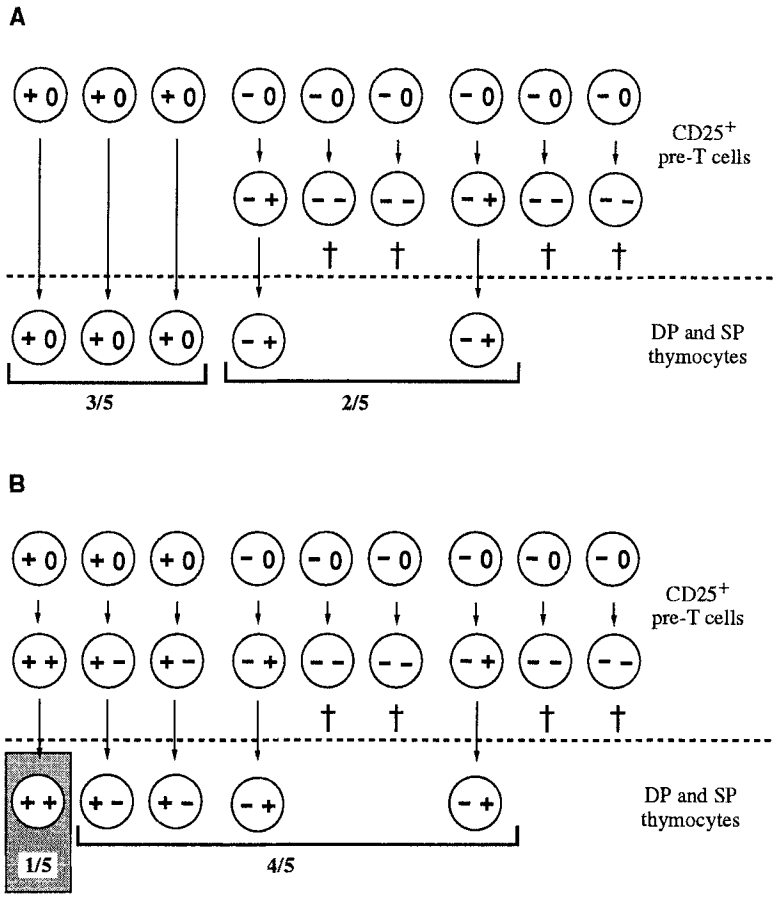


FIG. 3. Expected frequency of productive and nonproductive Vβ → (D)Jβ rearrangements in maturing T cells. This scheme is based on the premise that formation of a particular reading frame by Vβ → (D)Jβ rearrangements is completely random, i.e., that on average exactly one-third of all rearrangements are productive (in frame) and two-thirds are nonproductive (out of frame). The scheme further assumes that secondary rearrangements [rearrangements involving an allele that already bears a Vβ → (D)Jβ rearrangement] are not possible. The plus and minus symbols refer to in-frame and out-of-frame Vβ → (D)Jβ rearrangements, respectively; the symbol 0 indicates the absence of Vβ → (D)Jβ rearrangement (locus in germline configuration or Dβ → Jβ rearranged). (A) Perfect allelic exclusion. Out of nine pre-T cells that engage in Vβ → (D)Jβ rearrangements on the first allele, only three produce productive joints. The formation of a functional TCRβ chain allows these cells to mature and, at the same time, prevents further Vβ → (D)Jβ rearrangements at the second allele. The six remaining cells rearrange the second TCRβ locus. Two of these six cells are expected to generate an in-frame joint, allowing them to mature, whereas the remaining four cells have nonproductive rearrangements on both alleles and perish (unless they have the potential to enter the $\gamma\delta$ lineage). (B) Absolutely no allelic exclusion. Out of nine pre-T cells that engage in Vβ → (D)Jβ rearrangements on the first allele,

endogenous TCRβ rearrangements to a large degree even in the absence of pTα, the inhibition was significantly weaker, as bands specific for endogenous rearrangements involving all six functional Jβ2 segments were apparent at very substantial levels (Krotkova *et al.*, 1997). To allow a comparison of equivalent thymic subpopulations, the analysis was repeated with DNA from sorted $CD25^+CD44^{-/low}$ TN thymocytes representing the developmental stage at which TCRβ rearrangements predominantly occur. The result was virtually identical: although the transgenic TCRβ chain could inhibit endogenous V$\beta \rightarrow$ (D)Jβ rearrangements in the absence of pTα, the degree of inhibition was much less pronounced than in $CD25^+$ thymocytes expressing pTα (Krotkova *et al.*, 1997). Together, these results clearly demonstrated that the pTα chain and thus the pre-TCR were involved in allelic exclusion of endogenous Vβ rearrangements, at least in TCRβ-transgenic mice.

This conclusion has recently been extended to nontransgenic mice by comparing the rearrangement status of TCRβ alleles in sorted $CD25^+CD44^{-/low}$ TN thymocytes from pTα-deficient mice and wild-type littermates at the single cell level. Using a set of Jβ2-specific and several Vβ-specific primers and the single-cell PCR technique, V(D)J joints were amplified from a number of pre-T cells exhibiting V$\beta \rightarrow$ (D)Jβ rearrange-

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three produce productive joints. As the formation of a functional TCRβ chain does not prevent V$\beta \rightarrow$ (D)Jβ rearrangements on the second allele, one of the three cells with a productive rearrangement on the first allele is expected to generate another productive rearrangement on the second allele. Nonproductive TCRβ rearrangements on both chromosomes result in programmed death of four of the original nine cells. The frequency of cells with two productive V$\beta \rightarrow$ (D)Jβ rearrangements among the surviving T-cell population is therefore expected to be at most 1/5 (20%). This estimation rests on the following assumptions: (a) absolutely no feedback inhibition of V$\beta \rightarrow$ (D)Jβ rearrangements by a functional TCRβ chain, (b) sufficient time for TCRβ rearrangements on the second allele before cells progress to the next developmental stage where the TCRβ locus is recombinationally silenced, and (c) no developmental disadvantage of cells with two functional TCRβ chains. Any, even partial, violation of these assumptions would further reduce the percentage of cells with two productive TCRβ rearrangements in a corresponding, albeit unpredictable, fashion. Moreover, in some of the cells with two productive TCRβ rearrangements, the same V_β element is involved on both chromosomes. Cell surface staining with Vβ-specific antibodies fails to identify these cells. In practice, the percentage of cells with two distinguishable TCRβ chains is therefore clearly below 20%, even under conditions where allelic exclusion is completely compromised. The depicted scheme also predicts that cells with one TCRβ locus in germline or in a DJβ configuration are very infrequent in the absence of allelic exclusion, whereas such cells should constitute about 60% of the mature T-cell or SP thymocyte population when allelic exclusion is fully operating.

ments on both alleles. Subsequent sequence analysis revealed that only 1 out of 19 $CD25^+CD44^{-/low}$ cells obtained from wild-type mice had two productive TCRβ rearrangements. In contrast, functional TCRβ alleles on both chromosomes were found in 7 out of 20 pre-T cells from $pT\alpha^{-/-}$ mice, providing additional evidence for the important role of pTα in allelic exclusion at the level of gene rearrangements (Aifantis *et al.*, 1997). This evidence was of particular value because it was obtained with normal, nontransgenic mice.

These findings demonstrate that pTα and hence the pre-TCR are indeed involved in mediating the inhibitory effect of a functional TCRβ chain on V$\beta \rightarrow$ (D)Jβ rearrangements at the second allele. However, an important question remains: why does lack of pTα not result in a significant fraction of mature thymocytes or peripheral T cells with two functional TCRβ chains on the cell surface when, in the absence of pTα, at least 20% of all pre-T cells are carrying two productively rearranged TCRβ alleles? One possible explanation would be that there is another level of regulation preventing cells with two in-frame TCRβ loci to express both loci or preventing them to mature. This appears unlikely, considering that T cells with more than one TCRβ chain on the cell surface are very frequent in TCRβ double transgenic mice (Zal *et al.*, 1996). Thus, two functional TCRβ chains are not toxic, and maturing thymocytes with two functional TCRβ chains are not actively eliminated by some selection mechanism. It appears more likely that the failure to detect mature T cells with two distinct TCRβ chains on the cell surface, even in pTα-deficient mice, is a result of the tight association between allelic exclusion and maturation: pairing of a functional TCRβ chain with pTα and formation of a pre-TCR complex would result in rapid cell cycle induction, shut down of RAG gene expression, and progression to the next stage in differentiation. In the absence of pTα, β^+ pre-T cells are arrested at this stage and thus frequently rearrange the second allele. Unusually early TCRα rearrangements can provide functional TCRα chains in a few thymocytes at the $CD25^+$ stage (see Section IV,D,2). In cells with a productive TCRβ rearrangement, an $\alpha\beta$ TCR forms and inhibits rearrangement of the second allele. Such cells are expected to selectively overcome the $pT\alpha^{-/-}$ associated developmental block at the $CD25^+$ stage. Most of the SP thymocytes and mature T cells in $pT\alpha^{-/-}$ mice are therefore most likely derived from these few TN $CD25^+TCR\alpha\beta^+$ thymocytes. According to this view, one would expect that mature T cells in $pT\alpha^{-/-}$ mice do not carry more than one functional V(D)Jβ rearrangement, which would explain the absence of cells with two TCRβ chains on the cell surface, despite the fact that allelic exclusion is compromised at the level of DNA rearrangements in pTα-deficient mice.

F. Likely Components of the Pre-TCR Signal Transduction Machinery

1. Potential Contribution of the TCRβ and Pre-TCRα Chains to Signal Transduction

The TCRβ chain, like all other rearranging T-cell receptor and B-cell receptor molecules known to date, contains just a few cytoplasmic amino acids. A direct involvement of the intracellular portion of TCRβ in the transduction of pre-TCR-mediated signals therefore appears rather unlikely. In line with this view, deletion of the carboxy-terminal five residues that extend into the cytoplasm does not impair the ability of transgenic TCRβ chains to induce the generation of normal numbers of DP thymocytes in RAG-deficient mice or to mediate allelic exclusion in RAG^+ mice, demonstrating that these residues are not essential for normal pre-TCR function (Jacobs *et al.*, 1996b). Whether the much longer cytoplasmic tail of pTα (see earlier) actively participates in signal transduction was tested in transgenic mice expressing a tailless form of pTα under the control of the proximal lck promoter (Fehling *et al.*, 1997). A high copy number transgenic line was established and back-crossed onto $pT\alpha^{-/-}$ mice, generating animals that expressed the tailless pTα protein in the absence or presence of endogenous pTα. The analysis of these mice revealed that tailless pTα chains efficiently restored αβ T-cell development in pTα knockout mice: the severe blockage in the transition from $CD25^+CD44^{-/low}$ to $CD25^-CD44^{-/low}$ thymocytes was overcome, the proportion and cellularity of the DP compartment reconstituted, and the preferential expansion and maturation of thymocytes with functional TCRβ rearrangements (β selection) reinstated. All in all, tailless pTα chains normalized the proportion of thymic subsets, as defined by CD4 and CD8 expression, and augmented the total number of thymocytes to levels similar to those found in tailless transgenics with a $pT\alpha^+$ background (Fehling *et al.*, 1997). Although these experiments could not rule out a minor, quantitative contribution of the cytoplasmic residues of pTα to pre-TCR activity, the results clearly established that the cytoplasmic tail of mouse pTα was not essential for pre-TCR function (Fehling *et al.*, 1997).

2. Molecules of the CD3 Complex

The components of the CD3 complex are key players in the signaling circuit that links the pre-TCR to specific cytoplasmic targets (discussed in Tanaka *et al.*, 1995; Malissen and Malissen, 1996). In fact, cross-linking of CD3 complexes in TCR-deficient mice can bypass pre-TCR signaling. For instance, treatment of fetal thymic organ cultures derived from SCID, RAG-deficient, or TCRβ-deficient mice with anti-CD3ε antibodies (Levelt

et al., 1993) or injection of such antibodies into RAG-deficient mice (Jacobs *et al.*, 1994; Shinkai and Alt, 1994) triggers efficient expansion and maturation of DN thymocytes and thus restores early T-cell development in such mouse mutants. The striking effect of CD3ε-specific antibodies on T-cell development in $RAG^{-/-}$ mice implies that at least CD3ε must be exposed on the cell surface, despite the absence of mature T-cell receptor chains. Biochemical studies have shown that a large fraction, if not all, of such clonotype-independent CD3 complexes contain calnexin, an integral membrane protein that normally resides in the endoplasmatic reticulum (Wiest *et al.*, 1995). Interestingly, injection of anti-CD3ε-specific antibodies into $pT\alpha^{-/-} \times RAG^{-/-}$ doubly deficient mice restored the number of DP thymocytes to the same extent as anti-CD3ε treatment of $RAG^{-/-}$ singly deficient mice, demonstrating that pTα was not required for clonotype-independent surface expression of CD3ε (Fehling *et al.*, 1997).

The specific effects of anti-CD3ε antibody treatment on early T-cell development suggest that physical approximation of CD3ε-containing modules is sufficient for the induction of pre-TCR-like signals. This view has led to the attractive hypothesis that successful formation of a TCRβ–pTα heterodimer may be relayed to intracellular signal transduction pathways simply by bringing two CD3ε-containing modules in close proximity (Jacobs *et al.*, 1994). Approximation of the modules within a single pre-TCR/CD3 complex would then result in cross-phosphorylation of functional motifs, present in the cytoplasmic tails of these components. According to this view, the pre-TCR does not need to interact with any putative extracellular ligand, which would be in line with the finding that a TCRβ V-region domain is not essential for the signaling function of the pre-TCR (Krimpenfort *et al.*, 1989; Jacobs *et al.*, 1994, 1996a).

The ability of the CD3 complex to transduce TCR-derived signals depends on a shared functional sequence, the immunoreceptor tyrosine-based activation motif (ITAM), that is located within the intracellular portion of each CD3 component (reviewed by Weiss, 1993). Individual ITAMs consist of two Tyr-X-X Leu/Ile domains that are separated by six to eight X-type residues, where X denotes a nonconserved amino acid. A fully assembled TCR or pre-TCR complex contains a considerable number of different ITAMs: one in each CD3ε, δ, and γ chain and three in each CD3ζ chain. The variation in nonconserved residues between different ITAMs may constitute an important factor controlling the specificity and affinity of interaction with distinct signaling molecules.

To assess the function of individual CD3 components in pre-TCR-mediated events, Shinkai and colleagues (1995) generated chimeric transgenes encoding extracellular and transmembrane domains of the human interleukin 2 (IL2) receptor α chain (Tac) and the cytoplasmic domain of

either CD3ε or CD3ζ. These chimeric constructs, driven by the proximal lck promotor, were introduced separately into RAG-deficient mice. Injection of anti-Tac antibodies into both types of transgenic $RAG^{-/-}$ mice resulted in downregulation of CD25, upregulation of CD2, massive cellular expansion, and essentially complete reconstitution of the DP compartment, not different from $RAG^{-/-}$ control mice injected with anti-CD3ε-specific antibodies (Shinkai *et al.*, 1995). These *in vivo* cross-linking experiments thus demonstrated that both CD3ε- and CD3ζ-derived signals were able to trigger, to an equivalent extent, differentiation of $RAG^{-/-}$ TN thymocytes into DP cells, mimicking pre-TCR function. The fact that cross-linking of individual CD3ε or CD3ζ cytoplasmic domains was sufficient to mediate pre-TCR-specific effects further suggested that most of the ITAM motifs in a completely assembled pre-TCR complex might fulfill redundant functions, with the caveat that these experiments could not rigorously exclude the possibility that endogenous CD3 molecules participated in signal transduction. The observation that pre-TCR function was not significantly impaired in CD3ζ-deficient mice when reconstituted with tailless CD3ζ chains (Shores *et al.*, 1994) strongly supports the conclusion that not all ITAM motifs are required for efficient pre-TCR function.

3. *Nonreceptor Tyrosine Kinases $p56^{lck}$ and $p59^{fyn}$*

Tyrosine phosphorylation of specific ITAMs is one of the first signal transduction-associated events in mature T cells following stimulation of the TCR. Similar events are thought to occur after cell surface expression/stimulation of a pre-TCR. Tyrosine phosphorylation of CD3ε and CD3ζ chains has indeed been demonstrated in a pre-TCR- expressing, immature T-cell line after stimulation with antibodies against CD3ε (van Oers *et al.*, 1995). Which kinases mediate these events? Several lines of evidence indicate a major role of the nonreceptor tyrosine kinase $p56^{lck}$ in pre-TCR signal transduction (reviewed by Anderson and Perlmutter, 1995). For instance, in mice with a targeted disruption of the lck gene, maturation of αβ thymocytes is severely impaired at the same developmental stage as in mouse mutants, which fail to generate a functional pre-TCR complex (Molina *et al.*, 1992). This is also true for mice that overexpress dominant-negative Lck (Levin *et al.*, 1993). Moreover, transgenes encoding a catalytically activated form of $p56^{lck}$ restore the development of DP thymocytes in $RAG^{-/-}$ mice (Mombaerts *et al.*, 1994) and $pT\alpha^{-/-}$ mice (Fehling *et al.*, 1997). Finally, the generation of DP thymocytes after anti-CD3ε antibody treatment is severely hampered in $RAG^{-/-} \times lck^{-/-}$ doubly deficient mice, demonstrating the importance of Lck also in this experimental model of pre-TCR function (Levelt *et al.*, 1995).

Although the experimental evidence for a pivotal role of $p56^{lck}$ in pre-TCR signaling is convincing, the biochemical effectors on which Lck acts, as well as the mechanism of Lck activation, are still obscure. A role for $p56^{lck}$ in T-cell signaling was originally identified in more mature thymocytes and T cells by virtue of its physical association with the TCR coreceptors CD4 and CD8 (Glaichenhaus *et al.*, 1991; reviewed in Veillette *et al.*, 1991). Because these molecules are not available in pre-T cells, Lck must interact with other factors that are components of the pre-TCR complex or which can associate with the pre-TCR. However, specific molecules that provide binding sites for Lck have not yet been identified in $CD4^-CD8^-$ pre-T cells.

To study the effects of direct $p56^{lck}$ activation in immature $CD4^-CD8^-$ thymocytes, Norment and colleagues (1997) have generated mice expressing a CD4 transgene under the control of the proximal lck promoter, and thus predominantly during early thymopoiesis. When the transgene was bred on a $RAG^{-/-}$ background, early expression of transgenic CD4 induced downregulation of CD25, generation of DP thymocytes, and an approximately fivefold increase in thymocyte numbers, thus promoting thymocyte development in a manner similar to pre-TCR signaling. Notably, this process was dependent on the presence of the cytoplasmic portion of CD4, as transgenes encoding a tailless form of CD4 were ineffective, almost certainly reflecting an obligatory interaction of CD4 with Lck. Most interesting, the propagation of thymocyte development in CD4-transgenic $RAG^{-/-}$ mice required the presence of MHC class II ligands, as expression of the same CD4 transgene had no promoting effects on thymocyte development in $RAG^{-/-} \times$ MHC class $II^{-/-}$ doubly deficient mice (Norment *et al.*, 1997). Together, these results indicated that binding of CD4 to MHC class II molecules can trigger a physiologically relevant, Lck-dependent activation signal to thymocytes in the absence of TCRα or TCRβ chains. Most interesting, in CD3-deficient mice, early expression of transgenic CD4 had no appreciable effect on thymocyte maturation (Norment *et al.*, 1997). The inability of CD4/Lck to promote development in the absence of CD3 could indicate that CD3 components are located downstream of Lck in the pre-TCR signaling cascade, for instance serving as phosphorylation substrates of Lck kinase. Alternatively, it is also possible that CD4/Lck complexes must colocalize with the pre-TCR in order to approximate Lck with other components of the signal transduction machinery, which might not be available in an appropriate spatial arrangement in the absence of CD3.

Is $p56^{lck}$ the only tyrosine kinase directly downstream of the pre-TCR in the signal transduction cascade? Although Lck-deficient mice have a severe block at the TN to DP transition, a significant number of DP (and

SP) thymocytes are present (Molina *et al.*, 1992), comparable to the number of DP thymocytes in pTα- (Fehling *et al.*, 1995a) or TCRβ-deficient mice (Mombaerts *et al.*, 1992b). In contrast, transgenic mice expressing a dominant-negative form of Lck at high levels exhibit a complete arrest of thymopoiesis at the TN to DP transition (Levin *et al.*, 1993), such as $CD3^{-/-}$ (Malissen *et al.*, 1995) or $RAG^{-/-}$ mice (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1992), suggesting that a redundant kinase activity provided by another related kinase might be responsible for the leakiness in $lck^{-/-}$ mouse mutants. This kinase has been identified as $p59^{fyn}$, in that combined inactivation of the lck and fyn genes completely arrested T-cell development at the $CD4^-CD8^-$ stage (Groves *et al.*, 1996; van Oers *et al.*, 1996), whereas targeted disruption of the $p59^{fyn}$ gene alone had no appreciable inhibitory effect on T-cell development (Appleby *et al.*, 1992; Stein *et al.*, 1992). Together, these data indicate that Lck is the primary signaling kinase immediately downstream of the pre-TCR and that, in the absence of Lck, Fyn can at least partially substitute for this function.

4. The Receptor Tyrosine Phosphatase CD45

Both $p56^{lck}$ and $p59^{fyn}$ belong to the Src family of nonreceptor tyrosine kinases (reviewed by Neet and Hunter, 1996). Members of this family contain two conserved sites of tyrosine phosphorylation: an autophosphorylation site within the kinase domain and a carboxy-terminal regulatory site which, when phosphorylated, serves to decrease kinase activity. Several lines of evidence suggest a role for the receptor tyrosine phosphatase CD45 in regulating the activity of Src family kinases (reviewed in Trowbridge and Thomas, 1994). Studies of CD45-deficient T-cell clones and lines defective in T-cell receptor signal transduction revealed decreased kinase activity of $p56^{lck}$ and, to a lesser extent, $p59^{fyn}$, which was accompanied by a severalfold increase in the levels of phosphorylation at the negative regulatory site of the respective kinases (McFarland *et al.*, 1993). These findings, along with other *in vitro* studies, suggested that CD45 might be required *in vivo* to directly dephosphorylate the inhibitory C-terminal tyrosine residue and thus to activate both kinases. This conclusion would predict a negative effect of CD45 deficiency on early T-cell development, a prediction that has been tested in two different strains of CD45 gene-targeted mice. Kishihara and colleagues (1993) disrupted the alternatively spliced exon 6, generating mice in which most lymphoid cells failed to express all isoforms of CD45, probably due to interference with RNA processing. Byth and colleagues (1996) generated a genuine CD45 null strain by disrupting exon 9, which is common to all CD45 isoforms. The phenotypes of both strains of CD45-deficient mice were largely similar. TCR-mediated activation of mature T cells was completely abrogated,

and development of $\alpha\beta$ T cells was impaired at two stages, resulting in significantly reduced numbers of mature SP cells both in the thymus and in the periphery. The strongest effect of CD45 deficiency on thymopoiesis was seen at the transition from the DP to the SP stage, which was inhibited approximately four- to fivefold, at least partly due to impaired positive selection (negative selection was also shown to be impaired). The second developmental restraint was found at the TN to DP transition, in that the number of DP thymocytes was reduced by about 50%, whereas the number of TN thymocytes was increased concomitantly. Examination of the four TN compartments defined by the expression of CD25 and CD44 surface markers revealed an increase in the $CD25^{+}CD44^{-/low}$subset and a decrease in the percentage of $CD25^{-}CD44^{-/low}$ cells. The stage of this block strongly suggested that CD45 deficiency impaired pre-TCR-mediated maturation, as expected if CD45 was involved in the activation of Lck. However, this inhibition of pre-TCR-mediated effects was much less pronounced than in Lck-deficient (Molina *et al.*, 1992) or Lck/Fyn doubly deficient mice (Groves *et al.*, 1996; van Oers *et al.*, 1996). Taken together, these results clearly indicated an involvement of CD45 in pre-TCR signal transduction, most likely via dephosphorylation of Lck and Fyn, but the results also showed that CD45 was not essential for the functioning of pre-TCR-associated Src kinases. The relatively mild effect of CD45 deficiency on the efficacy of pre-TCR signal transduction may be related to the fact that hyperphosphorylation of Lck at the inhibitory site reduces but does not abolish kinase activity. Although the presumed residual level of Lck activity in the absence of CD45 might be compatible with pre-TCR signaling, it may not be sufficient for TCR-mediated activation of mature T cells. It is also possible that other hitherto unidentified tyrosine phosphatases expressed specifically during the early phase of T-cell development compensate for the lack of CD45 at the TN to DP transition. Additional studies are required to resolve this issue.

5. *Potential Components of the Pre-TCR Signaling Cascade Downstream of $p56^{lck}/p59^{fyn}$*

Very little is known about pre-TCR-specific signal transduction events downstream of $p56^{lck}$. The conclusive identification of pre-TCR-controlled signal transduction molecules is retarded by the fact that the role of candidate molecules potentially involved in pre-TCR signaling cannot be assessed in cell lines in suspension culture because it has been impossible so far to recapitulate the pre-TCR-mediated TN to DP transition outside of an intact thymus. However, many well-characterized components of the signal transduction cascade, operating in thymocytes or peripheral T cells after the engagement of mature $\alpha\beta$ TCRs, may also be utilized by the pre-

TCR. A promising approach to identify molecules involved in pre-TCR signaling therefore appears to be to determine whether factors known to be involved in signaling in mature T cells are required for pre-TCR-mediated events. This can be done in genetically manipulated mice or in thymic organ cultures derived from such mice.

a. Tyrosine Kinases ZAP-70 and Syk. In the mature $\alpha\beta$TCR, like in the pre-TCR, the initial step during signaling is tyrosine phosphorylation of CD3-associated ITAMs (see earlier). Once phosphorylated, ITAMs recruit the tyrosine kinase ZAP-70 to the activated receptor, which itself becomes a target of phosphorylation, also catalyzed, at least in part, by $p56^{lck}$ [the signal transduction pathways tapped by the mature $\alpha\beta$TCR have been reviewed in detail by Cantrell (1996)]. ZAP-70 plays an essential role in signal transduction during late thymopoiesis, as T-cell development is arrested at the DP stage in ZAP-deficient mice (Negishi *et al.*, 1995). Studies with TCR-transgenic, ZAP-$70^{-/-}$ mice have shown that ZAP-70 is indispensable for positive and negative selection (Negishi *et al.*, 1995). However, ZAP$^{-/-}$ mice do not show any defect in the generation of normal DP thymocytes, demonstrating that this kinase is not essential for pre-TCR function. A related tyrosine kinase, Syk, is also expressed in developing thymocytes, raising the possibility that this latter kinase might compensate for ZAP-70 during early thymopoiesis in ZAP-deficient mice. Early support for this notion has come from the finding that stimulation of a pre-TCR-expressing, immature T-cell line with anti-CD3ε-specific antibodies resulted in CD3ζ association and phosphorylation of both ZAP-70 and Syk (van Oers *et al.*, 1995). Syk-deficient mice die *in utero* or around birth, partly because of severe hemorrhage, but thymopoiesis is not noticeably effected, both in syk$^{-/-}$ mice and in radiation chimeras reconstituted with syk$^{-/-}$ fetal liver cells (Cheng *et al.*, 1995; Turner *et al.*, 1995). To uncover whether these two syk family kinases play complementary roles in pre-TCR signaling or whether they are both dispensable for pre-TCR function, ZAP$^{-/-}$ $\times$ syk$^{-/-}$ doubly deficient mice have been generated (Cheng *et al.*, 1997). The analysis of these double mutants revealed a complete arrest of thymopoiesis at the CD25^{+}CD44$^{-/low}$ stage, exactly as in RAG$^{-/-}$ or CD3$^{-/-}$ mice. Although V(D)Jβ rearrangements were shown to occur and TCRβ/CD3 complexes were found on the cell surface of DN thymocytes at low levels, DP cells were not generated and the total number of thymocytes remained as low as in RAG-deficient mice, indicating that the expression of a pre-TCR could no longer induce proliferation or differentiation of CD25^{+} pre-T cells. These data provide convincing evidence that ZAP-70 and Syk fulfill overlapping functions in the differentiation of DN to DP

cells, most likely as components of the pre-TCR signal transduction machinery.

b. GTPase p21^{ras}. The GTP-binding protein p21^{ras} defines a point of convergence for many tyrosine kinase-induced pathways. Numerous studies performed in cell lines and in transgenic animals have indicated a crucial role of Ras in T-cell activation (reviewed in Izquierdo Pastor *et al.*, 1995). Whether GTP-binding proteins are also involved in pre-TCR signal transduction has been studied in several interesting experimental approaches. Swat and colleagues (1996) introduced into RAG-1$^{-/-}$ ES cells an expression construct encoding constitutively activated Ha-ras under the control of the proximal lck promoter. Ras-transfected or nontransfected control ES cells, both lacking RAG-1, were then injected into blastocysts derived from homozygously RAG-2-deficient mice. Expression of activated Ras in RAG$^{-/-}$ pre-T cells was sufficient to induce the generation of normal numbers of DP thymocytes, despite the absence of a functional pre-TCR, demonstrating that activated Ras can fully mimic pre-TCR-derived signals (Swat *et al.*, 1996). Does p21^{ras} play a physiological role in pre-TCR signal transduction? To assess the importance of endogenous p21^{ras} in the control of thymocyte development, mice overexpressing a dominant-negative Ha-ras protein, also under the control of the lck proximal promoter, were generated and analyzed (Swan *et al.*, 1995). Although dominant-negative p21^{ras} severely inhibited positive selection and blocked TCR-mediated T-cell activation, it had no negative effects on early thymopoiesis or "β selection," as deduced from the normal number of DP thymocytes and the pattern of TCRβ staining on these DP cells. Considering the profound inhibitory effects of dominant-negative Ras on T-cell activation and positive selection and the fact that the transgene was expressed at even higher levels during early thymopoiesis, the results seemed to argue against an important role of endogenous Ras in pre-TCR-mediated events. However, at least two alternative explanations for this negative finding are conceivable, which are, in fact, by inference valid for many experiments involving dominant-negative transgenes. First, thymocytes express primarily N-ras and Ki-ras. It is therefore possible that dominant-negative Ha-ras may have failed to inhibit signaling processes mediated by other Ras isoforms. Second, it cannot be excuded that in mice expressing dominant-negative Ha-ras, residual endogenous Ras activity has been sufficient for p56^{lck}-mediated maturation events, but not for positive selection or T-cell activation. The question whether Ras participates in pre-TCR signaling therefore remains unanswered.

c. GTPase Rho. Several studies have suggested that members of another group of GTPases, the Rho family of proteins, have important signal-

ing functions in hematopoietic cells (discussed in Henning *et al.*, 1997). Interestingly, Rho proteins are inactivated by the enzyme C3 from the bacterium *Clostridium botulinum,* which selectively ADP-ribosylates Rho, thereby abolishing its biological function (Aktories and Just, 1995). To block Rho activity in thymocytes and thus assess its role in thymopoiesis, Henning and colleagues (1997) generated transgenic mice expressing the bacterial ADP-ribosylase C3 under the proximal lck promoter. The analysis of adult C3 transgenic mice revealed that although all major thymocyte subsets were present, total thymic cellularity was reduced by 90%. Cell cycle analysis indicated a severe reduction in the number of dividing thymocytes. Moreover, SP, DP, and all TN subsets, except the earliest $CD25^-CD44^+$ population, were significantly reduced. The phenotype therefore resembled that seen in mice lacking components of the IL-7 receptor signaling complex, suggesting that Rho was involved in the IL-7 signaling pathway (see Section V). The differentiation of TN thymocytes to DP cells and the proliferative expansion of late stage TN cells, controlled by the pre-TCR, were also severely inhibited, indicating a potential requirement for Rho in pre-TCR-mediated proliferative signals as well. These initial findings were substantiated in a subsequent study focusing on fetal thymopoiesis in the same C3 transgenic mouse strain (Galandrini *et al.*, 1997). The analysis of T-cell development in fetal thymi revealed even more dramatic defects than those seen previously in adult mice. Development of DP and SP thymocytes was completely abrogated until about 4 days after birth, when small numbers of these cells started to appear for the first time. Thymocyte numbers were severely reduced in all TN subsets following the $CD25^-CD44^+$ stage. Determination of the cell cycle status and of the percentage of apoptotic cells in each TN compartment revealed two discrete functions of Rho during early thymopoiesis. First, Rho was required for the survival of embryonic and adult $CD25^+CD44^+$ and $CD25^-CD44^{-/low}$ cells, but not for the cell cycle progression of these subpopulations. In line with this finding, expression of a bcl-2 transgene in C3 transgenic mice could reconstitute the $CD25^+$ TN subsets. Second, Rho function was shown to be required for cell cycle progression of $CD25^-CD44^{-/low}$ late pre-T cells, but not for the survival of this population. These findings have established distinct roles for Rho in the two key signaling pathways of early thymopoiesis, those emanating from the IL7 and/or c-kit/SCF receptor system and those emanating from the pre-TCR. Interestingly, activation of mature T cells and positive as well as negative selection of DP thymocytes were uncompromised in mice lacking Rho function (Henning *et al.*, 1997). The GTPases Rho and Ras therefore clearly act at different stages in T-cell development and T-cell function.

d. MAP Kinase Pathway. In metazoans, the transmission of signals from GTP-bound Ras to the cell nucleus is thought to occur predominantly via a protein serine/threonine kinase pathway, known as the mitogen-activated protein (MAP) kinase cascade (reviewed in Marshall, 1994). At the end of this pathway are MAP kinases, such as ERK-1 (extracellular signal-regulated kinase-1) and ERK-2, which, on activation, are translocated into the nucleus and control the activity of specific transcription factors via phosphorylation. The MAP kinases themselves are activated by phosphorylation mediated by MAP kinase kinases (MAPKK), such as MEK-1 (MAPK and ERK kinase-1) and MEK-2. These, in turn, are controlled by a MAP kinase kinase kinase (MAPKKK), which appears to be a direct target of activated Ras. In some cells, the kinase Raf-1 has been identified as the MAPKKK that is critically involved in coupling $p21^{ras}$, and hence receptors that act via $p21^{ras}$, to the MAP kinases (Howe *et al.,* 1992). GTP-bound Ras recruites Raf to the cell membrane, where the latter becomes activated by an unknown mechanism, possibly involving phosphorylation of a critical tyrosine residue (Van Aelst *et al.,* 1993; Vojtek *et al.,* 1993; Zhang *et al.,* 1993).

Several experiments have been performed to determine whether the MAP kinase cascade is involved in pre-TCR signal transduction. To assess the role of Raf in thymopoiesis, O'Shea and colleagues (1996) generated mice expressing either a dominant-negative or a constitutively active form of Raf under the control of the lck proximal promoter. The expression of activated Raf appeared to enhance the differentiation from DP to SP thymocytes. Conversely, dominant-negative Raf inhibited the generation of SP thymocytes from DP precursors and also impaired positive selection of thymocytes expressing a transgenic TCR (O'Shea *et al.,* 1996). However, expression of both transgenic Raf constructs had no obvious effect on pre-TCR-mediated events, suggesting that Raf is probably not involved in pre-TCR signaling. To assess the role of a more downstream component of the MAP kinase pathway, Alberola-Ila and colleagues (1995) generated transgenic mice expressing a dominant-negative form of the MAP kinase kinase MEK-1, again under the control of the thymocyte-specific lck proximal promoter. Although the MAP kinase pathway was efficiently blocked, as revealed by measuring *in vitro* phosphorylation of an appropriate substrate of MAP kinase, thymi in dominant-negative MEK-1 transgenic mice displayed a normal cellularity. Further analysis revealed that positive selection and the DP to SP transition were impaired, but not negative selection or pre-TCR-mediated events. In fact, the defect in thymopoiesis in mice expressing dominant-negative MEK-1 was very similar to the defect in thymopoiesis observed in mice bearing a dominant-negative ras transgene (Swan *et al.,* 1995), as expected if MEK-1 was a downstream element of

a signal transduction cascade controlled predominantly by Ras. Taken together, data obtained with transgenic mice therefore failed to provide any evidence for an involvement of the MAP kinase pathway in pre-TCR-controlled proliferation and maturation.

Different results were obtained in a study involving retrovirus-mediated gene transfer in fetal thymic organ culture. Using this more complex system, it was shown that dominant-negative MEK-1 inhibited the transition of TN thymocytes to the DP stage in fetal thymi from TCRα-deficient mice (Crompton, 1996), which were used instead of normal mice to limit possible CD3-mediated signaling to the pre-TCR, avoiding potential interference from mature $\alpha\beta$ TCR-derived signals. In the same study, dominant-negative MEK-1 also inhibited anti-CD3-induced thymocyte development in thymi from RAG-deficient mice. However, neither constitutively active MEK-1 nor constitutively active Ras was able to induce progression from TN to DP thymocytes in fetal thymi from RAG$^{-/-}$ mice, although previous data (Swat *et al.*, 1996) obtained with Ras-transfected ES cells in the "RAG complementation assay" described earlier had clearly shown that activated Ras can mediate this developmental transition very efficiently. At present, it is not clear why retrovirally transferred dominant-negative MEK-1 could inhibit pre-TCR-induced processes in FTOC, whereas a dominant-negative MEK-1 transgene, although highly expressed, was unable to do so. The apparent discordance could have a number of interesting reasons, e.g., the difference in the type of dominant-negative mutation introduced into MEK-1 in both experiments, potential differences in pre-TCR signal transduction pathways in fetal versus adult thymopoiesis, or construct-related differences in both experimental systems affecting the onset of dominant-negative MEK-1 expression relative to p56^{lck} expression (Crompton, 1996). Whatever the reason for the discrepancy, the potential role of the MAP kinase pathway in pre-TCR signal transduction is not yet fully understood.

e. Cyclic AMP. The second messenger cyclic AMP (cAMP) has been shown to repress the MAP kinase pathway in several distinct receptor/signal transduction systems (Burgering and Bos, 1995, and references therein). It has been suggested that a cAMP-dependent pathway can also interfere with signals delivered through the pre-TCR–CD3–p56^{lck} complex. Treatment of FTOCs with cell-permeable cAMP analogs mimicking the physiological activity of cAMP severely impaired the maturation of TN thymocytes and gave rise to a developmental profile that resembled the pattern found in TCRβ-deficient mice (Lalli *et al.*, 1996). Compounds that selectively activate β-adrenergic receptors, which are abundant on DN thymocytes, were found to produce the same effect. These results suggested that membrane receptors, which are able to activate or repress

adenylate cyclase activity, can modulate pre-TCR-mediated signals. It remains to be seen, however, whether these are purely pharmacological effects or whether they can also be observed in mice that have genetic alterations affecting cAMP signaling pathways in early thymocytes.

f. Calcium/Calcineurin Pathway. Activation of mature T cells cannot be induced via a single signaling pathway but requires the combined action of several pathways that integrate at the level of transcription factor activation (reviewed in Cantrell, 1996). One such pathway involves intracellular calcium (Ca^{2+}) as a second messenger. Triggering of the pre-TCR in a pre-T cell line with anti-TCRβ or anti-CD3ε-specific antibodies has been shown to induce a sustained flux of Ca^{2+} (Groettrup *et al.*, 1992). However, it is not yet clear whether Ca^{2+}-mediated signals constitute an obligatory branch in pre-TCR signal transduction. The best characterized downstream effector of calcium signals in T-cell activation is the calcium/calmodulin-dependent serine phosphatase calcineurin (reviewed by Alberola-Ila *et al.*, 1997). To assess the role of calcineurin in T-cell development, mice have been injected with cyclosporin A or FK506, two potent inhibitors of calcineurin. Whereas daily treatment with these drugs for 1 to 7 weeks efficiently inhibited positive selection and the development of SP thymocytes and mature T cells, there was no or only a very mild negative effect on the TN to DP transition (Gao *et al.*, 1988; Jenkins *et al.*, 1988; Wang *et al.*, 1995). These results suggest (1) that the Ca^{2+}/calcineurin pathway does not play an essential role in pre-TCR signaling or (2) that this pathway, although operating, is much less sensitive to inhibition than in later stages of T-cell development.

6. *Nuclear Targets of the Pre-TCR Signal Transduction Machinery*

The ultimate result of pre-TCR signaling is a change in gene expression patterns brought about by a modulation of the activity of transcription factors that control survival, proliferation, and/or differentiation of $CD25^+$ pre-T cells. No single transcription factor whose activity is controlled specifically by the pre-TCR has yet been identified with certainty. However, experiments with transgenic and gene knockout mice have provided two or three potential candidates. For instance, the fact that the absence of the p53 tumor suppressor gene on a $RAG^{-/-}$ background leads to the generation of DP thymocytes (Jiang *et al.*, 1996) could indicate a negative influence of pre-TCR signaling on p53 activity. Analysis of the expression pattern of the zinc finger transcription factor egr-1 has revealed higher levels of this transcriptional regulator in $CD25^-CD44^{-/low}$ compared to $CD25^+CD44^{-/low}$ TN thymocytes (Miyazaki, 1997). Overexpression of transgenic egr-1 in thymocytes of $RAG^{-/-}$ mice has been shown to result in the

generation of $CD8^+$ immature single positive (ISP) cells (Miyazaki, 1997), suggesting that upregulation of egr-1 can mimic pre-TCR signaling. However, egr-1 is not essential for T-cell development, as egr-deficient mice do not exhibit any defects in thymopoiesis (Lee *et al.*, 1995; Miyazaki, 1997). Interestingly, egr-1-overexpressing thymocytes did not proceed to the DP stage, but maturation stopped at the ISP stage, suggesting that additional factors were required to fully simulate pre-TCR-mediated maturation. Downregulation of CD25 and upregulation of CD5, two early differentiation events typical for the TN to DP transition, have also been reported in $RAG^{-/-}$ mice overexpressing the transcription factor Bmi-1 (Alkema *et al.*, 1997). Bmi-1 is another zinc finger-containing transcriptional regulator that was originally identified as a cofactor in c-Myc-mediated lymphomagenesis.

The characterization of genes that are specifically induced or suppressed on pre-TCR signaling is an important future goal. Injection of anti-CD3ε-specific antibodies into RAG-deficient mice as a model for pre-TCR signaling, in combination with new and powerful subtractive screening methods that have become available, should provide a number of candidate genes. The expression of some of these genes may turn out to be directly controlled by pre-TCR activity.

V. Growth/Differentiation Factors in Early Thymocyte Development

A. General Considerations

In addition to direct cell–cell contact between thymocytes and thymic stromal cells, soluble factors have long been implicated in the process of thymocyte development. This assumption was primarily based on the fact that thymocytes themselves and thymic stromal cells can produce cytokines (Fischer *et al.*, 1991; Wiles *et al.*, 1992; Wolf and Cohen, 1992; Moore *et al.*, 1993; Kelner *et al.*, 1994; Kelner and Zlotnik, 1995; Mossalayi *et al.*, 1995). In addition, thymocytes express constitutively or inducibly several cytokine receptors *in vivo* and are responsive to many growth factors *in vitro* (reviewed by Zlotnik and Moore, 1995). Before the advent of targeted mutants, the potential actions of cytokines and their receptors was analyzed by the addition of exogenous growth factors either to isolated thymocyte subsets in culture or to thymic organ culture systems (see earlier). It was only realized later, much to a surprise, that the vast majority of mutant mice lacking specific cytokines failed to reveal any obvious thymic phenotype as a result of the loss of growth factors or their receptors in the thymus.

B. Cytokines Not Affecting Thymocyte Development

To date, mice lacking the following cytokines, cytokine receptors, or cytokine-related gene products have been generated with no apparent

alteration of thymocyte development: IL-1β (Zheng *et al.*, 1995; Shornick *et al.*, 1996), IL-1 receptor (IL-1 R) (M. Labow, personal communication), IL-1β converting enzyme (ICE) (Kuida *et al.*, 1995; Li *et al.*, 1995), IL-2 (Schorle *et al.*, 1991), IL-2 R α chain (Willerford *et al.*, 1995), IL-2 R β chain (Suzuki *et al.*, 1997), IL-3 (Nishinakamura *et al.*, 1996), IL-4 (Kuhn *et al.*, 1991; Kopf *et al.*, 1993). IL-2 and IL-4 (Sadlack *et al.*, 1994), IL-5 (Kopf *et al.*, 1996), IL-8 (Cacalano *et al.*, 1994), IL-10 (Kuhn *et al.*, 1993), IL-12 p40 and p35 (Magram *et al.*, 1996; Mattner *et al.*, 1996), IL-12 R β 1 chain (J. Magram, personal communication), IL-13 (R. Murray, personal communication), tumor necrosis factor (TNF) R 1 (p55) (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993), TNF R 2 (p75) (Erickson *et al.*, 1994), lymphotoxin (De Togni *et al.*, 1994), TNF and lymphotoxin (Eugster *et al.*, 1996), transforming growth factor (TGF) β 1 (Shull *et al.*, 1992; Kulkarni *et al.*, 1993), interferon γ (Dalton *et al.*, 1993; Huang *et al.*, 1993), granulocyte/macrophage (GM) colony-stimulating factor (CSF) (Stanley *et al.*, 1994; Wada *et al.*, 1997), G-CSF R (Liu *et al.*, 1996), the common β subunit for receptors for IL-3, GM-CSF, and IL-5 (Nishinakamura *et al.*, 1995), and fetal liver kinase-2 (flk-2) (Mackarehtschian *et al.*, 1995).

C. Cytokines Affecting Thymocytes Development

In contrast to this long listing of cytokines apparently irrelevant for thymocyte development, lack of very few growth/differentiation factors was shown to affect the generation of thymocytes.

1. IL-6-Related Cytokine Family

Six distinct cytokines sharing structural and functional properties belong to the IL-6-related cytokine subfamily. These are IL-6, IL-11, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotropic factor, and cardiotrophin-1 (for further references, see Yoshimura *et al.*, 1996; Taga and Kishimoto, 1997). IL-6 related cytokines bind to cytokine receptors utilizing a common signal transducing receptor subunit termed gp130 (Gearing *et al.*, 1992; Taga and Kishimoto, 1997). In the case of IL-6, gp130 forms, together with the IL-6 R α chain (gp80), a functional IL-6 R. LIF binds with high affinity to a receptor complex formed from the LIF R and gp130 (reviewed by Taga and Kishimoto, 1997). With low affinity, LIF binds to the LIF R in the absence of gp130. OSM may also bind to LIF R/gp130 or to the OSM R/gp130 complex, but OSM can also bind to gp130 in the absence of another receptor molecule (Gearing *et al.*, 1992; Taga and Kishimoto, 1997).

Mice lacking IL-6 (Kopf *et al.*, 1994), LIF (Escary *et al.*, 1993), or the gp130 receptor subunit (K. Yoshida *et al.*, 1996), as well as transgenic mice expressing in T lineage cells LIF (Shen *et al.*, 1994) or OSM (Clegg *et al.*,

1996), have been generated. Interestingly, evidence has been derived from these mutants to suggest that at least two cytokines of the IL-6 family, i.e., OSM and LIF, can be involved, in addition to affecting the HSC compartment, in thymopoiesis. Data regarding the roles of IL-6, OSM, and LIF in T-cell development are summarized next.

a. IL-6. Numbers of thymocytes (and peripheral T cells) are reduced by 20–40% in IL-6-deficient mice (Kopf *et al.*, 1994) with normal expression of TCR, CD4, CD8, CD44, and HSA. Thus, IL-6 may provide proliferative signals to thymocytes and mature T cells, but the precise stage of action of IL-6 is not known. However, it should be kept in mind that IL-6 was also shown to be involved in survival or maintenance of HSC and/or committed progenitors as revealed by competitive reconstitution assays *in vivo* (Bernad *et al.*, 1994). Thus, the reduced number of thymocytes in IL-6 deficient mice may reflect a "HSC phenotype" of the IL-6 mutant rather than lack of intrathymic proliferation.

b. Leukemia Inhibitory Factor. LIF can affect the development and proliferation of various cell types, including hematopoietic cells (reviewed by Hilton and Gough, 1991). However, expression of LIF is dispensable for mouse development, as shown by the fact that LIF-deficient mice developed normally (Escary *et al.*, 1993). Within the hematopoietic system, however, mice lacking LIF showed clear alterations: Numbers of both CFU-S_{day12} as well as committed precursors for erythroid (BFU-E) and myeloid (GM-CFC) lineages were reduced ~10-fold in bone marrow and spleen compared to wild-type mice. In contrast, total cell numbers in bone marrow, spleen, and thymus were roughly normal, indicating that later hematopoietic compartments were "filled up" to compensate for the lower numbers of early progenitors in LIF-deficient mice. In contrast, transplantation of $LIF^{-/-}$ HSC into wild-type mice revealed normal donor-type reconstitution of hematopoietic lineages. Thus, LIF can act as an environmental (stroma)-derived factor to support the maintenance of the HSC compartments *in vivo*. These experiments give no indication that LIF is involved in early thymocyte development; however, thymocytes derived from $LIF^{-/-}$ or even $LIF^{+/-}$ mice responded at a much reduced level to mitogenic or allogeneic stimulation (Escary *et al.*, 1993).

In addition to pleiotropic alterations caused by LIF overexpression, such as splenomegaly, acute-phase response, and extramedullary hematopoiesis, dramatic effects were recognized in T-cell tissues (thymus and lymph nodes) in mice overexpressing transgenic LIF under the control of a T lineage-specific expression cassette (Shen *et al.*, 1994). Remarkably, flow-cytometric analysis revealed an apparent interconversion of phenotypes

between lymphocytes in thymus and mesenteric lymph nodes, i.e., $CD4^+CD8^+$ thymocytes were absent from the thymus, but $CD4^+CD8^+$ lymphocytes were found in the lymph nodes. The thymus contained only lymphocytes with a mature $CD4^+CD8^-$ and $CO4^-CD8^+$ single positive phenotype. The thymic architecture was entirely disrupted in LIF transgenic mice, such that epithelial cells apparently formed B-cell follicles but failed to reveal a normal medulla–cortex organization. Collectively, although LIF is dispensable for the generation of thymocytes (Escary *et al.*, 1993), its overexpression in T cells can cause an unprecedented conversion of phenotypes between thymus and lymph nodes (Shen *et al.*, 1994).

c. Oncostatin M. OSM is expressed in both hematopoietic lineages and stromal cells. OSM can inhibit proliferation or alter the morphology of various tumor cell types. Moreover, OSM can regulate cytokine production and is itself an immediate early gene induced by the JAK-STAT5 signal transduction pathway (for further references see Malik *et al.*, 1995; Yoshimura *et al.*, 1996). Expression of bovine OSM in transgenic mice under the control of the proximal lck promoter, which drives T-cell-specific expression, resulted in a phenotype resembling some findings reported for LIF transgenic mice (see earlier) (Malik *et al.*, 1995). In addition to abnormal bone growth and spermatogenesis, OSM transgenic mice displayed splenomegaly and, notably, a disrupted thymic architecture. Like LIF transgenic mice, mice overexpressing OSM showed ectopic, follicle-associated B cells in the thymus, a structure incompatible with normal medulla-cortex organization. Expression of OSM as a transgene, as well as infusion of recombinant OSM protein, can induce extrathymic T-cell development in mesenteric lymph nodes (Clegg *et al.*, 1996). Expression of early T lineage-specific mRNA encoding $pT\alpha$, as well as the presence of $CD4^+CD8^+$ lymphocytes in the lymph nodes of OSM transgenic mice, indicated "extrathymic (lymph node-associated) thymopoiesis." Moreover, OSM could induce the generation of mature T cells in athymic nude mice, and those extrathymically derived T cells behaved normally in tumor rejection experiments.

Although OSM-deficient mice have not yet been reported, mice lacking the common receptor subunit (gp130), which should lack high-affinity binding receptors for both OSM and LIF, have been generated (K. Yoshida *et al.*, 1996). These mice show very severe defects in multiple organs, causing prenatal death. The thymus size is greatly reduced in these mutants, but it is not known whether this is due to the strong reduction in hematopoietic precursor numbers as measured by CFU-S, BFU-E, and GM-CFC or whether lack of gp130 has a direct effect on thymic epithelium.

The molecular basis of these interesting phenomena remains to be determined. It is not known whether LIF and OSM act on thymic and lymph node epithelium directly or induce such functionally dramatic alterations in these tissues indirectly, e.g., via soluble factors generated on stimulation by LIF and OSM by another cell type. Nevertheless, the findings that LIF and OSM can induce a functional "thymic environment" ectopically in lymph nodes may open new avenues to examine the basis of epithelial cell-supported thymocyte development. In particular, insights into the mechanism of colonization of ectopic "thymic sites" by circulating precursors and the earliest events calling for induction of expression of the genes encoding RAG and pTα will be of interest.

It is remarkable that OSM-induced ectopic thymopoiesis is permissive in mice lacking the nude gene, the product of which is mandatory for the formation of a functional thymus from the thymus anlage *in vivo*. At present, the function of the nude locus-encoded winged-helix nude (whn) transcription factor for the development of the thymic epithelium is enigmatic (Nehls *et al.*, 1994). Moreover, it is not known whether whn is required primarily in thymus development or continues to be required for the maintenance of a functional thymic stroma once the thymus structure is established. By introducing a lacZ reporter gene into the nude locus, Nehls *et al.* (1996) showed that, in heterozygous mice (whn^{wt}/whn^{lacZ}), whn is also expressed in adult thymic stroma. If whn expression is essential for the function of the thymic environment in the adult, it should be surprising that an ectopic "thymus-like" environment is inducible by OSM or LIF in the absence of whn expression.

In a different experiment, i.e., overexpression of an IL-7 transgene in nude mice, functional T cells were also recovered (Rich and Leder, 1995). However, in this report, the site of T-cell production was not localized, and it seems possible that in this IL-7 transgenic nude mouse, IL-7-driven expansion acts on the low number of T cells present even in nude mice rather than stimulating *de novo* "thymopoiesis" in ectopic sites.

2. *IL-7*

Interactions of IL-7 (Namen *et al.*, 1988) with components of the IL-7 receptor complex are clearly involved in early T- and B-cell development. Indeed, of all cytokines examined so far, IL-7 appears to be the most important one. The IL-7 receptor complex contains the IL-7 R α chain (Goodwin *et al.*, 1990) and the common cytokine receptor γ chain (γ_c); the latter also participates in the formation of receptors for IL-2, IL-4, IL-9, and IL-15 (Noguchi *et al.*, 1993a; Kondo *et al.*, 1994; DiSanto *et al.*, 1995a; Leonhard *et al.*, 1995; Sugamura *et al.*, 1995). Germline mutations

in γ_c are a frequent cause of X-linked immunodeficiency in humans (Noguchi *et al.*, 1993b; Puck *et al.*, 1993; DiSanto *et al.*, 1994b; Fisher, 1996).

a. Expression of IL-7, IL-7 R α, and IL-7 γ_c. Intrathymically, both IL-7 R α and γ_c are expressed already at the DN stage in the thymus. DP thymocytes lack IL-7 R α, but mature CD4 and CD8 single positive thymocytes reexpress this chain (Sudo *et al.*, 1993; DiSanto *et al.*, 1994a; Hozumi *et al.*, 1994b). It is not known where precisely IL-7 R α expression is lost at the transition from the DN to the DP stage and whether this event is correlated with successful rearrangement of the TCR β chain. The finding that even fetal blood-derived pro-thymocytes (defined by a c-kit^{low}Thy-1$^+$CD44$^+$CD25$^-$ phenotype), but not fetal blood-derived HSC (c-kit$^+$Thy-1$^-$), are IL-7 R α^+ (Rodewald, 1995) suggests that, within the DN thymocyte compartment, IL-7 R α expression should be found as early as at the CD44$^+$CD25$^-$ pro-thymocyte stage. In agreement with these findings is an analysis for the expression of mRNAs encoding IL-7 R α and γ_c in purified bone marrow and thymocyte subpopulations (Orlic *et al.*, 1997). C-kit$^+$ bone marrow cells lacking lineage markers (lin$^-$) (a population containing uncommitted stem cells and potentially nonlymphocyte lineage-committed precursors), as well as DN thymocytes and pro-B cells, expressed γ_c mRNA. In contrast, IL-7 α mRNA, also present in DN thymocytes and pro-B cells, was not detected in c-kit$^+$lin$^-$ bone marrow cells. Thus, expression of the IL-7 R α chain, but not of the common cytokine receptor γ chain, is one of the earliest molecular markers of hematopoietic cells entering the lymphoid lineage pathways. In HSC and in committed precursors for nonlymphoid lineages, expression of γc-associated cytokine receptors may not be critical, as γ_c-deficient mice lack a hematological phenotype other than in lymphoid lineages (see later). IL-7 mRNA is produced intrathymically (Wiles *et al.*, 1992; Moore *et al.*, 1993), and, within the thymus, the IL-7 protein is located preferentially to the subcapsular zone (Tsuda *et al.*, 1996).

b. Functional role of IL-7. Numerous studies have shown that fetal or adult thymocytes proliferate in response to IL-7 *in vitro* (Conlon *et al.*, 1989; Murray *et al.*, 1989; Okazaki *et al.*, 1989; Watson *et al.*, 1989; Suda and Zlotnik, 1991; Godfrey *et al.*, 1993; Zlotnik and Moore, 1995). Furthermore, the addition of mAb directed against IL-7, IL-7 R α, or γ_c suppresses thymocyte development partially or completely in suspension cultures, in FTOC, or *in vivo* (Sudo *et al.*, 1993; Hozumi *et al.*, 1994b; Bhatia *et al.*, 1995; Tsuda *et al.*, 1996; He *et al.*, 1997). Critical roles for IL-7, the IL-7 R α chain, and γ_c in thymocyte development were also found in gene targeting experiments, which clearly demonstrated that TCR $\alpha\beta$ thymocytes are generated at strongly (20- to 70-fold) reduced levels, in mice

lacking IL-7 (von Freeden-Jeffry *et al.*, 1995), the IL-7 Rα chain (Peschon *et al.*, 1994; Maki *et al.*, 1996a), or γ_c (Cao et al., 1995; DiSanto *et al.*, 1995b; Ohbo *et al.*, 1996).

Differential results were obtained when TCR $\gamma\delta$ thymocyte and NK cell development were compared among IL-7, the IL-7 R α and γ_c mutant mice: γ_c deficiency caused the complete loss of NK cells and $\gamma\delta$ T cells, whereas $\alpha\beta$-cell development was permissive at reduced levels comparable to mice lacking IL-7 or IL-7 R α (Cao *et al.*, 1995; DiSanto *et al.*, 1995b). In contrast, $\gamma\delta$ T cell but not NK cell development is abrogated entirely in IL-7 R α chain- or IL-7-deficient mice, whereas $\alpha\beta$ T-cell development is reduced comparably in all mutants (He and Malek, 1996; Maki *et al.*, 1996a; Moore *et al.*, 1996).

c. Is IL-7 Involved in the TCR Rearrangement Process?

i. Experiments Addressing This Issue. Because IL-7 is a major growth factor for early thymocytes and IL-7 receptors are expressed at the earliest intrathymic stages showing signs of T lineage commitment, a stage preceding and leading to the recombination of TCR loci, many investigators have attempted to correlate the action of IL-7-mediated signaling with induction or regulation of TCR gene rearrangements in thymocytes. In two reports, putatively uncommitted fetal liver cells defined by the phenotype c-kit$^+$CD45$^+$ (Oosterwegel *et al.*, 1997) or total fetal liver cells (Tsuda *et al.*, 1996) were placed in suspension culture, or FTOC (Tsuda *et al.*, 1996) or RFTOC built up from selected primary thymic epithelial cells (Oosterwegel *et al.*, 1997). Subsequently, the cultures were scored for the presence of TCR β chain rearrangements by PCR. Tsuda and colleagues (1996) reported that both TCR β chain DJ and V(D)J rearrangements were generated from fetal liver cells in FTOC after 6 days. In contrast, in fetal liver cell suspension cultures supplemented with exogenous IL-7, only TCR β chain DJ rearrangements were detectable. Oosterwegel *et al.* (1997) found that TCR β and δ chain V(D)J rearrangements were generated in RFTOC composed of MHC class II$^+$, but not MHC class II$^-$, or IL-7-expressing, but not IL-7-deficient thymic epithelium. However, these exact conditions (MHC class II$^+$, but not MHC class II$^-$ thymic epithelium) were previously shown to be essential for thymocyte development to occur at all in RFTOC (Anderson *et al.*, 1993).

Maki *et al.* (1994b) attributed the lack of $\gamma\delta$, but not $\alpha\beta$ T-cell development in IL-7 R α chain-deficient mice to the inability to rearrange TCR γ loci. Because these mice lack $\gamma\delta$, but not $\alpha\beta$ T cells, this analysis obviously focused on $\gamma\delta$ rearrangements derived from $\alpha\beta$ T cells or from TCR$^-$ pro T cells and suggested that IL-7 acts on the recombination at the TCR γ locus. This result is in agreement with several reports implicating IL-7 mediated signaling

in the rearrangement process. However, in an analysis of TCR rearrangements in γ_c-deficient mice, TCR γ ($V_{\gamma 2}J_{\gamma 1}$) and TCR δ ($V\delta_1(D\delta)J\delta_2$) rearrangements were indeed present in both wild-type and mutant mice. Moreover, sequencing of the junctions revealed a normal level of diversity at these TCR γ and TCR δ joints in γ_c^- thymocytes (Tables I and II) (Rodewald and Haller, 1998) (see also later). These results clearly showed that γ_c-mediated signaling was not required to obtain diverse TCR γ rearrangements. If this discrepancy in data is not explained by differences in the sensitivity of the analyzes, it has to be assumed that lack of IL-7 R α causes a different phenotype than lack of γ_c. Incorporation of γ_c into the IL-7 R complex augments the IL-7-binding affinity (Noguchi *et al.*, 1993a; Kondo *et al.*, 1994). Nevertheless, signal transduction via the IL-7 R α chain in the absence of γ_c may cause signals qualitatively different from γ_c-mediated signals. In addition, ligands other than IL-7 [e.g., TSLP (Peschon *et al.*, 1994)] may bind the IL-7 R α chain and signal differentially from IL-7.

ii. Conclusions. The molecular basis of the developmental control of the recombination reaction, including opening of the TCR loci and induction of transcription of the RAG genes, remains elusive. Because of the nature of the experiments involved, i.e., coculture of progenitors and stromal elements, it is difficult to conclusively separate effects of growth factors (here IL-7) on cellular expansion vs survival vs putative direct effects on recombination. Therefore, in these experiments one cannot rule out the possibility that IL-7 drives thymocytes from an earlier into a more advanced stage of development in which, by an intrinsic program or by exogenously provided soluble or stroma-bound factors, the rearrangement process is initiated and maintained until successfully completed. This consideration implies that cell populations were compared that are most likely not comparable, as they represent different stages of development. To directly link IL-7 signaling with antigen receptor rearrangements would require to separate, experimentally, proliferation/survival from differentiation in lymphocyte development. In this context, one should also reemphasize the inverse correlation between cell cycle progression and RAG expression (reviewed by Lin and Desiderio, 1995; Desiderio *et al.*, 1996; Grawunder *et al.*, 1996). Rearrangement of TCR and Ig loci involves DNA cleavage at recombinational signal sequences. This process uses components of the general machinery used for double-strand break repair such as DNA-dependent protein kinase (DNA-PK) (Jeggo, 1997). If V(D)J recombination took place independently from the cell cycle phase, double-strand breaks would occur frequently during DNA replication. This, in turn, would be prohibited in order to prevent genomic instability. *In vivo,* RAG genes are transcribed in two waves during early T- and B-cell development (Wilson *et al.*, 1994; Grawunder *et al.*, 1995). Particularly for early B-

TABLE I

LACK OF GROWTH FACTOR RECEPTORS [C-kit AND THE COMMON CYTOKINE RECEPTOR γ CHAIN (γ_c)] BLOCKS T-CELL DEVELOPMENT BEFORE DIVERSITY AT TCRγ LOCI CAN BE GENERATED[a]

Germline: $V_{\gamma 2}$ $J_{\gamma 1}$	V CTGTTCCTACGGCTAAAG	N	J ATAGCTCAGGTTTT		F^b
c-kit$^-\gamma_c^-$ (Experiment 1)	CTGTTCCTACGGCTAA	CCTTGGG	ATAGCTCAGGTTTT	(6×)	−
c-kit$^-\gamma_c^-$ (Experiment 2)	CTGTTCCTACGGCTAA		TAGCTCAGGTTTT	(6×)	−
c-kit$^+\gamma_c^-$ (Experiment 1)	CTGTTCCTACGGCTA		TAGCTCAGGTTTT	(3×)	+
	CTGTTCCTACGGCTAAAG	GC	ATATAGCTCAGGTTTT	(1×)	−
	CTCTTCCTACGGCTAA	GGG	AGCTCAGGTTTT	(2×)	−
	CTGTTCCTACGGC		TAGCTCAGGTTTT	(1×)	−
c-kit$^+\gamma_c^-$ (Experiment 2)	CTGTTCCTACGGCT	T	ATATAGCTCAGGTTTT	(1×)	+
	CTGTTCCTACGGCTA		TAGCTCAGGTTTT	(1×)	+
	CTGTTCCTACGG	TCCC	ATATAGCTCAGGTTTT	(2×)	−
	CTGTTCCTACGGCTAAA	AGGG	ATATAGCTCAGGTTTT	(2×)	−
	CTGT		ATATAGCTCAGGTTTT	(1×)	−
c-kit$^+\gamma_c^+$	CTGTTCCTACGGCTAAAG		ATATAGCTCAGGTTTT	(1×)	−
	CTGTTCCTACGGC	AA	ATAGCTCAGGTTTT	(1×)	−
	CTGTTCCTACGGCTA	GA	AGCTCAGGTTTT	(1×)	−
	CTGTTCCTACGGCTA		ATAGCTCAGGTTTT	(2×)	−
	CTGTTCCTACGGCTA		TAGCTCAGGTTTT	(1×)	+

[a] Genomic sequences from PCR-amplified T-cell receptor junctions are shown for thymus DNA from c-kit$^-\gamma_c^-$, c-kit$^+\gamma_c^-$, or wild-type mice. TCR $V_{\gamma 2}J_{\gamma 1}$ rearrangements are depicted. Numbers shown in parentheses indicate frequencies of individual sequences among all sequences determined per mouse. Presumptive P (template- and TdT-independent) nucleotides are underlined (reprinted with permission from Rodewald and Haller, 1998). Lack of action of the common cytokine receptor γ chain alone clearly affects thymus cellularity (see text). Nevertheless, the observed TCR diversity in c-kit$^+\gamma_c^-$ mice is normal when compared to wild-type mice. This indicates that an apparently normal repertoire is still represented among fewer thymocyte in this mutant. In contrast, lack of diversity in c-kit$^-\gamma_c^-$ mice reflects the alymphoid thymus in such double mutants (see text). (Nomenclature according to Garman *et al.*, 1986.)

[b] Functional.

TABLE II

LACK OF GROWTH FACTOR RECEPTORS [C-kit AND THE COMMON CYTOKINE RECEPTOR γ CHAIN (γ_c)] BLOCKS T-CELL DEVELOPMENT BEFORE DIVERSITY AT TCRδ LOCI CAN BE GENERATED[a]

		V	N	D	N	J		F[b]
Germline:	$V_{\delta1}$	TGTGGGTCAGATAT						
	$D_{\delta2}$			ATCGGAGGGATACGAG				
	$J_{\delta1}$					CTACCGACAAA		
c-kit$^-\gamma_c^-$ (Experiment 1)		TGTGGGTCAGATAT		CGGAGGGA		TACCGACAAA	(6×)	−
c-kit$^-\gamma_c^-$ (Experiment 2)		TGTGGGTCAGATAT		CGGAGGG		CGACAAA	(11×)	−
c-kit$^+\gamma_c^-$		TGTGGGTCAGATAT		CGGAGGGATACGAG		CTACCGACAAA	(2×)	+
		TGTGGGTCAG	GTATC[c]	GAGGG	C	CTCTACCGACAAA	(1×)	−
		TGTGGGTCAGATAT		CGGAGGGATAC	AGGGCC	CCGACAAA	(1×)	+
		TGTGGGTCAGATA	CCTATT[c]	ATCGGAGGGATA		CGACAAA	(1×)	−
		TGTGGGTCAGAT		CGGAGGGATACGA		CCGACAAA	(1×)	+
		TGTGGGTCAGAT	CC	GAGGGATACGAG		CTACCGACAAA	(1×)	−
		TGTGGGTCAGAT		ATCGGAGGGATA		CCGACAAA	(2×)	−
c-kit$^+\gamma_c^+$		TGTGGGTCAGATAT		CGGAGGGATACGA		CAAA	(1×)	−
		TGTGGGTCAGATAT		ATCGGAGGGATA		TACCGACAAA	(1×)	+
		TGTGGGTCAGAT		AGGGAT	GAGCGA	CTACCGACAAA	(1×)	−
		TGTGGGTCAGATAT	CT	CGGAGGGATACGAG	CCCCCA	CTACCGACAAA	(1×)	−
		TGTGGGTCAGATAT	C	GGGATAC	AAG	CTACCGACAAA	(1×)	+
		TGTGGGTCAGA		GAGGGAT	G	CTACCGACAAA	(1×)	+
		TGTGGGTCAGATAT		CGGAGGGATACGAG		CTACCGACAAA	(1×)	+
		T		ATCGGAGGGATACGAG		CCGACAAA	(1×)	−

[a] Genomic sequences from PCR-amplified T-cell receptor junctions are shown for thymus DNA from c-kit$^-\gamma_c^-$, c-kit$^+\gamma_c^-$, or wild-type mice. TCR $V_{\delta1}D_{\delta2}J_{\delta2}$ rearrangements are depicted. Numbers shown in parentheses indicate frequencies of individual sequences among all sequences determined per mouse. Presumptive P (template- and TdT-independent) nucleotides are underlined (reprinted with permission from Rodewald and Haller, 1998). Lack of action of the common cytokine receptor γ chain alone clearly affects thymus cellularity (see text). Nevertheless, the observed TCR diversity in c-kit$^+\gamma_c^-$ mice is normal when compared to wild-type mice. This indicates that an apparently normal repertoire is still represented among fewer thymocyte in this mutant. In contrast, lack of diversity in c-kit$^-\gamma_c^-$ mice reflects the alymphoid thymus in such double mutants (see text).

[b] Functional.

[c] Possibly $D_{\delta1}$ derived.

cell development, it has clearly been demonstrated that RAG genes are expressed in resting cells, but not in cycling cells (Grawunder *et al.*, 1995). Because progenitor T and B cells proliferate in response to IL-7, growth factor withdrawal might be associated with rearrangement. In this scenario, IL-7 would expand certain progenitor populations before and after rearrangement, but not be involved in the rearrangement process itself. This proposal is supported by findings in an *in vitro* system supporting B-cell development where withdrawal, rather than addition of IL-7, is accompanied by active Ig rearrangements, a phenomenon more readily visible if cell death is prevented by overexpression of bcl-2 (Rolink *et al.*, 1993).

Collectively, despite the reports cited earlier and several other reports (Appasamy, 1992; Appasamy *et al.*, 1993; Muegge *et al.*, 1993; Maki *et al.*, 1996b), an involvement of IL-7 in the process of rearrangements has not been demonstrated conclusively. Thus, at present it appears safe to only conclude that IL-7 drives thymocyte expansion or serves as a survival factor *in vivo*. The latter possibility has received support from reports that introduction of a Bcl-2 transgene into an IL-7 R-deficient mouse rescues thymus cellularity to wild-type levels (Akashi *et al.*, 1997; Maraskovsky *et al.*, 1997).

3. *C-kit/Stem Cell Factor*

a. Genes and Mutants. C-kit, encoded at the W locus (Chabot *et al.*, 1988; Geissler *et al.*, 1988), is a receptor tyrosine kinase (Ullrich and Schlessinger, 1990; van der Geer *et al.*, 1994) widely expressed in HSC, committed hematopoietic progenitors, melanocytes, and germ cells. Soluble or transmembrane forms of stem cell factor, products of the steel locus (S1), have been identified as c-kit ligands (Anderson *et al.*, 1990; Copeland *et al.*, 1990; Flanagan and Leder, 1990; Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990; Flanagan *et al.*, 1991; reviewed in Fleischman, 1993; Galli *et al.*, 1994). Because W and S1 mutants reveal skin color phenotypes in the heterozygous state, a large number of mice bearing spontaneous point mutations or deletions at the W or S1 loci have been identified and analyzed. The most severe phenotypes are found in c-kit null (W/W; lethal in early postnatal life) and SCF null (S1/S1; lethal in late gestation) mice that bear mutations in c-kit and SCF genes, respectively, and entirely lack expression of c-kit or SCF proteins.

Before these loci and their products had been identified, transplantation experiments revealed that W mutations are intrinsic to melanocytes, germ cells, and HSC, whereas S1 mutants are defective in microenvironments that support development of those cell lineages (reviewed in Russell, 1979). In addition to their hematopoietic phenotypes, W or S1 mutants are characterized by developmental or functional abnormalities in other cell lineages,

including melanocytes (causing cutaneous white spots; H. Yoshida *et al.*, 1996), germ cells (causing sterility; Brannan *et al.*, 1992), and interstitial cells of Cajal, a gut associated pacemaker cell type of mesenchym origin (Lecoin *et al.*, 1996) (causing altered gut motility; Maeda *et al.*, 1992; Ward *et al.*, 1994; Huizinga *et al.*, 1995).

b. Expression of C-kit and SCF in the Thymus. C-kit serves as a very useful marker for early hematopoietic cells, including HSC, as shown by the fact that HSC activity, as revealed by multilineage developmental potential, long-term reconstitution, and spleen colony (CFU-S) formation, is exclusively contained within the c-kit$^+$ cell population in yolk sac, fetal liver, and bone marrow (Ogawa *et al.*, 1991, 1993; Ikuta and Weissman, 1992). In T lineage cells, c-kit is expressed on fetal blood-derived prothymocytes (Rodewald *et al.*, 1994) and in the most immature subsets of both fetal and adult intrathymic precursors. Specifically, c-kit expression has been reported on day 13 total fetal thymocytes (Godfrey *et al.*, 1992) and on fetal intrathymic stem cells defined as Thy-1^{low}CD44$^+$CD25$^-$ (Hozumi *et al.*, 1994a). In the adult thymus, the earliest intrathymic precursors, commonly referred to as the "CD4^{low}" population (Thy-1^{low}CD3$^-$CD4^{low} CD8$^-$CD44$^+$CD25$^-$Sca-2$^+$ phenotype) (Wu *et al.*, 1991b; Shortman and Wu, 1996) as well as their progeny, defined by the CD44$^+$CD25$^+$ phenotype, are c-kit$^+$ (Antica *et al.*, 1993; Godfrey *et al.*, 1993). C-kit expression appears to be lost in CD44$^-$CD25$^+$ thymocytes. Based on these expression studies and analysis of TCR rearrangements at various stages of development, c-kit is expressed in progenitor T cells before TCR V(D)Jβ chain rearrangements are detectable (Wu *et al.*, 1991b; Godfrey *et al.*, 1994; Hozumi *et al.*, 1994a; Shortman and Wu, 1996).

Transcripts encoding the c-kit ligand, SCF, are expressed, among other tissues, in thymic and bone marrow stromal cells (Zsebo *et al.*, 1990; McNiece *et al.*, 1991; Rolink *et al.*, 1991; Moll *et al.*, 1992; Wiles *et al.*, 1992; Williams *et al.*, 1992; Moore *et al.*, 1993). How important a growth factor/ligand pair is c-kit/SCF in the thymus?

c. Role of C-kit and SCF in Thymocyte Development. Evidence that SCF plays a role in the thymus came from morphological and quantitative analyses of SCF-mutant thymi *ex vivo,* following organ culture, and after transplantation of SCF-deficient fetal thymi into wild-type mice (Asamoto and Mandel, 1981). Fetal mice lacking SCF showed a three- to fourfold reduced number of total thymocytes. In addition, SCF$^{-/-}$ thymi harbored twofold fewer thymocytes when kept as FTOC or following grafting into wild-type (SCF-expressing) recipient mice (Asamoto and Mandel, 1981).

Analysis and comparison of both receptor (W/W) and ligand (Sl/Sl) deficient mice and of SCF-deficient or wild-type fetal thymi grafted into wild-type mice defined the phenotypic defects resulting from the lack of SCF/c-kit interactions in thymocyte development (Rodewald *et al.*, 1995). The size of the most immature thymocyte compartment was reduced ~40-fold in the W/W thymus and ~12-fold in the Sl/Sl thymus graft compared to wild-type levels. The fact that SCF-deficient thymus grafts were not colonized by SCF-producing (host-type) cells showed that, intrathymically, SCF is expressed strictly by thymic epithelial cells. Such grafting experiments are also informative because they provide a means to separate effects caused by lack of SCF in the thymus from effects at the HSC or prethymic (committed precursor) level. This is relevant, as it is well documented from both W/W and Sl/Sl mice (Russell, 1979; Ogawa *et al.*, 1991; Ikuta and Weissman, 1992) that absolute numbers of fetal liver HSC are reduced up to 10-fold.

Both by analysis of mitotic cells *in situ* (Asamoto and Mandel, 1981) and by bromodeoxyuridine labeling of thymocytes in SCF-deficient grafts *in vivo* (Rodewald *et al.*, 1995), lack of SCF was accompanied by impaired thymocyte proliferation. Accordingly, intrathymically expressed SCF was estimated to account for ~50% of the rate of expansion of the earliest intrathymic progenitors. The affected stage of development was characterized as a stage preceding TCR β chain V(D)J rearrangements (corresponding to a Thy-$1^{int}CD2^{-/low}$ phenotype, which includes the $CD44^{+}CD25^{-}$ population). More mature stages in T-cell development, including the generation of mature T cells, were not dependent on c-kit/SCF expression.

These *in vivo* findings are in agreement with studies demonstrating that thymic precursors are responsive to SCF *in vitro* (Godfrey *et al.*, 1992; Morrissey *et al.*, 1994). Although it is evident from the analysis of W/W mice that thymocyte development is not absolutely dependent on c-kit or SCF (see also later), complete inhibition of *in vitro* (FTOC) thymocyte development with mAbs against c-kit was reported (Godfrey *et al.*, 1992, 1994). However, no inhibition of FTOC reconstitution by $c\text{-}kit^{+}$ thymocytes was found by another group (Matsuzaki *et al.*, 1993). Finally, partial blocking (two- threefold reduction in cell number, no block in development) was reported from yet another experiment (Hozumi *et al.*, 1994a) which, in fact, gave the best reproduction of the *in vivo* situation. These results reemphasize the problem that neither *in vitro* proliferation assays nor mAb blocking studies can reliably predict the *in vivo* role of the molecules in question (see also Section II). Collectively, data show that thymocyte development is permissive in both c-kit and SCF mutant mice. Therefore, alternative growth factor receptors and their ligands, or direct stromal cell/thymocyte contact, can act in lieu of c-kit/SCF (see later).

4. *Two Distinct Receptor-Mediated Signaling Pathways in Thymocyte Development: Essential, Overlapping, and Synergistic Functions for C-kit and the Common Cytokine Receptor γ Chain*

a. Thymic Phenotype in Mice Lacking C-kit and γ_c. Mutations in either c-kit or γ_c reduce thymic cellularity, but neither mutation alone causes a complete block in thymocyte development (see earlier). However, both receptor type molecules act at similar (very early) stages of development in the thymus, suggesting that they represent complementing signaling pathways, which compensate for one another in the respective mutations. This possibility has been directly examined in mice lacking c-kit, γ_c, or both growth factor receptors (Rodewald *et al.*, 1997). In fact, in c-kit$^-\gamma_c^-$ mice, the thymus, but not the bone marrow and the spleen, was alymphoid, i.e., thymocytes were absent from thymic lobes as determined by cell counting, by flow cytometry (lack of lymphoid cells expressing Thy-1, CD4, CD8, CD25), and by histology (Rodewald *et al.*, 1997, and unpublished data).

At which level is T-cell development blocked in c-kit$^-\gamma_c^-$ mice? V(D)J β chain rearrangements were essentially lacking in thymus DNA from c-kit$^-\gamma_c^-$ mice, and only clonal or oligoclonal DJ β chain rearrangements were found in some double-mutant mice (Rodewald *et al.*, 1997). In addition, the impact of lack of c-kit, γ_c, or both on rearrangements and junctional diversity of TCR γ ($V_{\gamma 2}J_{\gamma 1}$), δ ($V_{\delta 1}(D_\delta)J_{\delta 2}$), and Ig $V_H(D_H)J_H$ loci has been examined (Rodewald and Haller, 1998). When analyzed by PCR, all rearrangements were present in wild-type and mutant mice. However, sequencing of the junctions revealed, similar to what was found for TCR β, only monoclonal TCR γ ($V_{\gamma 2}J_{\gamma 1}$) and TCR δ ($V_{\delta 1}(D_\delta)J_{\delta 2}$ joints in c-kit$^-\gamma_c^-$, but not c-kit$^+\gamma_c^-$ or wild-type thymocytes (Tables I and II). It is not known whether these rare recombination events are "true intrathymic" or extrathymic rearrangements as TCR rearrangements had also been identified previously in early ontogeny in extrathymic sites such as fetal blood (Rodewald *et al.*, 1994), fetal liver, and fetal gut (Carding *et al.*, 1990). Therefore, such extrathymic DJ β rearrangements may "contaminate" the DNA that was isolated from double-mutant thymi.

One possibility is that the block occurs at the level of the HSC. However, this appears not to be the case as hematological parameters were not altered in c-kit$^-\gamma_c^-$ compared to c-kit single-deficient mice. Moreover, B lymphopoiesis was only mildly reduced comparing c-kit$^-\gamma_c^-$ to c-kit$^+\gamma_c^-$ mice. Second, the generation of pro-T cell from HSC may be inhibited. If prethymic T lineage-committed progenitors depend on c-kit/γ_c for their generation or survival, thymus colonization by this type of precursor may be impaired. Alternatively, once pro-T cells colonize the thymus, they

might be dependent for their proliferation and/or survival on c-kit/γ_c-mediated signals.

The thymus phenotype of c-kit$^-\gamma_c^-$ mice stands in marked contrast to the phenotypes of mice deficient in the RAG genes (Mombaerts *et al.*, 1992; Shinkai *et al.*, 1992), TCR β (Godfrey *et al.*, 1994), CD3ε (Malissen *et al.*, 1995), or CD3γ (M. Haks, P. Krimpenfort, and A. Kruisbeek, personal communication) in which thymocyte development beyond the CD44$^{-/low}$CD25$^+$ stage is blocked or greatly reduced. Of note, in the latter mice, cell numbers are normal or even increased up to the stage of developmental arrest. For instance, the thymus in age-matched RAG-2$^{-/-}$ mice contained $\sim 3 \times 10^5$ thymocytes whereas c-kit$^-\gamma_c^-$ mice lacked such precursors. Thus, before structural components of the TCR are required for the formation of a functional pre-TCR complex, growth factor receptors are essential for further development. To a similar extent, thymocyte development was completely abrogated in mice deficient in the zinc-finger transcription factor GATA-3 (Ting *et al.*, 1996) and in mice expression Ikaros transcription factors lacking the DNA-binding domain (Georgopoulos *et al.*, 1994) (see later).

Collectively, data indicate that the earliest intrathymic "stem cell" pool needs to be expanded via proliferation and/or protection from cell death to generate larger numbers of pro-T and pre-T cells in which the somatic recombination machinery then generates the TCR repertoire. Progenitor expansion at this stage by growth factor receptors is essential. Similar mechanisms are likely to operate in early B-cell development, but lack of c-kit alone has apparently no effect on B-cell development *in vivo* (Takeda *et al.*, 1997), and lack of c-kit and γ_c does not completely abrogate the generation of IgM$^+$ B cells. In further support of this notion, and in contrast to TCR β, γ and δ loci, $V_HD_HJ_H$ junctions were diverse in c-kit$^-\gamma_c^-$ mice (Rodewald and Haller, 1998). Therefore, other factors such as flk-2 may be more critical in B-cell development. Indeed, mice lacking both flk-2 and carrying one c-kit null (W) allele and one c-kit viable allele harboring a point mutation in the kinase domain (W^v) showed a dramatic reduction in B lymphopoiesis (Mackarehtschian *et al.*, 1995). It would be interesting to determine whether W/W flk-2$^{-/-}$ mice can still generate B cells.

b. Considerations on C-kit and γ_c-Mediated Signal Transduction. The growth factor receptors identified to play critical roles very early in thymocyte development may drive differentiation, or proliferation, or protect from cell death (promoting survival). γ_c-associated cytokine receptors are known to signal via JAK3 and signal transducers and activators of transcription (STAT) to regulate cell growth and differentiation (Ihle, 1995). JAK-3 is the critical signal transducer for γ_c as shown by the fact that JAK-3-

deficient mice exhibit a very similar phenotype when compared to mice lacking γ_c. Thus, both T and B lymphopoiesis was strongly inhibited, and NK cells, $V_{\gamma 3}^+$ dendritic epidermal cells, and intestinal epithelial T cells were completely absent. In addition, JAK-3 is also expressed in thymic epithelium, and the thymic architecture was altered in JAK-3$^{-/-}$ mice when compared to γ_c-deficient mice, suggesting a role for JAK-3-mediated signal transduction also in the thymic environment. Like γ_c, JAK-3 is not involved in HSC development or function (Nosaka *et al.*, 1995; Park *et al.*, 1995; Thomis *et al.*, 1995).

STATs can regulate transcription, which, in turn, appears to drive functional changes rather than proliferation. In addition, JAKs can interact with other downstream signaling elements such as phosphatidylinositol-3-OH-kinase (PI-3-kinase), the $p21^{ras}$ pathway, and insulin response elements (IRS) (for a review, see Ihle, 1995). Little information is available regarding c-kit-mediated signal transduction in lymphoid cells. Experiments using nontransformed mast cells revealed that SCF-induced dimerization of c-kit causes tyrosine phosphorylation of several cellular substrates, including activation of PI-3 kinase and $p21^{ras}$, with subsequent activation of early response genes (Serve *et al.*, 1995). The antiapoptotic arm of the growth factor signals in early thymocytes may be provided via PI-3 kinase, which has been shown to mediate nerve growth factor-induced survival in PC-12 cells (Yao and Cooper, 1995). In fibroblasts, PI-3 kinase can activate a downstream molecule, the serine/threonine kinase Akt (protein kinase B), which can prevent apoptosis by inhibition of Ced3/ICE like protease activity (Kennedy *et al.*, 1997).

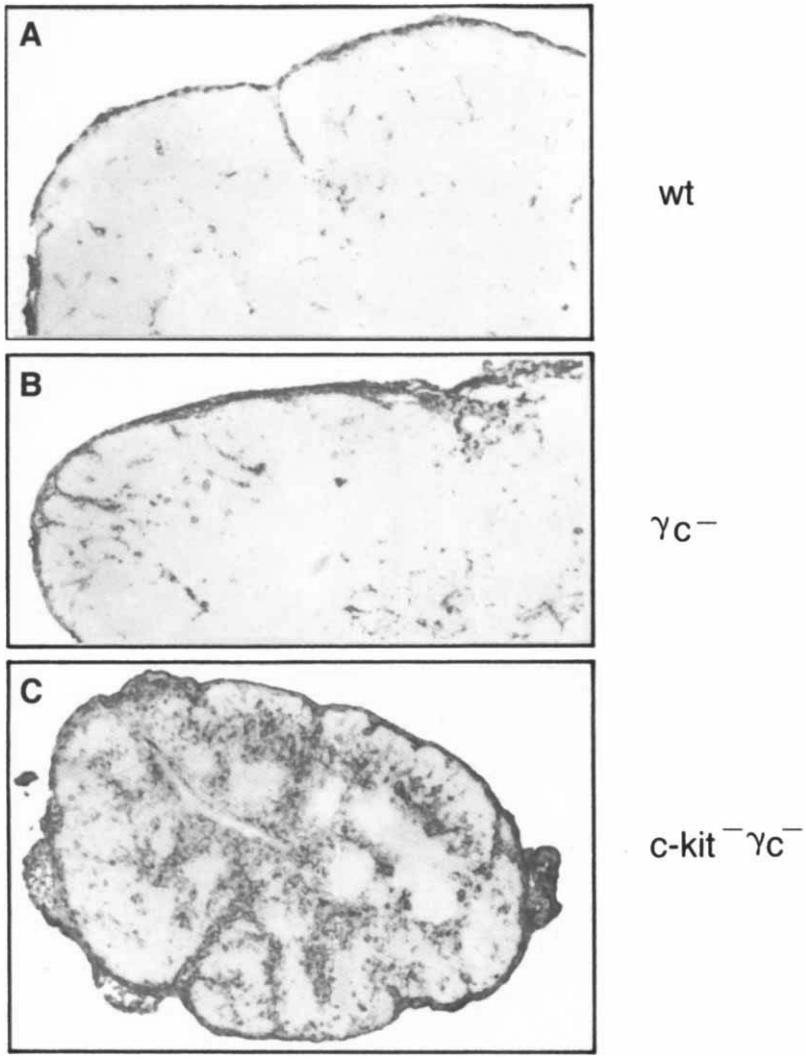
It is conceivable that c-kit and γ_c-associated receptors deliver qualitatively distinct signals in thymocytes, one mitogenic and one promoting survival. Absence of one, or the other, but not both pathways would be permissive for development.

c. Development of Thymic Dendritic Cells in C-kit$^-\gamma_c^-$ Mice. Another aspect of the thymus from c-kit$^-\gamma_c^-$ mice should be mentioned here: Experiments indicate that thymic dendritic cells (DC), a population of antigen-presenting cells of hematopoietic origin, are present, despite the absence of thymocyte progenitors, in c-kit and γ_c double-mutant mice (H. R. Rodewald, unpublished). It has been proposed that thymic DC are derived from the earliest intrathymic progenitor cell type, the "$CD4^{low}$" population. In adoptive transfer experiments *in vivo* (Ardavin *et al.*, 1993; Wu *et al.*, 1995) and by colony formation in the presence of various growth factors *in vitro* (Saunders *et al.*, 1996), the "$CD4^{low}$" population contained, in addition to precursor potential for T, B, and NK cells (Wu *et al.*, 1991a),

the potential to generate thymic DC. The presence of thymic DC in postnatal c-kit and γ_c double-mutant mice suggests (1) that thymocytes and thymic DC do not have to develop in parallel, (2) that the development of thymic DC can occur independently from c-kit$^-$ and γ_c-mediated signals *in vivo*, and (3) that pro-thymocytes, which were absent from c-kit$^-\gamma_c^-$ mice, may not be the obligatory precursors of thymic DC. The latter point may, however, differ among fetal, neonatal, and adult life.

d. Consequences of the Absence of Pro-Thymocytes on the Structure of the Thymic Stroma. The thymic architecture in c-kit$^-\gamma_c^-$ mice shall be briefly considered because it may offer some insights into the development of the thymic stroma. As mentioned earlier, thymi from c-kit$^-\gamma_c^-$ mice were almphoid and showed an architecture closely resembling RFTOC (Anderson *et al.*, 1993) prepared from purified fetal thymus stromal cells *in vitro* after the depletion of CD45$^+$ cells (Rodewald *et al.*, 1997). Thymic stroma from c-kit$^-\gamma_c^-$ mice was not organized into distinct medullary and cortical areas but showed strong infiltration with connective tissues as shown by collagen-like staining with mAb MTS 16 (Boyd *et al.*, 1993) (Fig. 4C). The MTS 16$^-$ stroma was mostly positive for the medullary marker MTS 10 (Fig. 5A). In addition, the thymus in c-kit$^-\gamma_c^-$ mice, in contrast to age-matched single mutant or wild-type controls, exhibited large numbers of thymic stromal cells expressing an embryonic phenotype as revealed by staining for expression of primordial markers MTS 20 and MTS 24 (R. Boyd and H. R. Rodewald, unpublished). This staining was in marked contrast to the structures found in RAG-deficient mice (Fig. 4D), mice lacking only γ_c (Fig. 4B), or wild-type mice (Fig. 4A), all of which revealed capsular and septum-associated staining with MTS 16, but lacked extended areas of connective tissues inside the thymus.

A requirement of normal thymocyte development for the formation of the regular thymic stromal architecture is well established (reviewed by van Ewijk, 1991; Boyd *et al.*, 1993). For instance, following radiation-induced ablation of thymocytes, the thymic epithelium shows a transient loss of cortically/medullary organization, and mutations that prevent or disturb thymocyte development also lead to abnormal thymic epithelium. Thus, it is generally accepted that interactions between the thymus epithelium and developing thymocytes (e.g., Lerner *et al.*, 1996) are essential for "induction" and/or maintenance of a functional thymic environment (van Ewijk, 1991; Ritter and Boyd, 1993), but there is very little information on the basis of this interplay. A transgenic mouse line carrying high copy numbers of human CD3ε showed an early arrest in thymocyte development at the Thy-1$^+$CD44$^+$CD25$^-$ stage. The cellularity was correspondingly



FIGS. 4 and 5. Thymic architecture in wild-type and growth factor receptor mutant mice. Thymic sections from c-kit$^{+}\gamma_c^{+}$ (Fig. 4A), c-kit$^{+}\gamma_c^{-}$ (Fig. 4B), c-kit$^{-}\gamma_c^{-}$ (Rodewald *et al.*, 1997) (Fig. 4C), and RAG-2$^{-/-}$ (Shinkai *et al.*, 1992) (Fig. 4D) mice or from thymus grafts (postnatal c-kit$^{-}\gamma_c^{-}$ thymus → nu/nu mice) (Fig. 4E) are shown. For histological analysis, tissue sections were stained with mAb MTS 16 recognizing the thymic capsule and septae (collagen-like staining) or with mAb MTS 10 binding to medullary type thymic stromal cells (Boyd *et al.*, 1993). Biotinylated sheep anti-rat Ig F(ab)$_2$ (1:100 diluted) (Boehringer Mannheim, Germany) and streptavidin–alkaline phosphatase (1:500 diluted) (Amersham Life Science, Buckinghamshire, UK) followed by ABC–aklaline phosphatase substrate (Vector Laboratories, Burlingame, CA) were used to develop the staining. Thymic stroma from c-kit$^{-}\gamma_c^{-}$ mice is not organized into distinct medullary and cortical areas, but shows strong

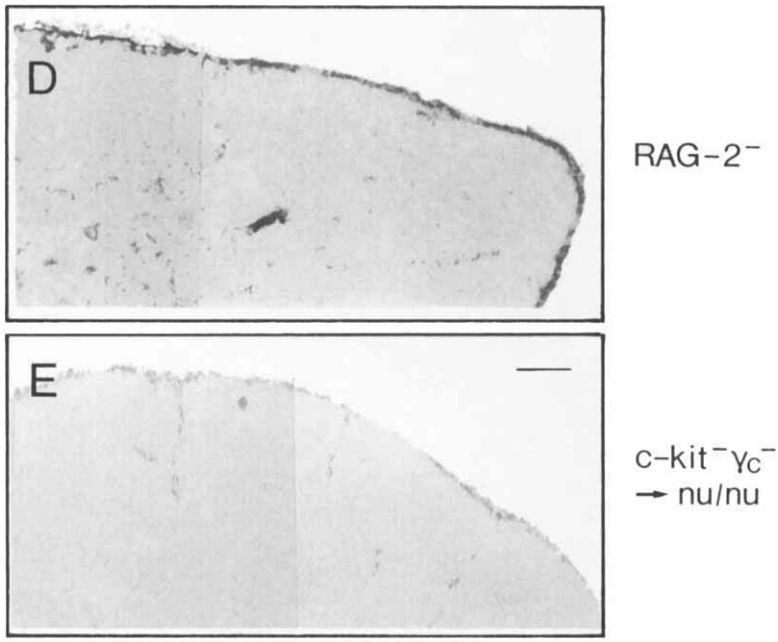


FIG. 4—*Continued*

reduced to 1–2% of the normal number (Wang *et al.*, 1994). Höllander and colleagues (1995) reported that the thymic architecture in these mice was irreversibly damaged as injection of bone marrow stem cells into adult mice failed to reconstitute a functional thymus. In contrast, transfer of wild-type stem cells *in utero* or grafting of the transgenic fetal thymus into wild-type mice led to the formation of a functional thymic environment. It was concluded that pro-thymocytes have to "induce" the thymic microenvironment during a certain developmental window (underfined between ~day 16 of fetal development and adult age).

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infiltration with connective tissues as shown by collagen-like staining by immunocytochemistry with mAb MTS 16 (Boyd *et al.*, 1993). MTS 16⁻ stroma in c-kit⁻γ_c^- thymi is mostly positive for the medullary marker MTS 10 (Fig. 5A). At the time of implantation, postnatal c-kit⁻γ_c^- thymus grafts lack pro-thymocytes. c-kit⁻γ_c^- thymus grafts are colonized by host progenitors, and thymic stroma unfolds into clearly separated cortical and medullary zones (not shown). Note that MTS 16 staining in c-kit⁻γ_c^- thymus grafts (Fig. 4F) is akin to wild-type thymus (Fig. 4A) and unlike *ex vivo*-isolated c-kit⁻γ_c^- thymus (Fig. 4C). Scale bar in E = 100 μm. See also color insert.

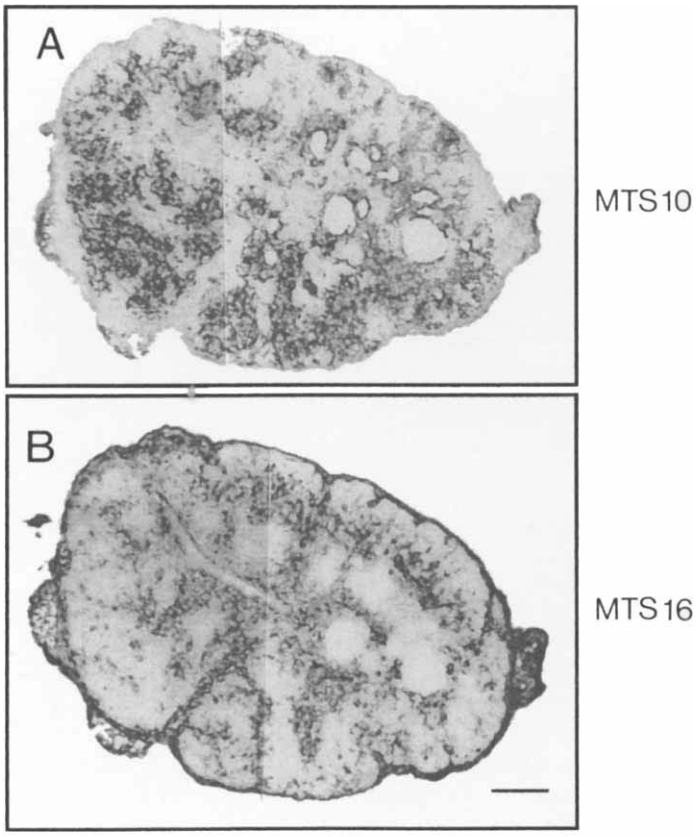


FIG. 5.

These data are in contrast to findings from experiments in which 1-week-old (postnatal) c-kit$^-\gamma_c^-$ thymi were grafted into nude mice (Fig. 4E). Although these grafts lacked thymocytes at the time of implantation and also pro-thymocytes at any appreciable level (see earlier), the grafts were colonized by host progenitors, and thymic stroma "unfolded" into clearly separated cortical and medullary zones, which lost, by and large, expression of embryonic markers MTS 20 and MTS 24, and supported functional thymocyte development with a regular pattern of thymic subsets as evaluated by expression of CD4 and CD8 (H. R. Rodewald and R. Boyd, unpublished). This system may be utilized to analyze the differentiation (and potential proliferation) of thymic stroma cells *in vivo*.

D. Cytokines Potentially Affecting Thymocyte Development

1. IL-15, TSLP

IL-15, a cytokine with functional similarity to IL-2, signals via a receptor complex composed of the IL-15 R α chain, the IL-2 R β chain, the common cytokine receptor γ chain (γ_c). Mutants lacking IL-15 or the IL-15 R α chain are not yet available. Lack of the IL-2 R β chain has no effect on thymocyte development, but perturbs extrathymic T and NK cell development (Suzuki *et al.*, 1997; for a review, see, e.g., DiSanto, 1997), whereas γ_c deficiency causes thymic hypocellularity (see earlier). Thus, a role of IL-15 or the IL-15 R α chain in thymocyte development is not known at present.

It will also be of interest to learn more about the potential role of thymic stromal cell-derived lymphopoietin (TSLP) (Peschon *et al.*, 1994) in thymocyte differentiation. TSLP may play a role in thymocyte development as it is thought to signal via the IL-7 R α chain and γ_c, respectively, the loss of either of which clearly affects thymocyte development (see earlier).

2. IL-1 α and TNF-α

Based on *in vitro* blocking studies in FTOC using mAbs directed against IL-1 α and TNF-α, it has been speculated that these two cytokines play, together, an essential role in early thymocyte development (Zuniga-Pflucker *et al.*, 1995). Addition of both mAb abrogated the appearance of $CD25^+$ fetal thymocytes and the subsequent generation of DP cells in FTOC. It is not trivial to test this proposal by genetic means because TNF may signal via two distinct receptors [TNF-R1 (p55) or TNF-R2 (p75)]. In parallel, the IL-1/IL-1 R pathway would have to be abrogated. Nevertheless, mice lacking both IL-1 R and TNF-R1 have been generated and fail to show any obvious phenotype in early thymocyte differentiation (H. Bluethmann, personal communication). This issue shall finally be answered in mice lacking IL-1 R and TNF-R 1 and 2).

VI. Transcription Factors in Early Thymocyte Development

A. General Considerations

Development progression from stem cells into a whole organism, including the development of the hematopoietic system is controlled, at the transcriptional level, by nuclear DNA-binding factors. Some transcription factors are specifically expressed in some but not other lineages, and their expression can be temporally restricted during ontogeny. Although it is clear that tissue-specific gene expression and differentiation can be controlled in this way, transcription factors themselves must be subject to regulation, and they may or may not be the commitment determination factors. In the strictest sense, one would expect that expression of a putative

commitment factor should dictate lineage decisions and that overexpression in the "wrong" lineage might preclude the development of this lineage.

Several transcription factor mutants have been reported that yielded interesting phenotypes with regard to thymocyte development. Findings derived from these mutants are summarized later. For detailed overviews, however, the reader should refer to reviews in which this topic has been covered first hand (Simon, 1995; Clevers and Grosschedl, 1996; Shivdasani and Orkin, 1996; Singh, 1996; Georgopoulos *et al.*, 1997). It is evident from these mutants that inactivation of single transcription factors can cause hematopoietic malfunction that may block development or function of some lineages, while leaving others unaffected. Before outlining the phenotype of the relevant mutants, it should be pointed out that these results could indicate that the affected lineages are derived from a common, oligopotent progenitor cell downstream from HSC or, alternatively, common regulatory mechanisms may be utilized in each lineage. Moreover, each transcription factor has the potential to regulate a large number of target genes. Hence, inactivation of a single factor may cause dysregulation of many substrate genes. Complex phenotypes (often lethal in early ontogeny) were indeed observed in many mutants. An understanding of the molecular basis for the observed phenotype, i.e., to elucidate target genes relevant *in vivo* and analyze their roles, would establish a direct relationship between the introduced mutation and the phenotype generated and clearly uncover novel molecular events in early lymphoid development.

B. GATA

Members of the GATA family of nuclear transcription factors bind to a consensus sequence (A/TGATAA/G) originally found in promoters and enhancers of erythroid-specific genes. Up until now, six different GATA genes (GATA 1–6) have been identified. All members of the family contain a conserved zinc-finger DNA-binding domain. GATA-1 to GATA-3, which belong to one subfamily, are primarily, but not exclusively, expressed in hematopoietic cells, whereas GATA-4 to GATA-6 are found in nonhematopoietic tissues, such as the developing heart, lung, liver, gut, or smooth muscle (reviewed by Simon, 1995).

1. GATA-1 and GATA-2

GATA-1 is essential for erythroid development, whereas other hematopoietic lineages appear unaffected by lack of GATA-1 (reviewed by Simon, 1995). GATA-2-deficient mice revealed an embryonic lethal phenotype (day, 9.5–11.5 of gestation) (Tsai *et al.*, 1994). Homozygous mutants were severely anemic. Hematopoiesis was perturbed at an early stage as formation of both erythroid and myeloid colonies from yolk sac hematopoietic

progenitors was strongly impaired. In mice chimeric for wild-type and GATA-2$^{-/-}$ tissues, GATA-2$^{-/-}$ cells failed to contribute at any significant level to fetal liver or bone marrow hematopoiesis. However, this block may not be absolute as in RAG-2 blastocysts complemented with GATA-2$^{-/-}$ ES cells very low levels of lymphopoiesis were observed. Collectively, these findings demonstrate that GATA-2 has a crucial function in hematopoiesis as early as at yolk sac stages. Lack of GATA-2 probably affects the HSC compartments and early progenitors for all lineages.

2. *GATA-3*

GATA-3 is expressed, among other tissues, in endothelium, kidney, and nervous tissues. The only hematopoietic cells expressing GATA-3 are T lineage cells. Thus, this transcription factor was a candidate for a key player in T-cell development. Because a null mutation in GATA-3, similar to GATA-2, is lethal on day 12 of gestation, generation of chimeric (GATA-3$^{-/-}$ ES into RAG-2-deficient blastocysts) mice was necessary for an analysis of T-cell development in the absence of GATA-3. In such chimeric mice (Ting *et al.*, 1996), GATA-3$^{-/-}$ ES cells contributed significantly to a variety of nonhematopoietic tissues, such as brain, lung, or liver, and to the formation of erythroid, myloid, and B-cell lineages. In marked contrast, both by flow cytometry and by genetic markers, GATA-3$^{-/-}$ ES cells failed to participate in thymopoiesis (Fig. 1). Because the earliest T-cell-committed progenitors express Thy-1 (Trowbridge *et al.*, 1985; Rodewald, 1995; Shortman and Wu, 1996) and GATA-3$^{-/-}$ Thy-1^{+} cells were not detectable in the thymus of chimeric mice, it is likely that GATA-3$^{-/-}$ ES cell-derived progeny was entirely absent from the thymus. It was not reported whether the thymus of chimeric mice (GATA-3$^{-/-}$ ES cells into RAG-2-deficient blastocysts) harbored any TCR rearrangements, which represents arguably the most sensitive way to address this question. Thus, Ting *et al.* (1996) could clearly show that GATA-3 is essential for thymocyte development.

GATA-3 is critical at stages later than HSC but as early as at the pro-T-cell stage, and perhaps cells with the potential to colonize the thymus are not generated from GATA-3$^{-/-}$ HSC. Can we speculate on the target genes that are regulated at these first stages of thymocyte differentiation by GATA-3? It is well established that the components of the pre-TCR, i.e., TCR β, pTα, CD3ε, CD3γ, or the RAG genes, are essential for thymocyte development beyond the CD4^{-} CD8^{-}CD44^{-}CD25^{+} stage (see Sections III and IV). However, the phenotype of chimeric mice (GATA-3$^{-/-}$ ES cells into RAG-2-deficient blastocysts) suggests that other gene products are essential for proliferation, survival, or differentiation prior to the CD4^{-} CD8^{-}CD44^{-}CD25^{+} stage. The only genes known to be essential at a similarly early stage are c-kit and the common cytokine receptor γ_c

chain. Loss of both of these receptors resulted in an alymphoid thymus (see Section V). However, mice lacking γ_c also showed defects in B-cell development. By further defining the targets genes regulated by GATA-3, as well as the regulation of expression of GATA-3 itself, molecules might be discovered that drive the very first stages of T-cell development.

C. PU.1

Disruption of the PU.1 gene (reviewed by Singh, 1996) caused a lethal phenotype in late gestation (day 18) in one mutant strain (Scott *et al.*, 1994), but was compatible with postnatal life in another (McKercher *et al.*, 1996). The distinct phenotypes may result from the different targeting strategies (see later). Scott *et al.* (1994) showed complete absence of committed B- and T-cell precursors on fetal day 16 in liver and thymus. In addition, granulocytes and macrophages were lacking in these mice. However, hematopoietic stem cell populations were not absolutely blocked as shown by the fact that numbers of megakaryocytes and red cell precursors appeared normal. To determine whether PU.1 acts in a cell autonomous manner or is also involved in the function of the hematopoietic environment, PU.1-deficient ES cell were used to generate fetal and adult chimeric mice (Scott *et al.*, 1997). Consistent with the findings in PU.1$^{-/-}$ fetal mice, PU.1-deficient ES cells failed to contribute at all to myeloid and lymphoid lineages, but showed, at low levels, participation in fetal erythropoiesis. However, in adult chimeras, PU.1 was essential for the generation of all hematopoietic lineages, including red cells, and PU.1$^{-/-}$ fetal liver cells failed to radioprotect lethally irradiated mice. These results suggest that PU.1 is a critical factor for myeloid and lymphoid progenitors, but, at least in the fetus, may not be essential for erythropoiesis and megakaryopoiesis.

Another PU.1 mutant strain was generated by McKercher *et al.* (1996). Both PU.1 mutant mice carry neo insertions in exon 5 (containing the DNA-binding domain); however, the deleted sequences differed by 35 amino acids. Although Scott *et al.* (1997) reported that PU.1 protein was absent from their mice and McKercher *et al.* (1996) found no evidence for PU.1–DNA complexes, results obtained with this mouse strain differed significantly. Aided by antibiotic treatment, mice generated by McKercher *et al.* (1996) survived until ~2 weeks postnatally. The defects were more restricted in these mice: Although macrophages and mature B cells were still lacking, thymocytes could now be found. However, thymocyte development was delayed and thymocyte numbers were reduced by a factor of 5–10. The fact that PU.1 is not detected in wild-type thymus (Scott *et al.*, 1997) would also argue against a role of this transcription factor in intrathymic development.

Collectively, PU.1 is essential for both lymphoid and myeloid progenitors in one study (Scott *et al.*, 1997), but not in another. In the latter study, absence of PU.1 clearly precluded the development of B cells and macrophages, but not T cells.

D. Ikaros

The Ikaros gene was originally identified by virtue of Ikaros protein binding to the CD3δ enhancer (Georgopoulos *et al.*, 1992). Ikaros gene transcripts are expressed as early as embryonic day 8 in the yolk sac. Later, expression is found in fetal liver and in the thymus. Based on cell fractionation experiments, Ikaros-RNA is expressed in HSC populations, thymocytes, B-cell progenitors in bone marrow, and mature T, B, and NK cells (Georgopoulos *et al.*, 1997). The Ikaros gene encodes a family of proteins containing zinc finger domains. Six distinct isoforms are generated from the Ikaros locus by alternative RNA splicing. The N-terminal region harbors DNA-binding domains, whereas the C terminus is critically involved in dimerization and functional activation of the proteins. Mouse mutants carrying a deletion of the C terminus ($C^{-/-}$) are therefore considered Ikaros null mutants (J.-H. Wang *et al.*, 1996), whereas Ikaros proteins lacking N-terminal sequences ($N^{-/-}$) appear to act as dominant-negative proteins (Georgopoulos *et al.*, 1994). The latter form may act as a dominant negative mutant by interferring via dimerization with other proteins with similar roles, such as Aiolos (Georgopoulos *et al.*, 1997).

The most drastic phenotype was found in homozygous $N^{-/-}$ mutant mice (Georgopoulos *et al.*, 1994). These animals lacked all fetal and adult T (Fig. 1) and B cells as well as NK cells, and thus bone marrow, thymus, and lymph nodes were strongly hypocellular. The defects were, however, not restricted to lymphocyte lineages as Mac-1^+Gr1^+ myeloid cells were missing in the bone marrow and the HSC compartment was affected as shown by impaired CFU-S activity. However, *in vitro* colony formation of mutant bone marrow in response to GM-CSF was normal.

Ikaros null mutant mice ($C^{-/-}$) yielded a markedly different phenotype when compared to $N^{-/-}$ mice (J.-H. Wang *et al.*, 1996). In $C^{-/-}$ mice, thymocytes were generated (all subsets, including mature stages) in postnatal and adult mice, but not at fetal stages (Fig. 1). In contrast, B lineage cells were completely missing at all stages. Corresponding to the late appearance of thymocytes, fetal thymus-derived Vγ3 T cells [dendritic epidermal cells (DEC)] were missing in the skin, but γδ T-cell subsets derived from postnatal stages were found in $C^{-/-}$ mice. It is remarkable that fetal and adult thymopoiesis differs in its requirement for Ikaros. Differences in genetic requirements between fetal and adult stages are, however, not without precedent as adult, but not fetal, thymopoiesis is affected in mice lacking TCF-1 (see later) or α4 integrins (see later) (Fig. 1).

Interestingly, thymic dendritic cells (and possibly also peripheral DC) were missing in these mice, demonstrating a requirement for Ikaros in the generation of DC lineages. Lack of intrathymic progenitors ("$CD4^{low}$" cells) proposed to be common to thymocytes and thymic DC (Ardavin *et al.*, 1993) cannot account for the absence of thymic DC as, in the adult, the former but not the latter was present in $C^{-/-}$ mice. Rather, the specific myeloid pathway giving rise to DC may be blocked in the absence of Ikaros. Interestingly, lymph nodes were lacking in these mice, suggesting that DC may be an essential component for lymph node formation. Some of the observed defects may be caused by lack of Ikaros in hematopoietic environments or may be indirect via lack of certain cell populations. Transplantation of mutant hematopoietic progenitors or tissues into wild-type mice would be required to address some of these issues.

Collectively, Ikaros proteins are critically involved in various stages of lymphopoiesis and potentially myelopoiesis. It remains to be determined whether Ikaros is a commitment determination factor that dictates lineage decisions or whether the actions of Ikaros are secondary to lineage commitment.

E. HMG-Box Family Members

1. TCF-1 and LEF-1

a. Expression of TCF-1. Tcf-1, a member of the HMG box family, was originally cloned by virtue of TCF-1 protein binding to the CD3ε enhancer. During embryonic development, Tcf-1 is expressed in both hematopoietic and nonhematopoietic tissues, but interestingly, in the adult mouse, Tcf-1 expression is confined to T lineage cells (Clevers and Grosschedl, 1996). Intrathymically, Tcf-1 expression can be detected as early as in the $CD44^+CD25^-$ progenitor stage (Verbeek *et al.*, 1995). However, Tcf-1 is probably already transcribed in prethymic T lineage cells as fetal blood pro-thymocytes expressed Tcf-1 RNA abundantly (H. Clevers and H. R. Rodewald, unpublished).

b. Tcf-1-Deficient Mice. Disruption of Tcf-1 in mice (Verbeek *et al.*, 1995) caused a T lineage-specific phenotype: In young mice (≤4 weeks of age), numbers of total thymocytes were strongly (10- to 100-fold) reduced but development was permissive (Fig. 1). DN populations appeared largely unaffected at this stage, but the transitory population termed "immature single positive" (ISP; phenotype $CD3^-CD4^-CD8^+$) and DP thymocytes were greatly reduced in absolute numbers and by their percentage. By ~6 month of age, however, an even earlier developmental arrest at the $CD44^+CD25^-$ progenitor stage became apparent (M. Schilham and H. Clevers, personal communication). Thus, TCF-1 is critical in adult, but

not fetal thymocyte differentiation (Fig. 1). Surprisingly, the phenotype observed in adult mice (blockade at the $CD44^{+}CD25^{-}$ stage) precedes even the block found in mice lacking CD3 ε (Malissen *et al.*, 1995), suggesting that the TCF-$1^{-/-}$ phenotype is not (only) caused by lack of CD3 ε expression. This is worthwhile considering given that the CD3ε enhancer is the original target sequence for TCF-1. Nevertheless, it would be interesting to know whether expression of CD3 subunits or formation of the pre-TCR complex is altered in Tcf-1-deficient mice.

c. LEF-1. Another member of this transcription factor family, LEF-1, is also expressed in addition to precursor B cells in early thymocytes. This transcription factor interacts with the TCRα enhancer. Unlike Tcf-1 mutations, disruption of Lef-1 does not cause a phenotype in the T-cell lineage (for further references, see Clevers and Grosschedl, 1996). Given the fact that lack of Tcf-1 is compatible with fetal, but not adult, thymopoiesis, one could speculate that Lef-1 might substitute for Tcf-1 in the fetus and postnatal period.

2. Sox-4

Another member of the HMG box family of transcription factors is Sox-4. Expression of Sox-4 in T and B cells in adult mice suggested a role for this gene in the development and/or function of lymphocytes. A null mutation in Sox-4 is lethal, probably due to cardiac malformation, in midgestation. To determine whether Sox-4 is involved in T- or B-cell development, mutant or wild-type fetal liver cells were transferred into irradiated recipient mice. Although early B-cell development was almost completely blocked, Sox-$4^{-/-}$ HSC gave rise to T cells, albeit at a delayed rate (Schilham *et al.*, 1997). In subsequent experiments employing competitive thymic reconstitution, Sox-$4^{-/-}$ fetal liver cells were inferior, but not incapable, to generate thymocytes when compared to wild-type progenitors. In addition, FTOC from Sox-$4^{-/-}$ mice contained up to 50-fold fewer thymocytes than wild-type FTOC, and the requirement for Sox-4 was intrinsic to thymocytes, as shown by the fact that similar defects were found when Sox-$4^{-/-}$ progenitors developed in wild-type FTOC (Schilham *et al.*, 1997). Thus, Sox-4 is clearly involved in thymopoiesis but is not essential.

F. Conclusions from Transcription Factor Mutant Mice

In conclusion, the following picture emerges from data generated from mutant mice lacking single transcription factors up until now. Several mutants affect the hematopoietic system at very early cell types (probably including HSC). These include SCL-TAL 1 (Shivdasani and Orkin, 1996) and GATA-2 (Tsai *et al.*, 1994). As a consequence of such mutations, the

establishment of hematopoiesis is hampered, which results in phenotypes characterized by severe anemia and lethality mostly by embryonic day 10 in ontogeny. A second type of mutation abrogates the development of several, but not all, hematopoietic lineages. Examples of this type of mutation are PU.1$^{-/-}$ progenitors (McKercher *et al.*, 1996; Singh, 1996) (which fail to produce B cells and myeloid cells and perhaps T cells, but not red blood cells) and mice carrying a mutation in the DNA-binding domain of Ikaros, causing a developmental block in T, B, and NK, but not erythroid and myeloid lineages (Georgopoulos *et al.*, 1994). Finally, the third group of mutations reveals lineage-specific phenotypes such as GATA-3 (Ting *et al.*, 1996) or TCF-1 (Verbeek *et al.*, 1995), mutations that affect exclusively thymopoiesis, but not B-cell development. The opposite, a block in B but not T-cell development, is true for several mutants lacking B-cell-specific transcription factors (e.g., Pax-5 or E2A) (for a review and further references, see Clevers and Grosschedl, 1996). These experiments have clearly established unique roles for some transcription factors in hematopoiesis *in vivo* and they open a way to further dissect molecular events underlying blood cell differentiation.

VII. Miscellaneous Mutations and Their Roles in Early Thymocyte Development

A. Adhesion Molecules

Migration of hematopoietic cells from their sites of origin to their peripheral locations, as well as their continuous trafficking, is mediated by adhesion molecules (for reviews and further references, see, e.g., Imhof and Dunon, 1995). For T cells, information on expression and function of adhesion molecules has accumulated primarily in the area of mature, peripheral T-cell migration, but experiments have also been reported that impinge on the critical role of cell adhesion molecules in hematopoiesis and, specifically, the generation or development of early thymocytes. Those new aspects are briefly reviewed next.

1. Integrins

Integrins are heterodimers formed from an integrin α and an integrin β chain, both of which are transmembrane glycoproteins. The heterodimers can be built from various combinations of α and β chains, giving rise to at least 21 distinct forms. α chains are termed $\alpha_{1\text{-}9}$, α_v, α_L, α_m, and α_x, and β chains exist as $\beta_{1\text{-}8}$.

a. β_1 Integrins. The integrin β_1 molecule can form dimers with at least 10 different α chains ($\alpha_{1\text{-}9}$, α_v). β_1 integrins [also referred to as "very late antigens" (VLA)] are widely expressed on hematopoietic cells and bind to

ligands, which are components of the extracellular matrix such as laminin, fibronectin, and collagen. In a multistep model of leukocyte/endothelial attachment (rolling or transient adhesion, leukocyte activation, activation-dependent adhesion, transmigration) (Butcher, 1993; Springer, 1994), β_1 integrins are mainly involved in adhesion steps.

Mice lacking β_1 integrins are early embryonic lethal (Fässler *et al.*, 1996). Nevertheless, by using β_1 integrin$^{-/-}$ ES cells to generate chimeric mice from RAG-deficient blastocysts, the role of β_1 integrins for the generation and migration of hematopoietic cells has been addressed (Hirsch *et al.*, 1996). ES-derived cells could not be identified in bone marrow, spleen, or thymus in such chimeric mice. In search for any possible contribution of β_1 integrin$^{-/-}$ hematopoietic cells, Hirsch *et al.* (1996) also analyzed yolk sac, fetal blood, and fetal liver. In addition, the potential of wild-type and β_1 integrin$^{-/-}$ ES cells to generate *in vitro* myeloid or erythroid or B lymphocytes was compared. The results showed that the absence of β_1 integrins is perfectly compatible with myelo-, erythro-, and B lymphopoiesis *in vitro*. However, lack of β_1 integrins prevents colonization of hematopoietic tissues such as fetal liver and thymus during ontogeny. HSC were present in the yolk sac (and presumably in the AGM region, although this was not tested), and hematopoietic cells were mobilized from these sites to circulate in the peripheral blood. Thus, neither generation/differentiation of hematopoietic cells nor their mobilization was hampered, but their entry into fetal liver and thymus was blocked in the absence of β_1 integrins. It is not known which α subunit (or combination of subunits) is responsible for this phenotype. It would also be interesting to know whether β_1 integrin-deficient progenitors that are present in fetal blood are capable of thymopoiesis on intrathymic transfer or whether β_1 integrins are also involved in intrathymic adhesion between thymocytes and stromal cells.

b. α_4 Integrins. The α_4 integrin can pair with either β_1 ($\alpha_4\beta_1$) or β_7 ($\alpha_4\beta_7$). To determine the *in vivo* role of these two adhesion receptors, Arroyo *et al.* (1996) generated α_4-deficient mice. Similar to the β_1 integrin, null mutations in α_4 integrin are embryonic lethal (Yang *et al.*, 1995). $\alpha_4^{-/-}$ ES cells were injected into wild-type or RAG-deficient blastocysts to generate chimeric mice (Arroyo *et al.*, 1996). Analysis of peripheral blood and spleens from young adult mice (<4 weeks) revealed that, initially, both wild-type and $\alpha_4^{-/-}$ ES cells contributed comparably to the generation of mature, peripheral T cells. These T cells were apparently thymus derived as the thymus in young adult (<4 weeks) mice contained ES-derived DP and SP thymocyte subsets. Strikingly, in $\alpha_4^{-/-}$ ES, but not wild-type ES chimeras, thymus cellularity was strongly reduced to $\sim 3 \times 10^6$ cells with concomitant loss of DP and SP thymocytes as mice grew older (>4 weeks).

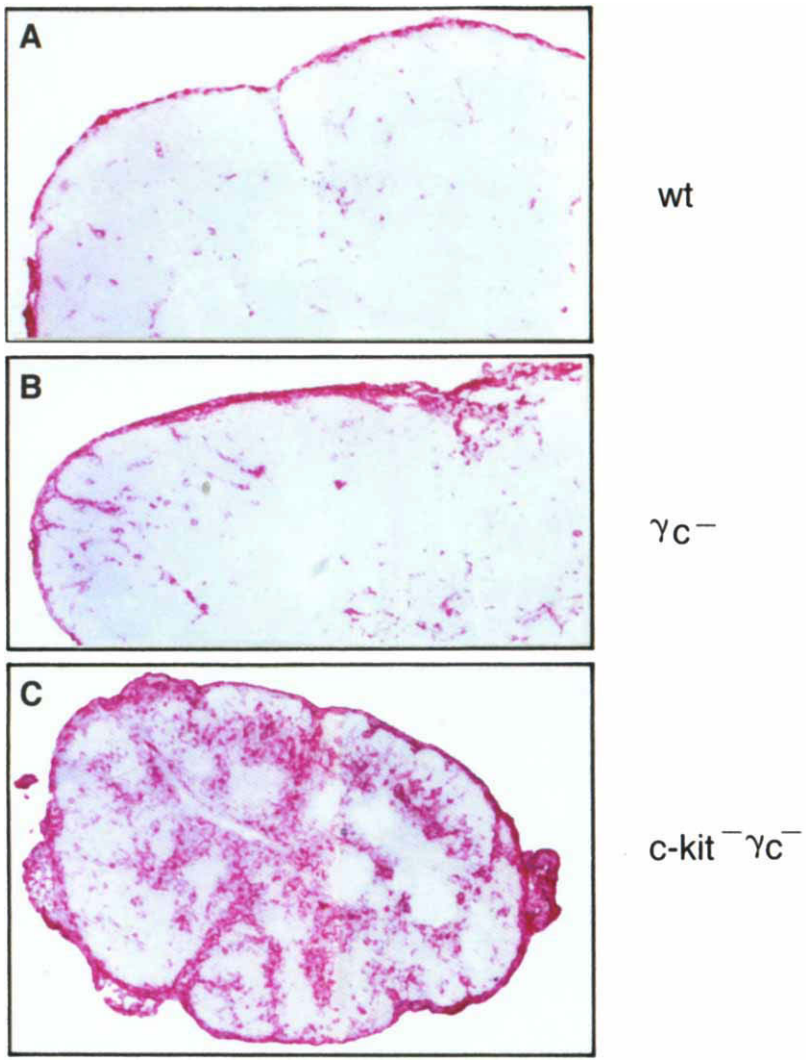
Finally, the residual DN thymocytes were all of blastocyst ($RAG^{-/-}$) and not $\alpha_4^{-/-}$ ES cell origin, suggesting that thymus seeding in the adult is α_4 dependent. However, this was not the case, as transplantation of bone marrow derived from chimeras made from either wild-type or $\alpha_4^{-/-}$ ES into $RAG^{-/-}$ blastocysts led to both T- and B-cell reconstitution in irradiated recipient mice. Even when these primary recipients served as donors in secondary bone marrow transplantations, T-cell reconstitution occurred.

These results imply several interesting points: (1) prenatal and juvenile thymopoiesis is unaffected by lack of α_4 integrins (Fig. 1), which is in contrast to an inhibitory effect on cell recovery in RFTOCs treated with mAb directed against α_4 integrin (Anderson *et al.*, 1997); (2) during adult life, supply of bone marrow-derived thymic progenitors declines completely such that the thymus is eventually devoid of such progenitors; (3) during adult life, the release of thymic progenitors from the bone marrow, rather than their colonization of the thymus, is dependent on α_4 integrins; (4) intrathymic T-cell development can occur in the absence of α_4 integrins; and (5) HSC colonization on bone marrow transplantation is α_4 independent.

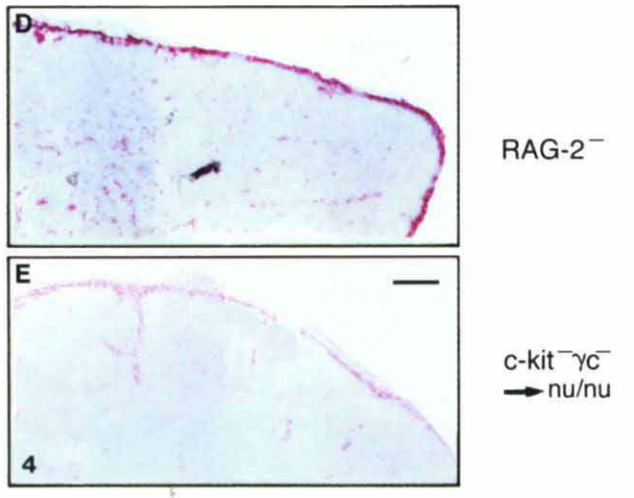
However, several findings in $\alpha_4^{-/-}$ ES into $RAG^{-/-}$ blastocyst chimeras are not T cell specific. In particular, B-cell development is drastically reduced, but not completely abrogated, at an early stage preceding the block found in RAG-deficient mice. This affects both conventional B-2 type and peritoneal B-1 type B cells. Even more striking is the inability of adult bone marrow derived from $\alpha_4^{-/-}$ ES into $RAG^{-/-}$ blastocyst chimeras to reconstitute B cells following transfer into irradiated recipient mice. This lack of reconstitution is obviously not due to failure of stem cells to colonize the host bone marrow as bone marrow from primary recipients could serve as donor tissue in secondary bone marrow transplantations (see earlier). How competitive are $\alpha_4^{-/-}$ HSC and their progeny to generate lineages other than T and B cells? Arroyo *et al.* (1996) described the presence of $\alpha_4^{-/-}$ Mac-1^+ monocytes in the blood of chimeric mice and also a small fraction of blood NK cells derived from $\alpha_4^{-/-}$ ES cells. However, these NK cells may be long lived and it is not known at which point in the animals life they were generated. Although the presence of $\alpha_4^{-/-}$-derived monocytes suggests that $\alpha_4^{-/-}$ HSC are functional and present in adult bone marrow derived from $\alpha_4^{-/-}$ ES into $RAG^{-/-}$ blastocyst chimeras, a more direct assessment of numbers and function of $\alpha_4^{-/-}$ HSC and their progeny would be informative. Colony assays in the presence or absence of neomycin to select for mutant and against wild-type cells might be useful (Hirsch *et al.*, 1996).

2. *E-cadherin*

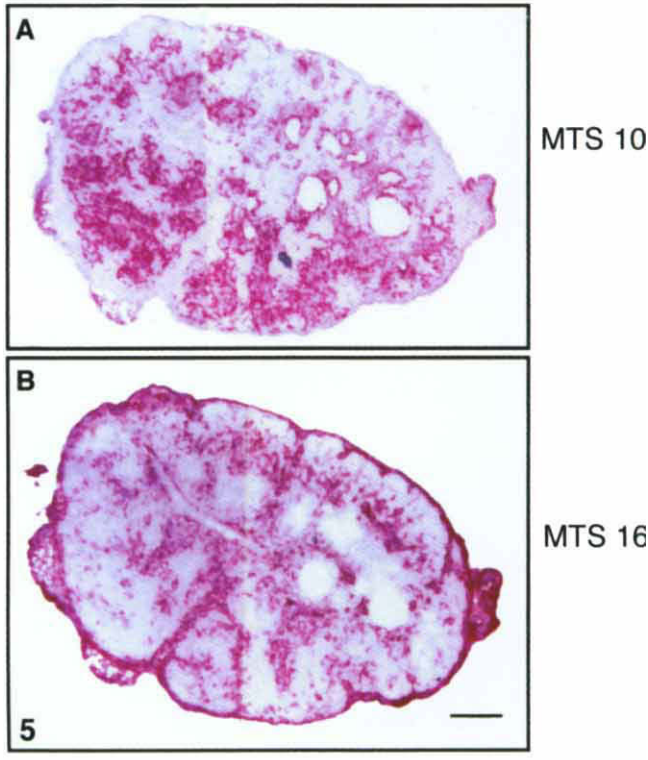
E-cadherin is an adhesion molecule mediating compaction of preimplantation embryos; E-$cadherin^{-/-}$ embryos fail to develop beyond the blasto-



CH. 1, FIGS. 4 AND 5. Thymic architecture in wild-type and growth factor receptor mutant mice. Thymic sections from c-kit$^{+}\gamma_c{}^{+}$ (Fig. 4A), c-kit$^{+}\gamma_c{}^{-}$ (Fig. 4B), c-kit$^{-}\gamma_c{}^{-}$ (Rodewald *et al.*, 1997) (Fig. 4C), and RAG-2$^{-/-}$ (Shinkai *et al.*, 1992) (Fig. 4D) mice or from thymus grafts (postnatal c-kit$^{-}\gamma_c{}^{-}$ thymus → nu/nu mice (Fig. 4E) are shown. Thymic stroma from c-kit$^{-}\gamma_c{}^{-}$ mice is not organized into distinct medullary and cortical areas, but shows strong infiltration with connective tissues as shown by collagen-like staining by immunocytochemistry with mAb MTS 16 (Boyd *et al.*, 1993). MTS 16^{-} stroma in c-kit$^{-}\gamma_c{}^{-}$ thymi is mostly positive for the medullary marker MTS 10 (Fig. 5A). At the time of implantation, postnatal c-kit$^{-}\gamma_c{}^{-}$ thymus grafts lack pro-thymocytes. c-kit$^{-}\gamma_c{}^{-}$ thymus grafts are colonized by host progenitors, and thymic stroma unfolds into clearly separated cortical and medullary zones (not shown). Note that MTS 16 staining in c-kit$^{-}\gamma_c{}^{-}$ thymus grafts (Fig. 4F) is akin to wild-type thymus (Fig. 4A) and unlike *ex vivo*-isolated c-kit$^{-}\gamma_c{}^{-}$ thymus (Fig. 4C). Scale bar in E = 100 μm.



CH. 1, FIG. 4 D & E



CH. 1, FIG. 5

cyst stage (Larue *et al.*, 1994; Riethmacher *et al.*, 1995). Because E-cadherin is involved in the maintenance and function of epithelial cells (Takeichi, 1990, 1995; Huber *et al.*, 1996), it is likely to also play a role in the development of the epithelial components of hematopoietic stroma, such as they are found in the thymus. E-cadherin can interact homotypically or heterotypically with integrin $\alpha_E\beta_7$.

To analyze the potential role of E-cadherin for the development of thymic stroma and for thymocyte/stromal cell interactions, Müller *et al.* (1997) used mAb blocking to interfere with RFTOC (see earlier). In this study, a mAb-blocking homotypic adhesion, but not a mAb-blocking heterotypic interaction, prevented the formation of functional RFTOC *in vitro*. It should be noted, however, that high concentrations (30–100 μg) of mAb were used, and it cannot be ruled out that some, but not other, mAbs are toxic to the epithelial cells. In addition, if reorganization of thymic stroma is perturbed in the presence of blocking mAb, it is difficult to conclude that the function of the epithelium is E-cadherin dependent. Intact thymic lobes could not be altered by mAb treatment, but reconstitution of lobes depleted of endogenous thymocytes, in particular with fetal liver cells but also with fetal thymocytes, showed inhibition or alteration of intrathymic development. Again, because the progenitors express E-cadherin, mAb blocking may affect this population directly. It should be mentioned that similar observations were made regarding CD44. The addition of anti-CD44 mAb to RFTOC, but not intact FTOC, inhibited, albeit not completely, thymocyte development (Anderson *et al.*, 1997), whereas mice lacking all splice variants of CD44 showed apparently normal thymocyte development (Schmits *et al.*, 1997).

Collectively, these results suggest that homotypic interactions of E-cadherin are involved in the organization of the thymic stroma and may also contribute to thymocyte/stromal cell interaction. The final answer to this issue could come from mutants lacking E-cadherin on thymic stroma, on hematopoietic cells, or on both.

3. *Vanin-1*

A novel molecule is involved in thymus seeding: Vanin-1 (Aurrand-Lions *et al.*, 1996). Vanin-1 (vascular noninflammatory molecule 1) was identified via a mAb (407) recognizing a thymic stromal cell line. mAb 407 shows a perivascular, and not luminal, staining pattern in thymic sections, but this staining was not restricted to thymus vessels. A cDNA encoding Vanin-1 was cloned from this stroma cell line. The polypeptide has a predicted size of 55 kDa, which, upon glycosylation, gives a mature protein of ~70 kDa. Although the Vanin-1 cDNA contains a potential cleavage site found in other glycosylphospatidylinositol (GPI)-anchored molecules, phospolipase C treatment did not cleave Vanin-1 from the membrane.

A variety of mAb-blocking experiments, both *in vivo* and *in vitro*, were performed using mAb 407. However, the results were somewhat variable, and, again, genetic experiments will determine whether Vanin-1 plays a role in thymus homing. Because mutants lacking integrin α_6 or CD44 (see earlier) failed to confirm the predicted roles of these molecules in thymus homing and because mice lacking Vanin-1 are not yet available, much has yet to be learned about the first molecule to be specifically mediating the colonization of the thymus. Nevertheless, integrins using the β_1 subunit are likely to play a role in the process as $\beta_1^{-/-}$ HSC or progenitors in fetal blood do not colonize the fetal thymus (Hirsch *et al.*, 1996). In addition, it is possible that mechanisms mediating thymic homing are redundant, and only mice bearing double or multiple mutations may reveal the role of such pathways.

B. CD81

CD81, also called TAPA-1, a member of the transmembrane 4 superfamily, is expressed on many cell types and forms a signaling complex on B cells in association with CD19, CD21, and leu13. CD81 had been implicated in thymocyte development based on mAb-blocking experiments and thymocyte development in "RFTOC" in which thymocytes were complexed, apparently in the absence of thymic stromal cells, with fibroblasts transfected with CD81. However, thymocyte development can proceed normally in the absence of CD81 as shown by two groups who generated CD81-deficient mice independently (Maecker and Levy, 1997; Miyazaki *et al.*, 1997). Thus, CD81 is not essential for thymocyte development.

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Regulation of Immunoglobulin Light Chain Isotype Expression

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I. Antibody Molecules and Immunoglobulin Gene Assembly

A. Historical Background

1. Selective Theories of Antibody Formation

Immunoglobulin (Ig) molecules, or antibodies, can aid in defending the host against an enormous array of foreign pathogens and substances (reviewed by Silverstein, 1993). At the 1967 Cold Spring Harbor Symposium on Quantitative Biology, Sir MacFarlane Burnet provided the introduction, tracing the development of his clonal selection theory of acquired immunity (Burnet, 1957, 1959), its origins in the selective theory of Dr. Neils K. Jerne (1955), and its recognition of a solution to a gap in Jerne's theory.

2. Jerne's Natural Selection Theory of Antibody Formation

Jerne's selective theory (Jerne, 1955; and reviewed by Burnet, 1967) proposed that Ig molecules of enormous diversity, generated by an unknown mechanism, are continuously being synthesized in small quantities, resulting in the presence of diverse antibodies in an individual's blood prior to exposure to antigen. Jerne proposed that these preexisting antibodies bind antigen and carry the antigen to the phagocytic cell, somehow stimulating the synthesis of additional antibody molecules of the same specificity.

3. Burnet's Clonal Selection Theory of Acquired Immunity

Drawing on emerging lines of evidence that immunological cells could actively proliferate (Mackay and Gajdusek, 1958), Burnet proposed that antibody could serve as a receptor on the cell (Fig. 1A). Unspecified genetic mechanisms would generate a repertoire of cells, each with a different receptor specificity. Contact with antigen by the cell surface receptor would then stimulate the cells to actively proliferate and secrete more antibody with the same specificity.

4. Structure of the Antibody Molecule

Studies concurrent with Burnet's work showed that the Ig molecule consists of two identical heavy (H) chain and two identical light (L) chain

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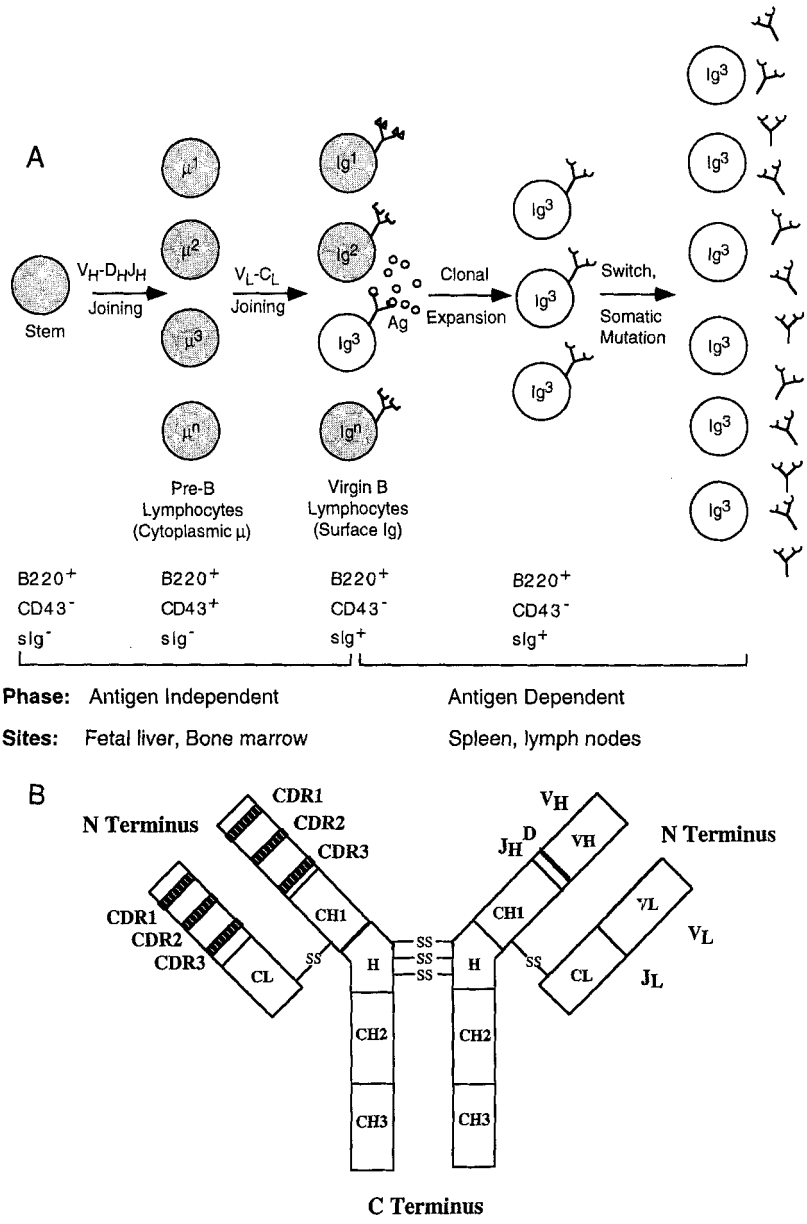

FIG. 1. (A) The clonal selection theory of acquired immunity. V, variable region gene; D, diversity segment; J, joining segment; C, constant region gene segment; H, heavy chain; L, light chain; μ, cytoplasmic μ HC; Ig, surface Ig molecule. Surface Ig molecules of different specificities are shown by different shaped molecules. A diverse repertoire of B cells bearing different Ig molecules is generated during the antigen-independent phase of

polypeptides (Fig. 1B), linked by disulfide bonds (Edelman, 1959; Porter, 1959). Each H and L chain consists of a portion that is constant (C) and a portion that is variable (V) between antibody molecules (Fig. 1B). V regions form the antigen-binding pocket, whereas C regions mediate effector functions, such as binding of F_c receptors by heavy chain (HC) C regions, and association with the HC molecule by light chain (LC) C regions. The amino acid sequence of V regions is most variable in the three specific complementarity-determining regions (CDRs), or "hypervariable" regions, that contact antigen. The sequence of the four interspersed "framework" regions is relatively conserved (Kabat *et al.*, 1987, 1991).

Based on the amino acid sequence and reactivity of the C regions with anti-Ig antisera, Ig HC molecules are divided into classes referred to as HC isotypes (IgM, D, G, E, and A), represented respectively by the Greek letters μ, δ, γ, ε, and α. Ig LC molecules are also divided into two isotypes, referred to as κ and λ. The HC, κ LC, and λ LC molecules are encoded by different gene families, located in separate loci on different chromosomes (reviewed by Max, 1993).

5. *The Twin Puzzles of Allelic and Isotype Exclusion*

In Burnet's theory, each Ig-producing cell expresses a unique antibody specificity. However, each immune cell has two HC loci and four LC loci per cell. If each chromosomal Ig locus could, through unknown diversity mechanisms, encode a distinct Ig HC or LC, how could a cell produce a single antibody specificity? Studies using antisera that could distinguish constant region allelic variants of HC and LC proteins found that more than 99% of individual B lymphocytes express only one HC allele and one

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B lymphocyte differentiation. Exposure to and binding of antigen result in clonal expansion of the B cells bearing Ig molecules bound by the antigen. Some B cells undergo HC isotype (class) switching and somatic mutation on exposure to antigen. Some surface markers are indicated (Hardy *et al.*, 1991). Adapted from Blackwell and Alt (1989a) and Lansford *et al.* (1996). (B) Structure of the murine antibody molecule. The antibody molecule pictured is of the IgG class. VH, heavy chain variable region; VL, light chain variable region; H, hinge region of heavy chain. The Ig molecule consists of two identical HCs, each of which is linked to one of two identical LCs by disulfide bonds. Disulfide bonds also convalently link the two HC molecules to each other. The three constant region domains are denoted CH1, CH2, and CH3. Complementary-determining regions (CDRs) are indicated in black on the left, and the variable protein portions encoded by the V, (D), and J gene segments are indicated on the right. Light chain proteins are shaded. Adapted from Lansford *et al.* (1996).

LC allele as surface Ig (sIg) receptors (Cebra *et al.*, 1966; Pernis *et al.*, 1965; reviewed by Blackwell and Alt, 1989a). The observation that almost all B cells produce the protein product from only one allele at each HC or LC locus was referred to as HC and LC allelic exclusion (Cebra *et al.*, 1966; Pernis *et al.*, 1965).

Even with strict allelic exclusion, an individual B cell expressing both κ and λ LC loci could express two Ig specificities. Indeed, the demonstration of three different functional λ subloci (see Section I,C,4) revealed that an individual B cell could potentially produce multiple Ig specificities if one allele from the κ and each of three different functional λ subloci were expressed in an individual cell. However, most B cells express Ig molecules containing only one LC isotype, a phenomenon known as "LC isotype exclusion" (Bernier and Cebra, 1964; Cebra *et al.*, 1966; Pernis *et al.*, 1965). Although the mechanisms of allelic exclusion and isotype exclusion are not completely understood (reviewed by Alt *et al.*, 1992; Okada and Alt, 1995; Willerford *et al.*, 1996), advances in the molecular genetics of antibody formation have provided new insights.

B. Assembly of Ig Genes from Multiple V, (D), and J Segments

1. Theories to Explain Antibody Diversity

The studies of Landsteiner (1945, 1962), Jerne (1955), and others showed that each individual possesses an extraordinary number of antibody specificities. Theories to explain antibody diversity had to explain how the constant region of H and L chains escaped diversification. Proposed models included the germline diversity theory, in which evolution had produced an extensive array of antibody genes in the germline, and the somatic diversification theory, in which a small number of germline genes encoding antibodies were diversified in ontogeny by a prolifically active somatic mutation mechanism (reviewed by Silverstein, 1993).

2. The Hypothesis of Dreyer and Bennet

Drawing on advances in protein sequence data and genetics, Dreyer and Bennett (1965) proposed that single germline genes encode Ig constant regions, whereas multiple separate V region genes encode V regions. They proposed that during the development of each immune cell, one of the multiple V region sequences could become physically associated with the C region sequence to form a complete (V + C) sequence that could then be expressed by the immune cell. Their revolutionary hypothesis envisioned actual rearrangement at the DNA level, resulting in individual immune cells that would then differ from other immune cells and from germline immune cells at the genomic level.

3. *The Discovery of V(D)J Gene Rearrangement*

The hypothesis of Dreyer and Bennett was subsequently confirmed (Hozumi and Tonegawa, 1976) with the demonstration that V and C region genes are widely separated in embryo DNA and are rearranged to be closely linked in adult B cells (Hozumi and Tonegawa, 1976; Lenhard-Schuller *et al.*, 1978; Maki *et al.*, 1980; Max *et al.*, 1979; Sakano *et al.*, 1979; Seidman *et al.*, 1978, 1979; Seidman and Leder, 1978; Tonegawa *et al.*, 1977, 1978). Ig HC V region genes are assembled from multiple germline variable (V_H), diversity (D), and joining (J_H) gene segments, whereas LC genes are assembled from multiple germline V_L and J_L, segments (reviewed by Blackwell and Alt, 1989a; Chen and Alt, 1993; Tonegawa, 1983). V(D)J rearrangement of Ig genes is both lineage specific, occurring only in B cells, and stage specific, occurring only in particular stages of B lymphocyte development.

4. *Mechanism and Proteins in V(D)J Rearrangement*

The variable region genes of all antigen receptor loci, including the single IgH chain locus and the two IgL chain loci (κ and λ), as well as the T-cell receptor (TCR) α and β loci, are assembled by a common V(D)J recombinase during early B-cell differentiation (reviewed by Blackwell and Alt, 1989a). The tissue-specific activities necessary and sufficient to confer recombination activity are encoded by the recombination activating genes (*rag*)-1 and -2 (Oettinger *et al.*, 1990; Schatz *et al.*, 1989). Several generally expressed activities also participate in the V(D)J recombination reaction (reviewed by Lieber *et al.*, 1997). The mechanism of V(D)J rearrangement involves the recognition of specific recombination signal sequences (RSS) found on either side of each germline gene segment, the introduction of double-stranded DNA breaks at the junction of the RSS and the germline gene segment, and the rejoining of coding segments and RSS (reviewed by Gellert, 1997).

5. *Deletional and Inversional Rearrangement*

Rearrangement can occur by either a deletional or an inversional mechanism (Fig. 2). The RSS that flank individual V, D, and J coding segments each consist of three parts: a conserved heptamer, a 12- or 23-bp spacer, and a conserved nonamer sequence (reviewed by Blackwell and Alt, 1989a).

Recombination usually occurs between one segment flanked by an RSS with a 12-bp spacer and another flanked by an RSS with a 23-bp spacer. Recombination between two genes flanked by RSS with opposite orientations, located in the chromosome between the two recombining segments, results in excision of the intervening sequences as a circle

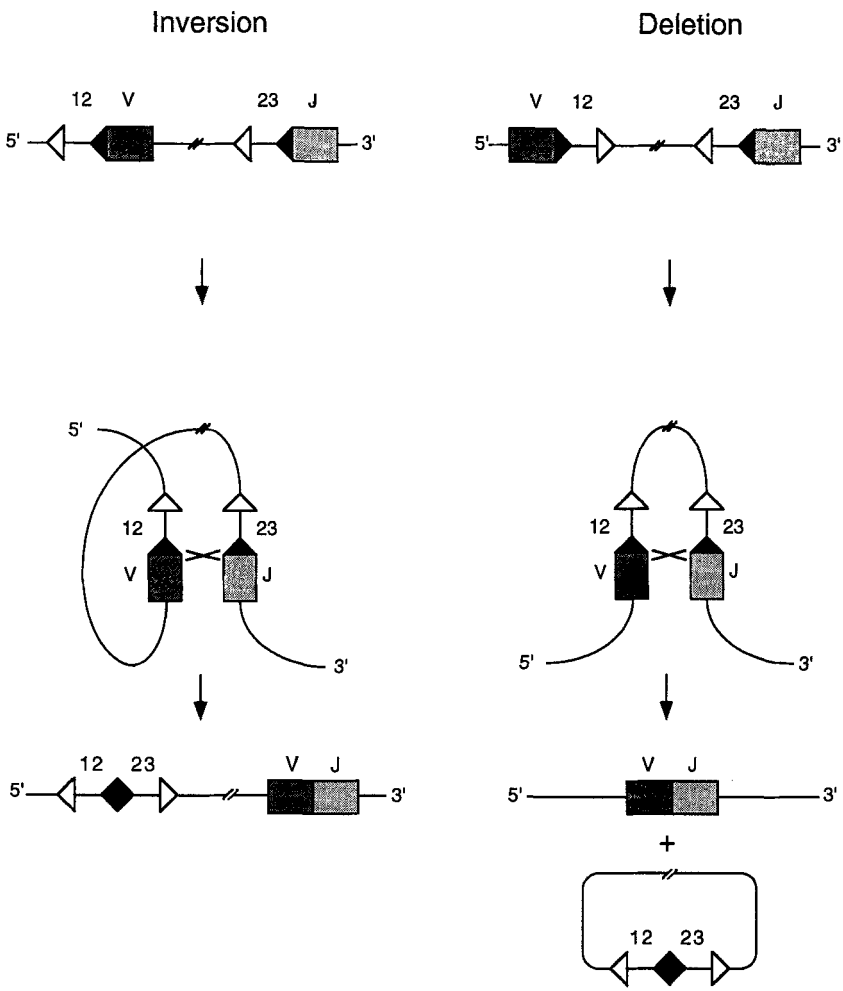


FIG. 2. Ig locus rearrangement by deletion or inversion. Shaded gray rectangles represent V_L and J_L coding sequences, filled triangles represent heptamer sequences, and open triangles represent nonamer sequences. Numbers 12 and 23 represent spacers between the heptamer and the nonamer sequences. Adapted from Lansford *et al.* (1996).

(Fig. 2, "Deletion"). However, recombination between gene segments flanked by two similarly oriented RSS results in inversion of the intervening sequences between the recombining gene segments (Fig. 2, "Inversion").

In some endogenous loci (see Section I,C), V gene segments and RSS are configured such that both deletional and inversional joining can occur. Human and murine κ loci, which contain V_κ segments in both transcriptional orientations, can rearrange by deletion or inversion (Lewis *et al.*, 1982; Selsing *et al.*, 1984), whereas murine λ loci, which contain V segments in a single orientation, rearrange only by deletion (Persiani *et al.*, 1987).

6. *Primary B-Cell Differentiation*

As B cells develop from hematopoietic stem cells in the fetal liver and the adult bone marrow (Figs. 1 and 6), they proceed through highly ordered stages involving Ig gene rearrangement and expression (reviewed by Blackwell and Alt, 1989a; Chen and Alt, 1993; Rolink and Melchers, 1991). Pro-B cells undergo D to J_H and V_H to DJ_H rearrangement. Pre-B cells have successfully completed V_H to DJ_H rearrangement and express μ chain. Immature B cells have completed rearrangement and expression of LC genes and express surface IgM. Mature B cells simultaneously express IgM and IgD by alternate splicing of the HC transcript. These cells migrate to the spleen, lymph node, and Peyer's patches, where they can undergo further antigen-dependent activation and differentiation (Fig. 1A).

B-cell precursors at different developmental stages can be studied through the use of surface markers detectable by flow cytometry (Hardy *et al.*, 1991). All B lineage cells express the markers CD45R/B220 (Fig. 1A). In the flow cytometric system of Hardy and colleagues (1991), sIg^- B-cell precursors in the mouse bone marrow can be resolved into populations that are $B220^+/CD43^+$ and $B220^+/CD43^-$. The $B220^+/CD43^+/sIg^-$ population includes pro-B cells undergoing D to J_H and V to DJ_H rearrangement (Ehlich *et al.*, 1994). Upon productive V(D)J rearrangement and expression of μ, the cells become $B220^+/CD43^-/sIg^-$. Upon rearrangement and expression of LCs, the newly generated immature B cells become $B220^+/CD43^-/sIg^+$.

7. *Generation of Repertoire Diversity*

In humans and mice the diverse primary Ig repertoire, generated primarily by the V(D)J joining process, develops prior to antigen exposure (Fig. 1A). Substantial diversity results from the use of 100 or more different germline V_H and V_κ gene segments, which encode the first three framework regions as well as CDR 1 and 2 (reviewed by Kabat *et al.*, 1991). The use of random combinations of V, (D), and J segments to assemble the CDR3-encoding portion of the V gene introduces tremendous additional diversity (Fig. 1B). The imprecision of the V(D)J

joining process, resulting in the loss or gain of nucleotides at the V(D)J junction, adds still more diversity. Addition of these short stretches of nucleotides, termed N regions, is mediated by activity of the enzyme terminal deoxynucleotidyltransferase (TdT), which is active during HC but not during LC gene rearrangement (Alt and Baltimore, 1982; Bollum, 1974; Gilfillan *et al.*, 1993; Komori *et al.*, 1993). Different LCs and HCs are associated with each other in different cells, adding another level of diversity to the final Ig repertoire.

During antigen-driven clonal expansion (Fig. 1A), somatic hypermutation results in a high frequency of additional individual nucleotide changes throughout the entire assembled V gene, including CDRs 1, 2, and 3 (reviewed by Rajewsky, 1996). The somatic hypermutation process results in altered antibody molecules with differing affinities for antigen. Cells producing antibodies with higher affinity are selected to expand in this process (Manser *et al.*, 1985).

8. Productive and Nonproductive Joining

Because the V(D)J joining mechanism is imprecise, rearrangement can result in joins that do not retain the proper translational reading frame, as determined by the translation initiation codon in the V gene segment. Although the triplet codon predicts that one in three joins will be productive, the fidelity of the joining process can be affected by small regions of homology between the ends of the rearranging segments, which may result in preferential usage of productive or nonproductive reading frames for joining reactions between specific segments (reviewed by Okada and Alt, 1994).

Many lines of experimental evidence have confirmed that about one in three joins are productive in the HC locus (reviewed by Blackwell and Alt, 1988). In the κ locus, early sequencing of joins confirmed that some cells harbor nonproductive joins (Altenburger *et al.*, 1980; Bernard *et al.*, 1981), and the prevalence of pseudogenes in the κ locus of both mice and humans has given rise to estimates that only one in five joins can encode a protein (Cohn and Langman, 1990). However, the occurrence of sequential $V_\kappa J_\kappa$ joining attempts on each κ chromosome (Levy *et al.*, 1989) means that the proportion of κ chromosomes harboring productive joins can be much higher than 0.3. The measured frequency of productive joins per κ chromosome has been estimated to be 0.6, suggesting that sequential κ locus rearrangements are the rule rather than the exception (Arakawa *et al.*, 1996). Sequencing of λ joins has likewise suggested that up to one-half of λ joins in peripheral B cells are productive (Alonso *et al.*, 1985; Zou *et al.*, 1993), possibly due to homology-directed joining.

C. Organization of Ig Loci

1. Overview of Ig Loci

HC and LC gene loci of humans, mice, and other species share common features (Fig. 3). The V gene segments are usually located in the 5′ portion of the locus, whereas the C region exons tend to be clustered in the 3′ end of the locus, with the J segments located between. λ loci have multiple (V) J-C cassettes rather than separate clusters of V, J, and C gene segments. HC loci contain additional segments, the D gene segments, between the V and the D clusters.

2. HC Locus Gene Segments

Murine and human IgH loci bear similar overall organization (reviewed by Honjo and Matsuda, 1995; Lansford *et al.*, 1996). Estimates of the number of mouse V_H segments vary from 100 to several thousand (Berman *et al.*, 1988; Livant *et al.*, 1986; and reviewed by Honjo and Matsuda, 1995). The human locus, which has been extensively mapped and sequenced, contains around 100 V_H gene segments (reviewed by Honjo and Matsuda, 1995), of which about one-third are nonfunctional pseudogenes. The majority of human V_H gene segments have the same transcriptional orientation as the D_H and J_H gene segments, in contrast to the organization of the human κ locus (reviewed by Honjo and Matsuda, 1995; Zachau, 1995). The mouse HC locus has 13 clustered D segments and 4 functional clustered J_H segments, whereas the human locus has 30 clustered D segments and 6 functional clustered J_H segments (Fig. 3).

3. κ Locus Gene Segments

The configurations of murine and human κ LC loci have been studied extensively (Max *et al.*, 1979; Seidman *et al.*, 1978; Tonegawa *et al.*, 1978; and reviewed by Max, 1993). The five murine J_κ segments identified are separated from each other by intronic sequences about 0.3 kb in length (Fig. 3A). One J_κ segment is probably nonfunctional due to a mutated splice donor site, which prevents the $J_\kappa C_\kappa$ intron from being removed.

Two hundred or more mouse V_κ segments are dispersed in the region upstream of the J_κ cluster (Cory *et al.*, 1981; and reviewed by Kofler *et al.*, 1992). Unlike in the mouse λ locus (Fig. 3A), V_κ gene segments lie in both transcriptional orientations with respect to the C_κ gene (reviewed by Zachau, 1989). Rearrangement can therefore occur by either deletion or inversion, as originally postulated based on observations in transformed cell lines undergoing rearrangement in culture (Lewis *et al.*, 1982). Inversions result in retention of intervening V_κ and J_κ coding sequences, possibly facilitating sequential κ locus gene rearrangements.

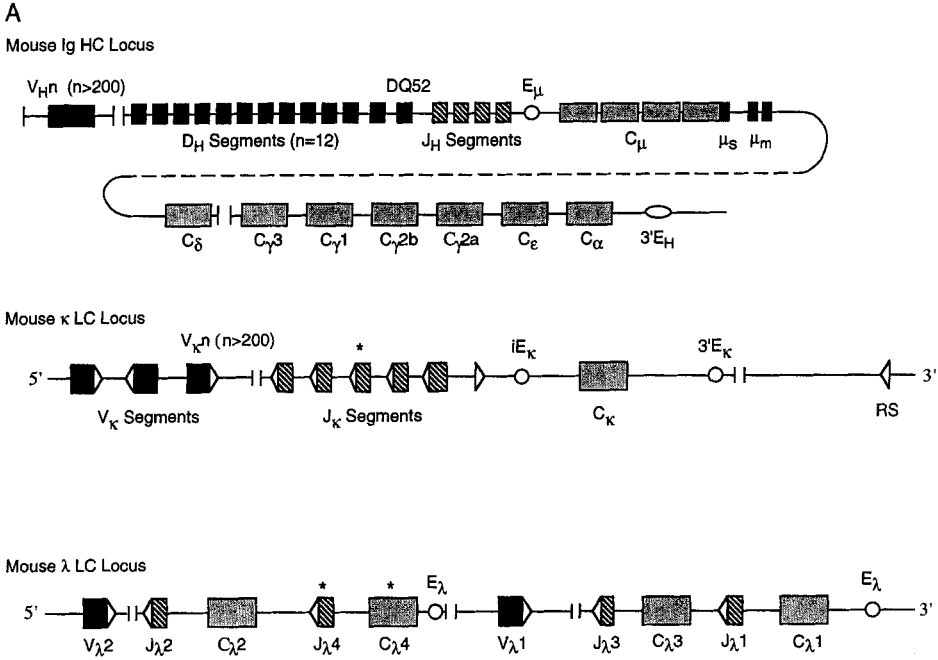


FIG. 3. Structures of (A) murine Ig loci [Adapted from Abbas *et al.* (1991) and Lansford *et al.* (1996)] and (B) human Ig loci. V, variable region gene; D, diversity segment; J, joining segment; C, constant region gene segment. All V gene segments are shaded black. J segments are hatched. D segments are darkly shaded with the most J_H-proximal D segment, DQ52, indicated. C region genes are speckled. Only the μ constant region is shown with its membrane and secreted exons. Enhancers are represented as open circles. Triangles represent RSS. Asterisks indicate pseudogenes. Distances are not drawn to scale. In human V_κ locus, asterisks represent pseudogenes, carats represent genes with single defects or allelic variants, arrows represent transcriptional orientation, and numbers 1 through 7 represent V_κ gene families (Zachau, 1995).

In human κ locus (Fig. 3B), the organization of the 1 C_κ and 5 J_κ gene segments resembles that seen in the mouse locus (Hieter *et al.*, 1980, 1982; and reviewed by Max, 1993). The entire region between J_κ and C_κ gene segments has been sequenced (Whitehurst *et al.*, 1992), and the human κ locus has been mapped extensively (Weichhold *et al.*, 1993; Zachau, 1995). Discovery of a duplicated V_κ gene segment (Bentley and Rabbitts, 1983), followed by more detailed cloning studies (Pech *et al.*, 1985), revealed that much of the V_κ region has undergone a duplication/inversion event (Fig. 3B).

The 76 human V_κ gene segments consist of 10 solitary gene segments and 33 gene segment pairs distributed in opposite orientations in the two duplication units (Weichhold *et al.*, 1990; Zachau, 1995). Thirty-two V_κ

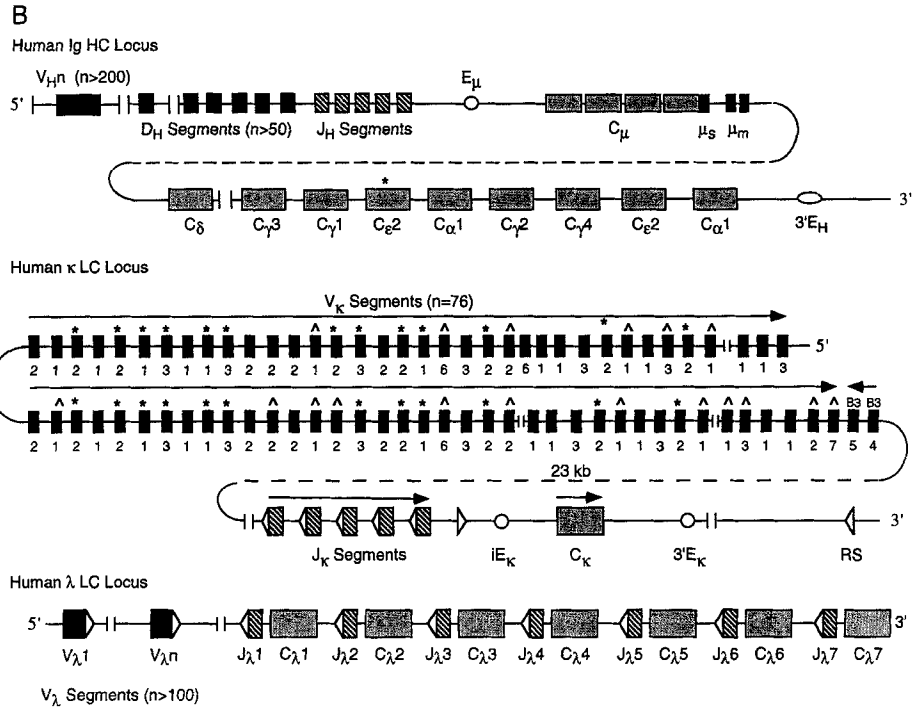


FIG. 3.—*Continued*

gene segments are potentially functional and 25 are clearly pseudogene segments with multiple defects. Of the remaining 19 segments, 3 are known to occur in functional and nonfunctional allelic forms and 16 have minor sequence defects and may therefore also have functional allelic forms. All human V_κ gene segments have been compiled, reviewed, and aligned (Schable and Zachau, 1993).

4. λ Locus Gene Segments

The structure of the murine λ locus (Fig. 3A) has been mapped (Bernard *et al.*, 1978; Dildrop *et al.*, 1987; Miller *et al.*, 1981; Reilly *et al.*, 1984; Sanchez and Cazenave, 1987; and reviewed by Selsing and Daitch, 1995). In the BALB/c mouse strain, in which λ genes have been most fully characterized, each C_λ region is preceded immediately upstream by a J_λ region, and a total of three V_λ and four C_λ gene segments are arranged in two apparent duplication units spanning approximately 200 kb (Miller *et al.*, 1981, 1988; Storb *et al.*, 1989). The two murine $J_\lambda C_\lambda$ clusters seen in most laboratory mice may have arisen by two sequential gene duplication events (Blomberg and Tonegawa, 1982; Selsing *et al.*, 1982). Some strains

of wild mice have larger arrays of λ genes, apparently acquired by additional duplication events (Scott and Potter, 1984a,b; Scott *et al.*, 1982).

The human λ locus (Fig. 3B) contains more than 200 V_λ gene segments (Anderson *et al.*, 1984). Downstream of the V locus are seven C_λ genes, each flanked by a 5′ J_λ gene segment, together spanning a 34-kb region that has been entirely sequenced (Combriato and Klobeck, 1991; Hieter *et al.*, 1981b; Udey and Blomberg, 1987; Vasicek and Leder, 1990). Three C_λ genes are functional, with protein products observed in sera (Hieter *et al.*, 1981b), and three C_λ genes are nonfunctional, containing frameshift mutations or stop codons (Vasicek and Leder, 1990). The seventh C_λ gene appears to be functional (Bauer and Blomberg, 1991; Vasicek and Leder, 1990). Additional polymorphic variants of the human C_λ locus with one to three additional $J_\lambda C_\lambda$ cassettes have been detected in some individuals (Taub *et al.*, 1983).

Unlike the κ locus, the human λ locus appears to be organized with its approximately 100 V_λ gene segments in the same transcriptional orientation as the C_λ genes (Combriato and Klobeck, 1991). In the 60 kb upstream of the $C_\lambda 1$ gene, only one of the six linked V_λ gene segments appears to be functional (Combriato and Klobeck, 1991).

D. Transcriptional Regulation of IgL Loci

1. Transcriptional Regulatory Elements of Ig Loci

Transcription of Ig loci is under multiple levels of control (reviewed by Calame and Ghosh, 1995; Ernst and Smale, 1995a,b; Staudt and Lenardo, 1991). One level involves interactions between *cis*-acting DNA control elements such as promoters and enhancers, with *trans*-acting DNA-binding factors that influence the ability of RNA polymerase II (pol II) to initiate RNA synthesis. Another level involves interactions among chromatin structure, chromosomes, the nuclear matrix/nuclear scaffold, and specialized *cis*-acting control elements including matrix attachments regions (MARs), locus control regions (LCRs), and silencers.

Promoters, located adjacent to each V region, regulate transcription in a distance- and orientation-dependent manner. Promoter elements help determine the start site and direction of transcription, organize formation of the preinitiation complex, including pol II and other general transcriptional components, bind regulatory proteins, and carry out transcriptional activation by these regulatory proteins. Enhancers, found in all antigen receptor loci usually at a distance from the promoter, stimulate increased transcription initiation by promoters in a distance- and orientation-independent manner (reviewed by Dillon and Grosveld, 1993).

In the "rate" model of enhancer action, enhancers increase the rate of transcription from linked promoters by increasing the density of RNA

polymerase on each expressed template. This model is based on nuclear run-on quantitation of transcript production in cells transiently transfected with plasmid constructs either linked or not linked to a simian virus 40 (SV40) enhancer (Treisman and Maniatis, 1985; Weber and Schaffner, 1985). In the "probability" model of enhancer function, based on quantitation of construct expression in single transfected cells rather than in cell populations, enhancers, increase the percentage or number of cells expressing a given locus, rather than the level of locus expression per expressing cell (Magis *et al.*, 1996; Moon and Levy, 1990; Walters *et al.*, 1995, 1996; Weintraub, 1988). Thus, Ig locus enhancers may act to increase the percentage or number of B lymphocytes expressing the HC (or κ or λ) locus at a given stage of development.

Between the J and the C regions, the κ locus and the HC locus each have an intronic enhancer closely associated with MARs, termed E_μ in the HC locus and iE_κ in the κ locus (Cockerill *et al.*, 1987; Emorine *et al.*, 1984; Gimble and Max, 1987; Parslow and Granner, 1982; Picard and Schaffner, 1984a). Additional B-cell-specific enhancers have been described in the 3′ regions of HC, κ, and λ loci (Figs. 3 and 4; Dariavach *et al.*, 1991; Judde and Max, 1992; Lieberson *et al.*, 1991; Meyer and Neuberger, 1989; Muller *et al.*, 1990; Pettersson *et al.*, 1990). The 3′ IgH enhancer ($3'E_H$) is located at the most 3′ end of the HC locus. The 3′ κ enhancer ($3'E_\kappa$, Fig. 3) lies 9–12 kb downstream of C_κ (Judde and Max, 1992; Meyer and Neuberger, 1989; Muller *et al.*, 1990), and a λ enhancer lies downstream of each major duplication unit of the λ locus (Fig. 3A).

2. *Tissue- and Stage-Specific Enhancers in the Ig HC Locus*

In the HC locus (Fig. 3), E_μ is active throughout B-cell development, including at progenitor B-cell stages, and has been used to target transgene expression to lymphoid cells (Adams *et al.*, 1985). E_μ contains a promoter for germline HC transcripts (also termed "sterile transcripts" because they do not appear to encode a protein) and activates transcription of these germline transcripts prior to the initiation of V(D)J rearrangement (Chen *et al.*, 1993c; Ferrier *et al.*, 1990; Su and Kadesch, 1990). Eμ appears to permit or promote HC V(D)J rearrangement (reviewed by Sleckman *et al.*, 1996). Specific deletion of Eμ results in a modest diminution of D to J_H rearrangement and a substantial diminution in V_H to DJ_H rearrangement (Serwe and Sablitzky, 1993), whereas replacement of Eμ with a drug resistance gene results in dramatic inhibition of D to J_H rearrangement (Chen *et al.*, 1993c), suggesting that other elements in addition to Eμ can promote V(D)J rearrangement in the absence of Eμ. Eμ also appears to play an important role in activating transcription from rearranged HC genes through its interaction with V_H promoter elements (Calame and

Ghosh, 1995). Thus, E_μ appears to play an important role prior to, during, and after V(D)J rearrangement, in stimulating transcription of the unrearranged locus, in promoting rearrangement, and in driving transcription of the rearranged locus.

In cells that lack E_μ, HC expression is still observed (Aguilera *et al.*, 1985; Klein *et al.*, 1984; Wabl and Burrows, 1984), apparently stimulated by $3'E_H$ (Lieberson *et al.*, 1995), which appears to be active at mature B and plasma cell stages (Dariavach *et al.*, 1991; Lieberson *et al.*, 1991; Pettersson *et al.*, 1990). Gene targeted deletion of the $3'E_H$, unlike deletion of E_μ, does not affect V(D)J rearrangement, but rather appears to play a role at later stages of B-cell development, possibly affecting transcription of and rearrangement to downstream Ig constant regions involved in HC isotype switching (Cogne *et al.*, 1994).

3. *Tissue- and Stage-Specific Enhancers in the IgL κ Locus*

In the κ locus (Fig. 4), the iE_κ element becomes active during the pre-B to B-cell transition (Atchison and Perry, 1987; Lenardo *et al.*, 1987; Sen and Baltimore, 1986a,b). Of the various binding sites in the iE_κ, only the 10-bp κB site (Fig. 4) is developmentally regulated in B-cell lines. κB binds factors of the *rel* family of transcription factors, together referred to as NF-κB, which are constitutively active in the nuclei of mature B cells and plasma cells. In pre-B cell lines, NF-κB is found in an inactive complex in the cytoplasm, bound to the inhibitor IκB, until a variety of stimuli causing posttranslational modification result in dissociation from IκB, and transfer to the nucleus in an active form (Lenardo *et al.*, 1987; Nelson *et al.*, 1984; Sen and Baltimore, 1986a,b). Stimulation of transformed pre-B-cell lines with bacterial lipopolysaccharide (LPS) results in activation of NF-κB, increased germline transcription, and $V_\kappa J_\kappa$ rearrangement (Atchison and Perry, 1987; Lenardo *et al.*, 1987; Schlissel and Baltimore, 1989). Similarly, in a pre-B-cell line transformed with a temperature-sensitive virus, growth at the restrictive temperature results in a removal of viral suppression of NF-κB, accompaned by κ locus germline transcription and rearrangement (Y. Y. Chen *et al.*, 1994; Klug *et al.*, 1994). Thus, developmental regulation of NF-κB activity may play a crucial role in activation of the iE_κ, induction of κ locus germline transcription, and $V_\kappa J_\kappa$ rearrangement. However, gene targeted disruption of the rel family members p50, c-rel, or RelB does not affect B-cell surface κ expression in mutant mice (Kontgen *et al.*, 1995; Sha *et al.*, 1995; Weih *et al.*, 1995). Whether this is due to functional redundancy between different Rel family members or whether other elements can substitute for the NF-κb interaction with iE_κ is unclear from these studies.

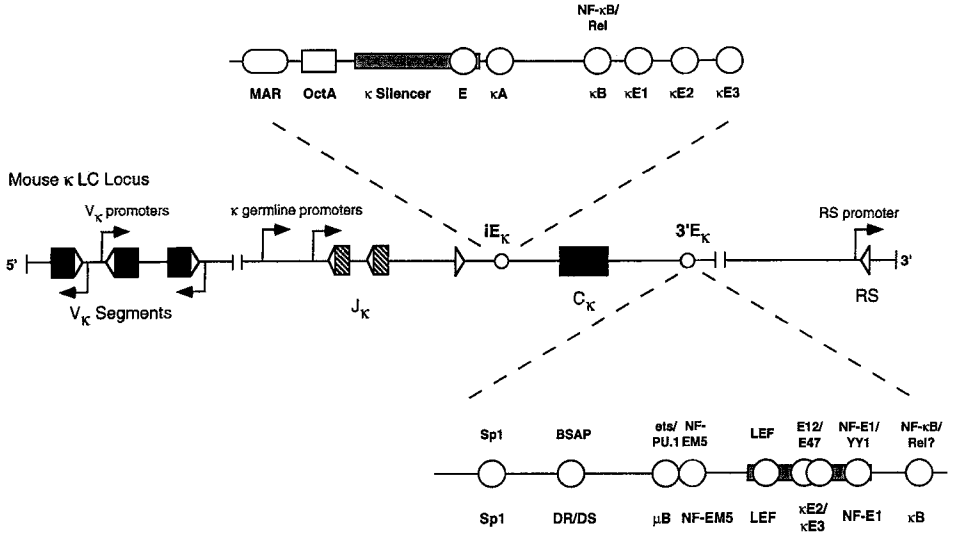


FIG. 4. Enhancers in the Ig LC κ locus. Schematic diagram of promoters and enhancers in the Ig LC κ locus (Max, 1993). Promoters are shown as arrows indicating transcriptional orientation. Enhancers are shown as open circles. Representative V segments are shown as black boxes, J segments as cross-hatched boxes, and C segments as white dots on a black background. Schematics of selected motifs in iE_κ and $3'E_\kappa$ are located above and below the locus, respectively. Names of DNA sequence motifs are indicated below the relevant motif. Proteins discussed in text that may bind motifs are indicated above some motifs. Silencer regions are shown as gray-shaded rectangles. Adapted from Calame and Ghosh (1995) and Max (1993).

The observation of high-level κ chain production in the S107 plasmacytoma line, which does not express NF-κB (Atchison and Perry, 1987), led to the identification of $3'E_\kappa$ (Meyer and Neuberger, 1989). The importance of $3'E_\kappa$ for transcriptional enhancement has been demonstrated in transient and stable transfection assays (Blasquez *et al.*, 1989, 1992; Fulton and Van Ness, 1993; 1994; Meyer and Neuberger, 1989; Meyer *et al.*, 1990; Pongubala and Atchison, 1919) and in mice carrying transgenic κ expression constructs (Betz *et al.*, 1994; Meyer *et al.*, 1990; Xu *et al.*, 1989). The activity of $3'E_\kappa$ is weakly indicible by LPS in pre-B-cell lines and is constitutively active in mature B-cell and plasma cell lines (Pongubala and Atchison, 1991). $3'E_\kappa$ is weaker than iE_κ in pre-B-cell lines and stronger than iE_κ in plasma cell lines, suggesting that $3'E_\kappa$ may be inactive at the pre-B-cell stage and active at the B-cell and plasma cell stages (Klug *et al.*, 1994; Pongubala and Atchison, 1991).

The 132-bp core of $3'E_\kappa$ does not contain NF-κB sites (Pongubala and Atchison, 1991), but does bind a B-cell-specific complex consisting of the

ets-family member PU.1 and the associated factor NF-EM5 (Pongubala *et al.*, 1992, 1993). The 3′E_κ also contains a direct repeat, which binds the B-cell-specific transcription factor BSAP/Pax-5 (Roque *et al.*, 1996). BSAP is found in both pre-B and B cells and can both enhance and inhibit transcription of various pre-B and B-cell-specific genes, including CD19, λ5, V_{preB}, *blk*, and some HC constant region germline transcripts (reviewed by Busslinger and Urbanek, 1995).

Flanking the core 3′E_κ enhancer element is a "silencer," the deletion of which increases the transcription of reporter constructs (Park and Atchison, 1991). This sequence is bound by the ubiquitous protein NF-E1/YY-1 and by two lymphoid-specific proteins, LEF-1 (Meyer and Ireland, 1994), and an E12/E47-like helix-loop-helix transcription factor (Meyer and Ireland, 1994; Park and Atchison, 1991). LPS stimulation decreases LEF-1 binding to the silencer, a finding that may suggest a second pathway by which LPS can induce κ locus germline transcription and activation (Meyer and Ireland, 1994).

4. *Gene Targeting Studies of the IgL κ Locus*

IgL κ rearrangement has been completely inactivated by gene targeted replacement of the $J_\kappa C_\kappa$ region (Chen *et al.*, 1993a), of the iE_κ element (Takeda *et al.*, 1993; Xu *et al.*, 1996a), or of the C_κ region (Zou *et al.*, 1993) with a *neo*^{r} gene (Fig. 5). $J_\kappa C_\kappa$ mutant mice (Chen *et al.*, 1993a) and

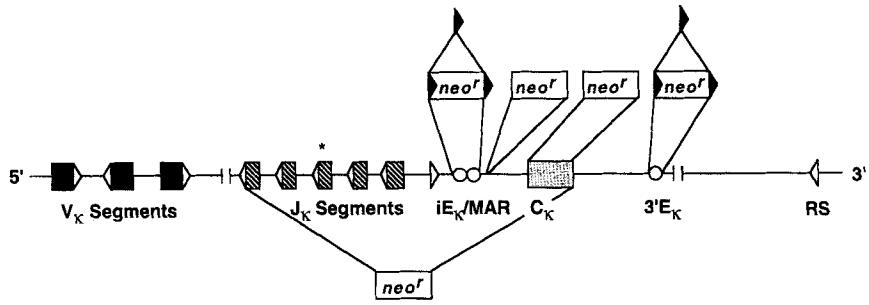


FIG. 5. Murine Ig LC κ locus gene targeting studies. V, variable region gene; D, diversity segment; J, joining segment; C, constant region gene segment. Enhancers are shown as open circles. Representative V segments are shown as black boxes, J segments as cross-hatched boxes, and C segments, as gray dots on white background. Triangles represent RSS. Asterisks indicate pseudogenes. Distances are not drawn to scale. Black triangles represent *loxP* sites. White rectangles represent the *neo*^{r} gene segment, with lines drawn to endogenous elements that have been replaced by the *neo*^{r} gene segment. Black triangles above the *loxP*-flanked *neo*^{r} gene segment indicate initial replacement of the endogenous element by the *loxP*-flanked *neo*^{r} gene, followed by the Cre-*loxP*-mediated deletion of the *neo*^{r} gene.

iE_κ *neo*^{r} replacement mutant mice (Takeda *et al.*, 1993; Xu *et al.*, 1996a) do not rearrange, delete, or express the κ locus, whereas mutant mice in which iE_κ is replaced by a *loxP* site undergo $V_\kappa J_\kappa$ rearrangement at a reduced level (Xu *et al.*, 1996b). C_κ replacement with a *neo*^{r} gene (Zou *et al.*, 1993) eliminates κ chain production and surface expression, but not $J_\kappa C_\kappa$ rearrangement. Replacement of $3'E_\kappa$ by a *loxP* sequence (Fig. 5) altered the $\kappa:\lambda$ ratio, significantly decreased preferential κ versus λ rearrangement, and partially inhibited κ locus expression in resting B cells (Gorman *et al.*, 1996). $3'E_\kappa$ replacement by a *neo*^{r} gene led to a similar but more pronounced phenotype (Gorman *et al.*, 1996). These mutational studies and their implications are discussed in more detail later.

II. Allelic Exclusion of Immunoglobulin Loci

A. Regulated and Stochastic Models of Allelic Exclusion

Among antigen receptor loci, allelic exclusion has been observed at HC, LC, and TCRβ loci (reviewed by Willerford *et al.*, 1996). Various models have attempted to explain how allelic exclusion occurs at antigen receptor loci (reviewed by Alt *et al.*, 1992; Blackwell and Alt, 1989b). Proposed models included regulated models (F. Alt *et al.*, 1981; F. W. Alt *et al.*, 1980), unregulated models, which included stochastic models (Beck-Engeser *et al.*, 1987; Coleclough, 1983; Coleclough *et al.*, 1981), and other models, such as counterselection models (Wabl and Steinberg, 1982).

The regulated model of LC allelic exclusion, as originally proposed, suggested that V(D)J rearrangement is ordered, proceeding on one LC chromosome at a time, and that protein products expressed as a result of the V_LJ_L rearrangement process play a critical role in mediating LC allelic exclusion (see Section II,F). A similar model was subsequently proposed linking ordered HC gene rearrangement with HC allelic exclusion (F. Alt *et al.*, 1981; F. W. Alt *et al.*, 1984). Together, these models proposed that regulation of Ig gene assembly actively prevents assembly of multiple productive and functional HC or LC gene segments in a single B lymphocyte (F. Alt *et al.*, 1981; F. W. Alt *et al.*, 1980, 1984; and reviewed by Yancopoulos and Alt, 1986).

Stochastic models, unlike regulated models, proposed that inefficient V(D)J rearrangement mediates allelic exclusion (Coleclough, 1983; Coleclough *et al.*, 1981). In these models, although V(D)J rearrangement proceeds simultaneously on both HC and LC alleles, both alleles are rarely expressed in an individual B cell because the generation of productive and functional joins occurs infrequently (Coleclough *et al.*, 1981; Walfield *et al.*, 1981). Counterselection models proposed that apparent HC allelic exclusion occurs, even though HC rearrangement proceeds simultaneously

on both alleles because expression of both HC loci is toxic, resulting in counterselection of double HC-expressing cells (Wabl and Steinberg, 1982).

In contrast to the predictions of stochastic models based on inefficient V(D)J joining, many lines of experimental evidence have confirmed that the V(D)J joining process is relatively efficient. About one in three Ig HC joins are productive (reviewed by Blackwell and Alt, 1988). Counterselection models were tested by the production of transgenic mice expressing multiple HC and LC transgenes. The resultant double-expressing cells did not appear to undergo any inherent counterselection (Gollahon *et al.*, 1988; Goodhardt *et al.*, 1987; Neuberger *et al.*, 1989; Stall *et al.*, 1988). Gene targeted mice bearing two productively rearranged HC genes in endogenous Ig HC loci have shown that biallelic HC expression is not inherently toxic (Sonoda *et al.*, 1997). Thus, neither counterselection models nor purely stochastic models explain allelic exclusion.

B. Ordered HC Rearrangement and HC Allelic Exclusion

HC rearrangement precedes LC rearrangement during B-cell differentiation (Alt *et al.*, 1981; Korsmeyer *et al.*, 1981; Maki *et al.*, 1980; Perry *et al.*, 1981; Siden *et al.*, 1981). During HC gene rearrangement, DJ_H rearrangement precedes V_H to DJ_H rearrangements (Alt *et al.*, 1984). These DJ_H rearrangements can serve as a target for V_H to DJ_H rearrangements (Alt *et al.*, 1984) or can be deleted by joining of an upstream D segment to a downstream J_H segment (M. G. Reth *et al.*, 1986). Because DJ_H rearrangements were found on both chromosomes in most normal and transformed pre-B and B lymphocytes (Alt *et al.*, 1981; Coffman and Weissman, 1983; Coleclough *et al.*, 1981; Nottenberg and Weissman, 1981; Sakano *et al.*, 1981), as well as in some pre-T lymphocytes (Alt *et al.*, 1982; Kurosawa and Tonegawa, 1981), whereas V_H to DJ_H rearrangements were found only in B cells and productive V_HDJ_H joins were generally found on only one chromosome (Alt *et al.*, 1984), the V_H to DJ_H joining process was proposed to be the regulated step in HC gene assembly (Alt *et al.*, 1984).

A regulated model of allelic exclusion provided a mechanistic link between the observations of ordered HC rearrangement and HC allelic exclusion (F. Alt *et al.*, 1981; F. W. Alt *et al.*, 1984). In this model, HC V_H to DJ_H rearrangement proceeds on one chromosome at a time, and successful V_H to DJ_H rearrangement results in μ HC protein expression, thereby shutting down further HC gene rearrangement. After a nonproductive V_H to DJ_H rearrangement on the first HC allele, the cell can undergo V_H to DJ_H rearrangement on the other allele. A significant percentage of sIg-expressing B lymphocytes retain one allele in the DJ_H configuration, consistent with this proposal (Alt *et al.*, 1984). HC protein expression was

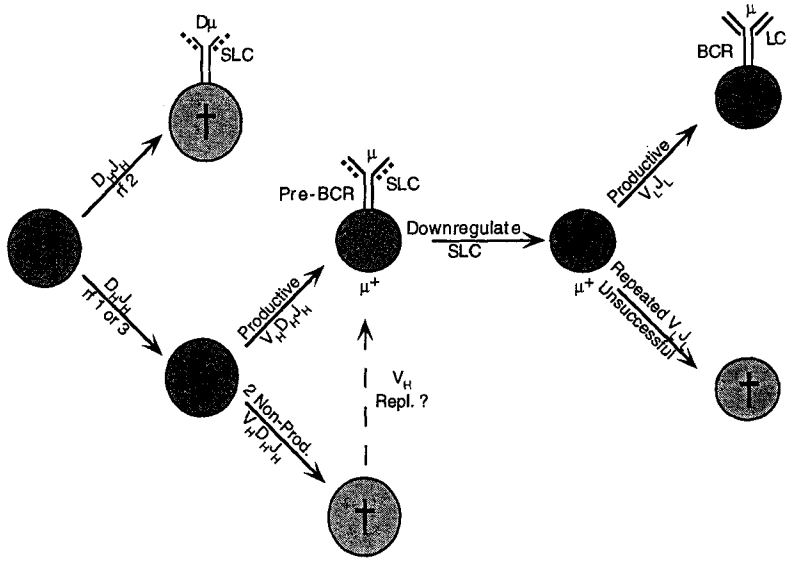
further proposed to activate LC locus gene rearrangement (Alt *et al.,* 1981). The requirement that HC be expressed prior to LC was postulated to fix the HC component of an Ig molecule, thereby providing the cell with a means to test for a functional LC gene rearrangement through combination of the LC with the preexisting intracellular HC (Alt *et al.,* 1981). If a given LC could not associate, LC rearrangement would continue until a LC was produced that could pair in a functional manner with the HC molecule or until no further LC rearrangements were possible.

C. Role of μ Protein and the Pre-BCR in HC Allelic Exclusion and Prelymphocyte Developmental Progression

Introduction of a surface μ HC transgene into A-MuLV cell lines resulted in cessation of rearrangement of HC V gene segments and initiation of κ LC gene rearrangement (Reth *et al.,* 1985). Similarly, rearrangement of mouse endogenous HC genes is significantly inhibited by transgenes directing expression of a μ or δ HC to the B-cell surface (Herzenberg *et al.,* 1987; Manz *et al.,* 1988; Nussenzweig *et al.,* 1987, 1988; Rusconi and Kohler, 1985; Stall *et al.,* 1988; Weaver *et al.,* 1985; and reviewed by Storb, 1987). Secreted IgM did not inhibit rearrangement or expression of endogenous HC genes in transfected cell lines or HC-transgenic mice (Nussenzweig *et al.,* 1987; Reth *et al.,* 1987; Storb *et al.,* 1986). These data supported the model that the membrane form of μ and/or δ HC inhibits further rearrangement of HC variable region genes.

Prior to LC gene rearrangement, the μ HC is thought to associate with the products of the $\lambda 5$ and V_{preB} genes (Karasuyama *et al.,* 1990; Nishimoto *et al.,* 1991; Pillai and Baltimore, 1987; Tsubata and Reth, 1990), which can form a surrogate LC (SLC; Fig. 6). In both pre-B and B cells, surface μ HC also associates with two other transmembrane proteins, Igα and Igβ (reviewed by Borst *et al.,* 1996), to form the pre-B-cell receptor (pre-BCR) in pre-B cells and the BCR in B cells. Although pre-BCR surface expression is not consistently detected on pre-B cells *in vivo* (Cherayil and Pillai, 1991; Karasuyama *et al.,* 1994; Lassoued *et al.,* 1993; Spanopoulou *et al.,* 1994; Young *et al.,* 1994) and the signaling function of the pre-BCR in untransformed mouse and human pre-B cells has not been conclusively demonstrated *in vitro,* the role of the HC in allelic exclusion has been proposed to be mediated through its participation in the pre-BCR (Bauer and Scheuermann, 1993).

Direct evidence for the role of the pre-BCR in mediating HC allelic exclusion has come from mice that can produce secreted but not membrane μ HC due to gene targeted disruption of the μ chain membrane exon (Kitamura and Rajewsky, 1992; Kitamura *et al.,* 1991). B cells in the bone marrow of these mice show substantially increased HC allelic inclusion



Stage:	**stem cell/ pro-B**	**pro-B**	**large pre-B**	**small pre-B**	**immature B**
Rearrangement:	D_H to J_H	V_H to D_HJ_H			V_L to J_L
Other regulation:			**proliferation, transient Rag downregulation**		**exit from cell cycle**

FIG. 6. Ordered rearrangement and B-cell development in the bone marrow. V, variable region gene; D, diversity segment; J, joining segment; C, constant region gene segment; rf, reading frame; SLC, surrogate light chain; μ, surface μ HC; BCR, B-cell receptor; Non-Prod., nonproductive $V_HD_HJ_H$ rearrangement. Cells with a cross represent cells destined to die in the bone marrow. Dashed arrows represent possible pathways. Expression of the pre-BCR inhibits further HC gene rearrangement, resulting in HC allelic exclusion, transiently downregulates Rag expression, induces pre-B-cell proliferation, and may activate LC gene rearrangement. Expression of the BCR generally inhibits further LC gene rearrangement, resulting in LC allelic and isotype exclusion. Cells with two nonproductive $V_HD_HJ_H$ rearrangements may be rescued by rearrangement of an upstream VH gene to a partial RSS in the assembled $V_HD_HJ_H$ segment (M. Reth *et al.*, 1986). Adapted from Rajewsky (1996).

(productive rearrangement on both HC alleles) rather than allelic exclusion, supporting the critical role of the μ HC in the normal mediation of HC allelic exclusion (Kitamura *et al.*, 1992). The role of the pre-BCR in mediating allelic exclusion was also apparent in gene targeted mice lacking

the λ5 gene (Loffert *et al.*, 1996). Developing but not peripheral B lymphocytes in λ5 mutant mice, which cannot express a functional μ-containing pre-B surface receptor, are allelically included rather than excluded (Loffert *et al.*, 1996). The failure of allelically included B cells cannot be explained by simple counterselection because B cells in gene targeted mice carrying two productively rearranged HC genes appear to undergo normal development (Sonoda *et al.*, 1997). Thus, expression of the Pre-B cell receptor inhibits further rearrangement of HC genes.

The product of a DJ_H rearrangement is sometimes expressed as a truncated protein (Fig. 6) referred to as D_μ (Reth and Alt, 1984) when DJ_H rearrangement occurs in one of the three possible DJ_H reading frames, termed rf2 (Gu *et al.*, 1991; Kaartinen and Makela, 1985; Meek, 1990). The D_μ protein can be expressed on the cell surface and can also associate with the λ5 and V_{preB} gene products (Tsubata *et al.*, 1991). B cells expressing the D_μ protein appear to be arrested in further development by a shutdown of V_H to DJ_H rearrangement (Loffert *et al.*, 1996), as had been hypothesized earlier (Gu *et al.*, 1991; Reth *et al.*, 1985). This arrest of B-cell development by the D_μ-containing surface receptor appears to resemble allelic exclusion mediated by the μ-HC-containing pre-B cell receptor.

In cell lines the HC can, through its association with Igα and Igβ, stimulate aggregation of nonreceptor tyrosine kinases (NRTKs), including Lyn, Fyn, Blk, Syk, and Lck (reviewed by Chan and Shaw, 1996; Owen and Venkitaraman, 1996; Weiss and Littman, 1994). These NRTKs initiate further intracellular signaling, thereby inhibiting endogenous gene rearrangement and mediating allelic exclusion. The strongest evidence for the role of signaling in mediating allelic exclusion has been found in T cells, where surface expression of β chain from a productive TCRβ transgene suppresses endogenous Vβ to DJβ rearrangement to an undetectable level (Uematsu *et al.*, 1988). Overexpression of a catalytically active form of Lck results in a similar inhibition of endogenous Vβ to Dβ rearrangement, suggesting that Lck signals allelic exclusion at the β locus (Anderson *et al.*, 1992). In contrast, in a doubly transgenic mouse line expressing a TCRβ transgene and a catalytically inactive (dominant negative) form of the Lck kinase (Anderson *et al.*, 1993), substantial rearrangement of endogenous Vβ gene segments occurred, suggesting that Lck may normally signal allelic exclusion at the β locus.

Antigen receptor gene products and their accessory signaling molecules also appear critical for orderly lymphocyte developmental progression. In mice lacking the *rag-1* or *rag-2* gene, inability of the prelymphocytes to undergo V(D)J rearrangement completely blocks lymphocyte development at the pro-B and double-negative thymocyte stages (Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1992). Addition of a TCRβ transgene allows develop-

ment of double-positive thymocytes (Shinkai *et al.*, 1993), whereas a μ HC transgene allows appearance of pre-B cells (Spanopoulou *et al.*, 1994; Young *et al.*, 1994). An Ig LC transgene with the μ HC transgene results in the appearance of surface IgM-expressing B cells (Spanopoulou *et al.*, 1994; Young *et al.*, 1994), and a TCRα transgene with the TCRβ transgene results in the appearance of single-positive thymocytes as well as TCR-expressing peripheral T cells (Mombaerts *et al.*, 1992a; Shinkai *et al.*, 1993). Thus, expression of antigen receptor gene products permits prelymphocytes to differentiate into lymphocytes in an orderly fashion (reviewed by Willerford *et al.*, 1996).

Targeted disruption of the *Ig*β gene blocks the pro-B to pre-B-cell transition (Gong and Nussenzweig, 1996), severely inhibiting V_H to DJ_H but not D to J_H joining. In contrast, gene targeted disruption of the C-terminal cytoplasmic tail-encoding portion of the *Ig*α gene dramatically decreases the number of peripheral B cells, without significantly affecting the pro-B to pre-B and the pre-B to B-cell transitions, pre-BCR or BCR assembly, or allelic exclusion. In T lymphocytes, accessory molecules similar to Igα and Igβ associate with the TCR and are referred to as the CD3 complex, comprising CD3 γ, δ, ε, and ζ. They mediate signaling by the pre-TCR and the TCR (reviewed by Owen and Venkitaraman, 1996). A profound block in early thymocyte development is seen in mice lacking components of the CD3 complex (Liu *et al.*, 1993; Love *et al.*, 1993; Malissen *et al.*, 1995).

The signaling portions of the BCR and the TCR can activate Syk in B cells or ZAP-70 in T cells (reviewed by Chan and Shaw, 1996), resulting in Ras activation and calcium mobilization (reviewed by Chan and Shaw, 1996). *Syk* gene disruption blocks development at the pre-B to B-cell transition in mice (Cheng *et al.*, 1995; Turner *et al.*, 1996) and renders BCR signaling defective in chicken B cells (Takata *et al.*, 1994). An analogous block in lymphocyte development occurs in mice lacking the Lck tyrosine kinase (Molina *et al.*, 1992), the λ5 SLC (Kitamura *et al.*, 1992; Loffert *et al.*, 1996), or the analogous gp33/pre-Tα component of the pre-TCR (Fehling *et al.*, 1995; Groettrup *et al.*, 1993; Saint-Ruf *et al.*, 1994; Xu *et al.*, 1996b) and in mice or humans lacking ZAP-70 (Arpaia *et al.*, 1994; Chan *et al.*, 1994; Elder *et al.*, 1994; Gelfand *et al.*, 1995; Negishi *et al.*, 1995; reviewed by Chan and Shaw, 1996). These studies indicate that the BCR and the TCR, including the subunits that initiate intracellular signaling, as well as the Syk, Lck, and ZAP-70 NRTKs that associate with them on activation, play critical roles in the development of pre-B and pre-T cells.

D. RAG Gene Expression and HC Allelic Exclusion

Ordered rearrangement appears to result because HC expression inhibits V_H to DJ_H rearrangement and helps activate LC gene rearrangement. This

shift must require decreased HC locus and increased LC locus accessibility to V(D)J recombinase, mediated by *cis*-acting elements, including enhancers and silencers (reviewed by Sleckman *et al.*, 1996). The cessation of LC gene rearrangement after expression of functional Ig may be mediated in part by a downregulation of V(D)J recombinase activity. For example, cross-linking of surface IgM on a Rag-expressing B-cell tumor line or of surface TCR on CD4/CD8-expressing double-positive thymocytes results in a rapid downregulation of Rag expression (Brandle *et al.*, 1992; Ma *et al.*, 1992). *Rag* gene expression is transiently decreased in large μ-expressing pre-B cells (Fig. 6), but is again increased in μ-expressing small pre-B cells, in which LC gene rearrangement predominantly occurs (Grawunder *et al.*, 1995; Loffert *et al.*, 1996). This transient downregulation may inhibit further HC gene rearrangement while downregulation of accessibility is being affected at the HC locus.

E. Role of HC and Pre-BCR in LC Locus Activation

Because HC rearrangement and expression tend to occur before that of LC and because most LC-rearranging Abelson murine leukemia virus (A-MuLV) transformants produce or formerly produced μ HC, the μ protein was proposed to activate LC locus rearrangement (F. Alt *et al.*, 1981; F. W. Alt *et al.*, 1984; Reth and Alt, 1984). A-MuLV transformants expressing μ_m from a productive V_H to DJ_H rearrangement (Reth *et al.*, 1985) or from introduction of a μ_m but not a μ_s expression vector into A-MuLV transformants harboring two nonproductive V_HDJ_H rearrangements (Era *et al.*, 1991; Iglesias *et al.*, 1991; Reth *et al.*, 1987) can undergo $V_\kappa J_\kappa$ rearrangement (Schlissel and Morrow, 1994; Shapiro *et al.*, 1993; Tsubata *et al.*, 1992). In transgenic mice, μ_m but not μ_s expression led to endogenous LC gene rearrangement (Nussenzweig *et al.*, 1987; Ritchie *et al.*, 1984).

Although HC appears to play a major role in promoting activation of the LC locus, its requirement for LC locus activation is not absolute. For example, rearrangement of V_κ gene segments has been described in pre-B cells lacking surface HC, including transformed cell lines (Blackwell *et al.*, 1989; Kubagawa *et al.*, 1989; Schlissel and Baltimore, 1989) and transformed and untransformed bone marrow-derived pre-B cells (Grawunder *et al.*, 1993). In some of these pre-B-cell lines, μ HC may have been expressed and then lost. In gene targeted mice lacking surface μ chains (Kitamura and Rajewsky, 1992), the $\lambda 5$ gene (Kitamura *et al.*, 1992), or the J_H locus (Chen *et al.*, 1993b; Gu *et al.*, 1993), neither the pre-BCR complex nor HC gene rearrangement is absolutely required for the induction of low levels of κ LC gene rearrangement (Chen *et al.*, 1993b; Ehlich *et al.*, 1993). However, this pathway may be enhanced by the developmental arrest imposed by these targeted mutations and likely

represents a minor pathway in normal B-cell development. Thus, substantial evidence supports the role of μ_m expression in stimulation of LC locus activation.

F. LC Allelic Exclusion

As in Ig HC allelic exclusion, the protein products of productive Ig LC gene rearrangements appear to play, through the Ig molecule, a critical role in mediating LC allelic exclusion. Characterization of B lineage cell lines that produce multiple LC proteins, but express only one LC protein on the surface, suggested that regulation of Ig LC gene rearrangement, feedback, and allelic exclusion requires more than productive $V_\kappa J_\kappa$ or $V_\lambda J_\lambda$ gene rearrangement and protein production (Alt *et al.*, 1980; Bernard *et al.*, 1981; Kuehl *et al.*, 1975; Kwan *et al.*, 1981). The LC protein must also associate with the μ HC in a functional manner in order to mediate feedback inhibition of further LC gene rearrangement (Alt *et al.*, 1980). In support of this model, studies of transgenic mice have revealed that high level expression of a functional transgenic κ or λ chain generally inhibits the rearrangement of endogenous LC genes (Brinster *et al.*, 1983; Doglio *et al.*, 1994; Hagman *et al.*, 1989; Neuberger *et al.*, 1989; Ritchie *et al.*, 1984; Sharpe *et al.*, 1991; Storb *et al.*, 1984, 1986; Xu *et al.*, 1989; and reviewed by Storb, 1987, 1995; Storb *et al.*, 1992). These transgenic results suggest that feedback from expressed endogenous LC in normal lymphocytes plays a critical role in mediating allelic exclusion.

G. Possible Mechanisms of Asynchronous HC or LC Gene Rearrangement

Although feedback mechanisms can explain how rearrangement ceases following protein expression from a productive join, feedback alone cannot explain allelic exclusion. If most developing B lymphocytes initiated V_H to DJ_H rearrangement simultaneously at both allelic Ig loci, a substantial percentage of B lymphocytes would exhibit allelic inclusion. The fact that allelic exclusion is almost always observed suggests that in a given B lymphocyte, the two alleles at each Ig locus may not initiate rearrangement simultaneously. The mechanism by which HC or LC locus alleles rearrange one at a time is not known. Asynchronous rearrangement, like the control of locus expression by enhancers, could be accomplished by either stochastic or regulated mechanisms.

In a stochastic mechanism of asynchronous rearrangement, allelic loci would be regulated independently, and each cell could potentially express both loci simultaneously. In this model, enhancer activity would need to increase the probability of expression of a given Ig locus such that most cells would express one allele. However, increasing the likelihood of expres-

sion of a given locus to the degree that most cells express at least one allele would likely result in a substantial proportion of cells expressing both alleles. Alternately, chromosomal accessibility could be regulated such that only one allele at a time at each locus could potentially become accessible for transcription or recombination in a given cell. Enhancers might then increase the percentage of cells actually expressing the single potentially expressible allele so that most or all cells express one allele. The very high percentage of B cells exhibiting allelic exclusion rather than inclusion supports a regulated rather than a stochastic model of asynchronous rearrangement control, suggesting an active mechanism making one allele at a time accessible to the action of the transcriptional and/or recombinational machinery.

Asynchronous replication of Ig loci might allow transcriptional and/or recombinational accessibility in each cell to be confined to one chromosome at a time. Some imprinted loci are embedded in large domains that undergo asynchronous replication, with domains of paternal origin replicating earlier than those of maternal origin, thereby allowing monoallelic expression (Kitsberg *et al.*, 1993; and reviewed by Efstradiatis, 1994, 1995). If V(D)J rearrangement can occur only in certain stages of the cell cycle (Li *et al.*, 1996; Lin and Desiderio, 1994; and reviewed by Lin and Desiderio, 1995), then asynchronous replication of homologous Ig loci might expose one Ig locus allele to the recombinase at a given time, thereby preventing allelic inclusion. Other mechanisms for making one Ig allele at a time accessible to the action of the transcriptional and/or recombinational machinery have been proposed, such as interaction of *cis*-acting elements in the receptor loci with proteins or nuclear matrix-binding sites that might be available in limited quantities (Alt *et al.*, 1992).

III. Tissue- and Stage-Specific Control of Immunoglobulin Light κ Expression

A. Germline Transcription of Murine IgL κ Loci

Some HC and LC variable region gene segments are transcribed prior to rearrangement (Blackwell *et al.*, 1986; Picard and Schaffner, 1984b, 1985; Schlissel and Baltimore, 1989; Yancopoulos and Alt, 1985), as are HC and LC constant region genes (Alt *et al.*, 1982; Kemp *et al.*, 1980; Lennon and Perry, 1985; Nelson *et al.*, 1983, 1984, 1985; Van Ness *et al.*, 1981). In the murine κ locus, one type of germline κ transcript initiates about 3.5 kb upstream of $J_\kappa 1$ (Fig. 4), producing an 8.4-kb primary transcript extending through the Cμ region (Perry *et al.*, 1980; Van Ness *et al.*, 1981), and a 1.1-kb processed transcript (Kelley *et al.*, 1988). A second type initiates 50–100 bp upstream of $J_\kappa 1$ (Leclercq *et al.*, 1989; Martin

and van Ness, 1990) and results in a 0.8-kb processed transcript extending through the Cμ region (Martin and Van Ness, 1990).

B. Germline Transcription of Human IgL κ loci

The human κ locus contains a germline promoter and a transcriptional start site located about 4 kb 5′ of the $J_κ1$ gene, as demonstrated by the finding of spliced $J_κC_κ$ transcripts without an associated V region (Frances *et al.*, 1994; Martin *et al.*, 1991). Although these transcripts did not initially appear to encode a protein (Martin and Van Ness, 1989; Thompson *et al.*, 1992), subsequent analysis revealed a non-AUG initiation codon followed by an open reading frame encoding a 15-kDa protein that associates with μ HC in human pre-B cells (Frances *et al.*, 1994). Mice and human germline transcripts are highly conserved, with equivalent potential to encode a protein using GUG translation initiation codons (Frances *et al.*, 1994). This $C_κ$ protein might play a role in pre-B-cell signaling, perhaps as an alternative to λ5 in the pre-B receptor.

C. Possible Functions of IgL κ Germline Transcripts

$J_κC_κ$ germline transcription is correlated with κ locus rearrangement in transformed pre-B-cell lines (Lennon and Perry, 1990; Schlissel and Baltimore, 1989). Despite this correlation in the κ locus and in other antigen receptor loci (Fondell and Marcu, 1992; Goldman *et al.*, 1993; Holman *et al.*, 1993; Yancopoulos and Alt, 1985), the role of germline transcripts in the rearrangement process is not known. Germline κ transcripts are inducible in pre-B cells by specific signals such as LPS (Nelson *et al.*, 1984, 1985). The regulation of these germline κ transcripts in early B cells by the $iE_κ$ and $3'E_κ$ elements is poorly understood, but could be clarified by assays of germline transcription in pro- and pre-B cells from normal and κ enhancer mutant mice (Gorman *et al.*, 1996; Xu *et al.*, 1996a).

D. Role of κ Enhancers in Expression of Rearranged κ Genes

Studies of transgenic κ loci have suggested that $3'E_κ$, but not $iE_κ$, is required for high-level κ expression in Ig-secreting cells and for efficient exclusion of endogenous L chain gene rearrangement (Betz *et al.*, 1994; Meyer *et al.*, 1990; Xu *et al.*, 1989). Most initial κ transgenic constructs included $iE_κ$ and fortuitously either did or did not include the subsequently identified $3'E_κ$. Some of these κ transgenic mice expressed the κ transgene at high levels and repressed rearrangement of endogenous genes (Brinster *et al.*, 1983; Ritchie *et al.*, 1984; Sharpe *et al.*, 1991; Xu *et al.*, 1989), whereas others did not (Carmack *et al.*, 1991; Rusconi and Kohler, 1985; Sharpe *et al.*, 1990).

Discovery and functional characterization of the $3'E_\kappa$ (Meyer and Neuberger, 1989) revealed that most transgenic κ constructs with the $3'E_\kappa$ express well and repress rearrangement of the endogenous LC genes. For example, in one transgenic experiment, a transgenic μ chain was expressed at a high level, whereas the transgenic κ chain, which lacked the $3'E_\kappa$ element, was expressed at a 10-fold lower level (Rusconi and Kohler, 1985). A similar experiment utilizing a κ construct containing the $3'E_\kappa$ element resulted in high level κ expression and inhibition of endogenous gene rearrangement (Ritchie *et al.*, 1984). Another construct did not express well and did not inhibit rearrangement of endogenous LC genes without $3'E_\kappa$ (Sharpe *et al.*, 1990), but did with the downstream $3'E_\kappa$-containing sequence (Sharpe *et al.*, 1991). Of the two reported exceptions to the correlation between $3'E_\kappa$ and high level transgenic κ expression, one probably arose due to the integration site of the transgene (Carmack *et al.*, 1991) and one may have resulted from poor pairing of the particular transgenic LC with endogenous HCs (Storb, 1995). Thus, inclusion of $3'E_\kappa$ is necessary and probably sufficient for high level transgenic κ locus expression.

Two series of experiments with transgenic constructs omitting the iE_κ element found that inclusion of the downstream region, including $3'E_\kappa$, was sufficient to mediate high level κ expression and efficient allelic exclusion of endogenous LC genes, even when iE_κ was omitted from the construct (Betz *et al.*, 1994; Xu *et al.*, 1989). In contrast, inclusion of iE_κ with omission of $3'E_\kappa$ gave no detectable κ expression (Betz *et al.*, 1994) or a modest increase (Xu *et al.*, 1989). Together, these studies suggest that $3'E_\kappa$ is critical for high level expression of a rearranged transgenic κ locus and for allelic exclusion of endogenous LC genes, whereas iE_κ is dispensable for these processes.

Studies in κ locus gene targeted mice (Fig. 5) have revealed a more complex picture. In the endogenous locus, the iE_κ/MAR is dispensable for normal κ protein expression in resting B cells (Xu *et al.*, 1996a), whereas the $3'E_\kappa$ is required for normal κ protein surface expression in resting B cells (Gorman *et al.*, 1996). Absence of the $3'E_\kappa$ decreases κ expression to about one-third its normal level in resting B cells, indicating that the $3'E_\kappa$ element functions to maintain baseline expression at this stage. However, neither the iE_κ/MAR nor the $3'E_\kappa$ is required for normal κ expression in activated B cells, at least when the other is present (Gorman *et al.*, 1996; Xu *et al.*, 1996a). This finding is surprising given that the $3'E_\kappa$ element is required for high level expression of rearranged κ transgenic constructs, even when the construct includes iE_κ (Betz *et al.*, 1994; Blasquez *et al.*, 1989, 1992; Carmack *et al.*, 1991; Meyer *et al.*, 1990; Rusconi and Kohler, 1985; Xu *et al.*, 1989).

These apparently contradictory results can be explained if an additional endogenous element, absent from the transgenic constructs, cooperates with iE_κ to stimulate endogenous κ locus expression in the absence of $3'E_\kappa$. In this context, B-cell activation and terminal differentiation may stimulate multiple, distinct signaling pathways acting through $3'K_\kappa$ or iE_κ, each sufficient to stimulate high level κ expression. LPS stimulation probably activates iE_κ, primarily by mobilization of NF-κB (Lenardo *et al.*, 1987), which may allow iE_κ to compensate for the absence of $3'E_\kappa$ in LPS-activated B cells. A distinct $3'E_\kappa$-activating pathway may be required for normal κ expression in resting B cells in which the iE_κ/NF-κB pathway has not been activated.

E. Stage Specificity of $3'E_\kappa$ Activity

Previous discussions of the activity and function of the $3'E_\kappa$ have focused on its activity in mature and secreting B cells (Calame and Ghosh, 1995; Klug *et al.*, 1994; Meyer and Neuberger, 1989; Meyer *et al.*, 1990). However, $3'E_\kappa$ mutant mice had an altered ratio of newly generated to precursor B cells similar to that observed in mutant mice in which the κ locus was completely inactivated by targeted mutation (Chen *et al.*, 1993a; Takeda *et al.*, 1993; Zou *et al.*, 1993). The altered $\kappa:\lambda$ ratio of newly generated B lymphocytes, the altered ratio of newly generated to precursor lymphocytes, and the altered κ versus λ rearrangement in $3'E_\kappa$ mutant mice indicate that the $3'E_\kappa$ is normally active during the pre-B to B-cell transition (Gorman *et al.*, 1996). The activity of the $3'E_\kappa$ at this stage could include stimulation of germline transcription, direct stimulation of rearrangement, or stimulation of expression of the rearranged κ locus. Interference with any of these processes by deletion of the $3'E_\kappa$ could result in the observed alteration in the $\kappa:\lambda$ ratio.

In this context, two studies have provided additional evidence that $3'E_\kappa$ is active in early B-cell development (Roque *et al.*, 1996; Shaffer *et al.*, 1997). One study assessed *in vivo* occupancy of the κ enhancers in primary pro- and pre-B cells (Shaffer *et al.*, 1997). Unlike the differential developmental modulation seen in transformed cell lines, the NF-κB site of the intronic enhancer was found to be occupied in both pro- and pre-B cells. Given that deletion of the iE_κ blocks $V_\kappa J_\kappa$ rearrangement, the NF-κB-binding site may therefore be necessary but not sufficient for efficient activation of $V_\kappa J_\kappa$ rearrangement (Shaffer *et al.*, 1997). In contrast, striking changes were found in the occupancy of PU.1 and BSAP sites within the $3'E_\kappa$ during the pro-B to pre-B transition, with the BSAP site occupied at the pro-B stage, and the PU.1 site occupied at the pre-B stage. These data suggest a mechanism by which $3'E_\kappa$ might mediate stage-specific changes in κ locus activation between pro- and pre-B cell stages.

A second study found that two DNase hypersensitivity sites within the core $3'E_\kappa$ element become accessible at the pro-B cell stage in cultured B-cell lines (Roque *et al.*, 1996). This chromatin arrangement persists in mature B-cell lines undergoing subsequent dramatic alteration in cell lines representing the mature B to plasma cell transition. These data provided additional evidence that the $3'E_\kappa$ is active throughout the pre-B and B-cell stages and suggested that a major shift in function for the $3'E_\kappa$ occurs during the mature B-cell to plasma cell transition, possibly related to somatic mutation and high level κ expression in B cells undergoing terminal differentiation (Roque *et al.*, 1996).

IV. Regulation of Immunoglobulin Light Chain Gene Rearrangement

A. Models for Preferential LC Rearrangement and Isotype Exclusion

1. LC Isotype Exclusion

In assays of allotypic markers, more than 99% of B lymphocytes express a single surface LC isotype (Bernier and Cebra, 1964; Cebra *et al.*, 1966; Pernis *et al.*, 1965) and more than 99% of monoclonal myelomas secrete a single LC isotype (Morse *et al.*, 1976). Similarly, 1% or less of mouse or human B lymphocytes express multiple LC isotypes (Giachino *et al.*, 1995; Gollahon *et al.*, 1988). In normal mice, fewer than 1% of splenic B lymphocytes carry productive rearrangements of κ and λ LC genes in the same B cell (Zou *et al.*, 1993), and only rare mouse or human tumors and cell lines express two LC isotypes (Cuisinier *et al.*, 1992; Hardy *et al.*, 1986; Oriol *et al.*, 1974; Pauza *et al.*, 1993). Most B lymphocytes thus express either a functional κ or a λ LC, but not both.

2. Preferential Rearrangement of κ versus λ LC Genes

Similar to HC gene rearrangement, LC gene rearrangement appears ordered. In mice and human λ-expressing cell lines, κ LC genes are usually nonproductively rearranged or deleted (Alt *et al.*, 1980; Coleclough *et al.*, 1981; Hieter *et al.*, 1981a; Korsmeyer *et al.*, 1981, 1982). Conversely, in κ-expressing cell lines, λ LC genes remain unrearranged (Alt *et al.*, 1980; Coleclough *et al.*, 1981; Hieter *et al.*, 1981a; Korsmeyer *et al.*, 1981; Reth *et al.*, 1985). The contrasting rearrangement status of κ versus λ loci in mice and human B lineage cell lines suggests that κ rearrangement occurs preferentially to λ rearrangement in both species.

In mice, preferential κ versus λ gene rearrangement has been confirmed in large populations of normal cells (Gorman *et al.*, 1996; ten Boekel *et al.*, 1995; Zou *et al.*, 1993). In purified λ-expressing B cells assayed by

quantitative Southern blot analysis, only 2–5% of J_κ loci are retained in the germline configuration (Gorman *et al.*, 1996; Zou *et al.*, 1993). Similarly, in 36 splenic λ-expressing B cells assayed, only 2 retained a germline κ locus (ten Boekel *et al.*, 1995). On 80% of assayed κ chromosomes, rearrangement to the most 3′ J_κ segment precludes further $V_\kappa J_\kappa$ rearrangement. In contrast, the majority of λ LC genes in κ-expressing cells remain in the germline configuration (ten Boekel *et al.*, 1995; Zou *et al.*, 1993).

These studies in normal B lymphocyte populations confirm the findings of most studies of κ and λ genes in B-cell lines (Alt *et al.*, 1980; Coleclough *et al.*, 1981; Hieter *et al.*, 1981a). One exception is a study of λ-expressing hybridomas derived from a mouse enriched for λ B cells by treatment with anti-κ antiserum, in which 45% of λ hybridomas retained at least one κ locus in the germline configuration (Berg *et al.*, 1990). The reason for this contrasting finding is unclear. However, data from normal cell populations convincingly confirm that κ LC rearrangement is strikingly preferred to λ rearrangement.

In humans, λ-expressing cells purified from one individual had rearranged or deleted almost 100% of κ LC genes (Korsmeyer *et al.*, 1982), whereas the germline status of λ LC genes in κ-expressing cells has not been assayed in large cell populations. In human B-cell tumors and cell lines, κ rearranged preferentially to λ. For example, in 8 human κ cell lines, no λ alleles were rearranged. Conversely, in 10 λ cell lines, 19 of 20 κ alleles were deleted and 1 was aberrantly retained, leaving no germline κ alleles (Hieter *et al.*, 1981a). In 8 non-T/non-B human acute lymphocytic leukemias, no tumor line with an unrearranged κ locus showed a rearranged λ locus. In humans, κ rearrangement therefore appears to occur preferentially to λ rearrangement (Korsmeyer *et al.*, 1981). The extent of preferential κ versus λ rearrangement in normal human B cells could be further quantified by testing λ rearrangement in large populations of purified κ-expressing cells from several individuals.

Although the majority of B cells undergo κ rearrangement prior to λ rearrangement, this ordering appears strongly biased, or "preferential," rather than absolute. Accordingly, some cells rearrange λ before κ (Beishuizen *et al.*, 1991; Berg *et al.*, 1990; Felsher *et al.*, 1991; Giachino *et al.*, 1995; Tang *et al.*, 1991). Three to four percent of κ-expressing B cells appear to harbor nonproductive λ joins, suggesting that pre-B cells undergoing a nonproductive λ rearrangement may rearrange κ (Zou *et al.*, 1993).

3. *Sequential (Ordered) and Probabilistic (Stochastic) Models*

Ordering of LC gene rearrangement might result from either a "sequential" mechanism that affects "ordered" activation of the κ and λ loci or a

"probabilistic" ("stochastic") mechanism in which these loci are activated simultaneously, with the probability of κ gene rearrangement greatly exceeding that of λ genes (Alt *et al.*, 1980; and reviewed by Chen and Alt, 1993). In a sequential model, LC rearrangement initiates at the κ locus, and λ rearrangements generally occur in cells that have attempted and failed to assemble productive κ genes, perhaps because κ and λ loci rearrange at different times or in different locations in the bone marrow or because genetic activation of the λ loci requires κ protein. For example, the κ locus could gain initial access to recombination-promoting sites such as nuclear matrix attachment sites or to factors such as recombinase or transcriptional machinery. Strictly sequential models proposed that rearrangement proceeds in an obligatory sequence through both κ loci, and only then to λ loci (Hieter *et al.*, 1981a; Korsmeyer *et al.*, 1982).

The probabilistic model, in its simplest form, suggests that κ and λ loci are simultaneously, rather than sequentially, activated for rearrangement and that rearrangements at each locus occur independently of rearrangements at other loci. A strict probabilistic model emphasizes the independence of events at the two loci, in which λ rearrangements proceed at a constant rate, simultaneous with and independent from rearrangements at κ loci. Proposed mechanisms that might mediate probabilistic ordering of LC gene rearrangement include differential rearrangement probabilities in the different loci due to the relative number of V_κ to V_λ gene segments (Alt *et al.*, 1980; Coleclough *et al.*, 1981) or to the increased efficiency of κ locus recombination signal sequences compared to RSS in λ loci (Ramsden and Wu, 1991). Such differences in RSS efficiency have been reported in extrachromosomal plasmic rearrangement substrates, in which a single κ and a single λ RSS were assayed and compared for rearrangement efficiency (Ramsden and Wu, 1991). Whether these findings extend to other specific κ and λ locus RSS is not known.

4. A-MuLV Transformants Show Strikingly Preferential κ versus λ Rearrangement

Most Abelson murine leukemia virus-transformed pre-B-cell lines exhibit strikingly ordered Ig gene rearrangement. Most A-MuLV transformants bear stably rearranged HC genes, but germline LC genes (Alt *et al.*, 1981; Sugiyama *et al.*, 1983), and therefore make HC and not LC (E. Siden *et al.*, 1981; E. D. Siden *et al.*, 1979). During maintenance in culture, some A-MuLV lines undergo active HC gene rearrangement (Alt *et al.*, 1981). Occasional cell lines are capable of rearranging κ LC genes in culture (Alt *et al.*, 1981; Lewis *et al.*, 1982; Reth *et al.*, 1985; Riley *et al.*, 1981; Whitlock *et al.*, 1983). Most κ-rearranging A-MuLV transformants were found to retain their λ loci in the germline configuration (Alt *et al.*, 1981; Lewis *et*

al., 1982; Reth *et al.*, 1985). A smaller subset of A-MuLV-transformed pre-B-cell lines was identified that rearranges λ genes in culture (Muller and Reth, 1988; Persiani *et al.*, 1987). These λ-rearranging A-MuLV transformants had first rearranged their κ loci, and they continued to undergo κ locus rearrangement and deletion while undergoing λ rearrangement (Persiani *et al.*, 1987). Artifacts could arise from the transformation process itself (Alt *et al.*, 1981; Y. Y. Chen *et al.*, 1994; Klug *et al.*, 1994). However, strikingly preferential κ versus λ rearrangement was also found in cell lines conditionally transformed with a temperature-sensitive A-MuLV variant (Y. Y. Chen *et al.*, 1994). In these studies, inactivation of the v-*abl* tyrosine kinase by growth at the restrictive temperature triggered active rearrangement of κ and λ LC genes. All subclones with λ rearrangements had undergone rearrangement at the κ locus, whereas λ genes were predominantly unrearranged in cells with κ locus rearrangement. Thus LC gene rearrangements in A-MuLV-transformed pre-B-cell lines show strongly preferential κ versus λ rearrangement, consistent with a sequential mechanism or with a strongly biased probabilistic mechanism.

5. *Role of Enhancers in Control of V(D)J Rearrangement*

Rearrangement of different antigen receptor loci is mediated by differential accessibility to the recombinational machinery (reviewed by Alt *et al.*, 1992; Sleckman *et al.*, 1996). Enhancers appear important in regulating transcription and rearrangement of Ig loci. Transcription of antigen receptor genes has been found to precede or to accompany gene rearrangement during lymphocyte development. This close correlation between recombinase accessibility and transcription could result because transcription is a byproduct of accessibility or could imply that transcription plays a role in initiating or maintaining accessibility. Enhancer elements might affect rearrangement via stimulation of transcription from unrearranged ("germline") Ig gene segments or by playing a more direct role in attracting the recombinational machinery.

Analysis of transfected and transgenic recombination substrates has demonstrated that enhancer elements can mediate accessibility of substrate V(D)J segments (reviewed by Sleckman *et al.*, 1996). However, because of possible confounding variables, such as integration effects and copy number effects, recombination substrate findings cannot unequivocally establish a role for enhancer elements in mediating accessibility of endogenous antigen receptor loci. Direct evidence for such a role has come from application of gene targeting technology to the study of *cis*-acting DNA control elements (Chen *et al.*, 1993c; Cogne *et al.*, 1994; Serwe and Sablitzky, 1993; Takeda *et al.*, Zou *et al.*, 1993; and reviewed by Sleckman *et al.*, 1996).

Studies in the Ig HC locus demonstrated that specific deletion of E_μ partially inhibited IgH gene rearrangement (Serwe and Sablitzky, 1993), whereas replacement of E_μ by a *neo*^{r} gene led to more complete inhibition (Chen *et al.*, 1993c), suggesting that the *neo*^{r} gene may inhibit additional IgH elements that promote rearrangement in the absence of E_μ. Finally, replacement of part of the IgH 3′ enhancer with a *neo*^{r} gene blocked transcription of and class switching to IgH constant region genes up to 120 kb away, perhaps due to deletion of $3'E_H$, inhibition of other elements by the *neo*^{r} gene, or both (Cogne *et al.*, 1994). Contrasting results obtained when comparing deletions in the presence and absence of drug resistance genes have led to the conclusion that deletion of inserted drug resistance genes is required in order to draw definitive conclusions about the function of deleted enhancer elements (Chen *et al.*, 1993c; Serwe and Sablitzky, 1993).

For this reason, alternative strategies have been used to allow initial replacement of an element by a drug resistance gene, followed by subsequent deletion of the drug resistance gene (Fiering *et al.*, 1995; Gu *et al.*, 1993; Hasty *et al.*, 1991; Serwe and Sablitzky, 1993). The most widely used approach (Gu *et al.*, 1993; Saint-Ruf *et al.*, 1994) utilizes a site-specific phage P1 recombinase called Cre and its 34-bp *loxP* target sites (Abremski *et al.*, 1983) to simultaneously delete a *cis* element and introduce a *loxP*-flanked drug resistance gene, either *in vitro* (Fiering *et al.*, 1995; Gu *et al.*, 1993) or *in vivo* (Gu *et al.*, 1994; Lakso *et al.*, 1992, 1996; Pichel *et al.*, 1993). By allowing deletion of the expressed marker after targeting of a locus, Cre-*loxP*-mediated excision permits the locus to be studied both in the presence and in the absence of the constitutively expressed drug resistance gene.

6. Gne Targeting Studies and Ordered LC Rearrangement

Data from κ locus mutant mice (Fig. 5) have been interpreted as arguing against both strictly ordered and strictly probabilistic models (Chen *et al.*, 1993a; Takeda *et al.*, 1993; Zou *et al.*, 1993). Data from gene targeted $J_\kappa C_\kappa$, iE_κ, and C_κ mutant mice are not consistent with a strictly probabilistic model of preferential κ versus λ rearrangement (Chen *et al.*, 1993a). Compared to wild-type mice, all three types of homozygous κ locus mutant mice show at least a fivefold increase in the pool of newly generated λ cells (Chen *et al.*, 1993a; Takeda *et al.*, 1993; Zou *et al.*, 1993). However, the pre-B-cell pool of these mutant mice shows at most a twofold increase, suggesting a significantly increased rate of λ-expressing B-cell production in mutant mice. Because the λ B-cell production rate is independent of the κ B-cell production rate in a strictly probabilistic model, increased

size of the newly generated λ pool would require a corresponding increased size of the pre-B cell pool, which is not found in κ locus mutant mice.

Data from κ locus gene targeted mice have also been interpreted as arguing against a strictly ordered model because neither κ locus rearrangement nor κ locus expression per se is required for λ locus rearrangement and expression (Zou *et al.*, 1993). However, data from these mice would be consistent with a strictly sequential/ordered model if a sequence(s) within the κ locus that normally suppresses λ-locus rearrangement is deleted from the κ locus by gene targeting of the κ locus (Chen *et al.*, 1993a; Takeda *et al.*, 1993; Zou *et al.*, 1993). Such inhibitory sequences could include the iE_κ and/or the $3'E_\kappa$. The finding that absence of the $3'_\kappa$ or the iE_κ significantly diminishes preferential κ versus λ rearrangement is consistent with this hypothesis (Gorman *et al.*, 1996; Xu *et al.*, 1996a) and suggests that the two κ locus enhancers function to target rearrangement preferentially to the κ locus.

7. *Relative Ordered Model*

To account for the increased rate of λ production in κ locus mutant mice, a "relative ordered model" was proposed (Chen *et al.*, 1993a). This model postulates that in newly generated pre-B cells, the intrinsic probability of κ rearrangement is high, and that of λ rearrangement is low or zero. Use of the term "relative ordered model" emphasizes the dependence of λ rearrangement rate on rearrangement at the κ locus. The probability of λ rearrangements progressively increases as pre-B cells accumulate nonproductive κ locus rearrangements or κ locus deletions, which may eliminate κ-locus elements favoring κ rearrangement or inhibiting λ rearrangement.

8. *Positive Regulation of κ Locus Rearrangement by Transcriptional Enhancers*

In the chromosomal κ locus, gene targeted replacement of the iE_κ with a *neo*^{r} resistance gene appears to completely inhibit J_κ rearrangement, whereas *neo*^{r} gene insertion just downstream of iE_κ reduces but does not completely inhibit rearrangement (Takeda *et al.*, 1993; Zou *et al.*, 1993). Cre-*loxP*-mediated deletion of the iE_κ/MAR sequences reduced but did not abolish $V_\kappa J_\kappa$ rearrangement, altered the κ: λ ratio to 1:1, and completely inhibited RS rearrangement (Xu *et al.*, 1996a). These data suggest that the iE_κ element promotes $V_\kappa J_\kappa$ rearrangement, but that other elements can promote $V_\kappa J_\kappa$ rearrangement in the absence of iE_κ. In addition, deletion of the iE_κ either permits or promotes λ rearrangement, whereas RS rearrangement per se is not required for λ rearrangement. Finally, an inserted

neo^r gene can itself inhibit $V_\kappa J_\kappa$ rearrangement, via interference with iE_κ, $3'E_\kappa$ or other unknown elements by the *neo*^{r} gene.

Replacement of $3'E_\kappa$ by a *neo*^{r} gene or by a *loxP* sequence yields mice in which λ B cells show increased retention of germline J_κ and RS sequences (Gorman *et al.*, 1996). Thus $3'E_\kappa$, like iE_κ, stimulates preferential κ versus λ rearrangement by inhibiting κ rearrangements, by promoting the initiation or rate of λ rearrangements, or both. Increased accumulation of λ cells in $3'E_\kappa$ mutant mice could also occur if $3'E_\kappa$ normally contributes to inhibition of λ gene rearrangement, and its absence stimulates this process. Potential mechanisms would include competition between κ and λ LC loci for limited recombinational machinery or sites.

Compared to $3'E_\kappa$, deletion, iE_κ/MAR deletion resulted in more pronounced inhibition of J_κ rearrangement, implying that iE_κ/MAR promotes J_κ rearrangement and that $3'E_\kappa$ or other elements promote J_κ rearrangement in the absence of iE_κ/MAR. Thus, the $3'E_\kappa$ and iE_κ/MAR elements likely interact, potentially with additional elements such as the KI–KII sequence upstream of $J_\kappa 1$ (Ferradini *et al.*, 1996), to promote the full rearrangement potential of the κ locus. Deletion of the iE_κ/MAR or $3'E_\kappa$ element via RS rearrangements could promote the accumulation of λ cells, perhaps by increasing the rate of λ rearrangement per progenitor cell, as suggested in the relative ordered model (Chen *et al.*, 1993a).

9. *Positive Regulation of κ Locus Rearrangement by Elements Other than Transcriptional Enhancers*

Elements other than transcriptional enhancers have also been implicated in the positive regulation of rearrangement of the mouse Ig κ locus. In a transgenic chicken λ LC rearrangement construct, an element located in the V–J intronic region reduced rearrangement of the construct in mouse pre-B cells (Bucchini *et al.*, 1987; Lauster *et al.*, 1993). This "silencer" element is flanked by one or more "antisilencer" elements counteracting its effect (Lauster *et al.*, 1993). Together these elements may mediate allelic exclusion at the chicken λ locus (Ferradini *et al.*, 1994). Binding of silencer proteins to the silencer element is hypothesized to suppress rearrangement; transient binding of antisilencer proteins is hypothesized to displace the silencer proteins, transiently counteracting the silencer effect, and thereby allowing rearrangement of one λ allele.

Although two palindromic motifs upstream of the murine $J_\kappa 1$ gene segment, termed KI and KII, are similar in sequence and location to the chicken antisilencer element, the exact function of these sequences in mouse B cells is unknown. The shorter κ germline transcript initiates in the region of the KII site, which is bound by a DNA-binding protein in pre-B and early B cells (Leclercq *et al.*, 1989; Weaver and Baltimore,

1987). The influence of the KI and KII sites on rearrangement of the murine κ locus was tested by gene targeted mutation of the KI and KII sites (Ferradini *et al.*, 1996). Rearrangement of the targeted allele was inhibited significantly. Although rearrangement appeared to be reduced by 80% on the targeted allele, the mutation did not prevent accessibility to the locus, at least as measured by production of one germline transcript, by the unaltered methylation status relative to the normal allele, and by the occurrence of baseline levels of rearrangement on the targeted allele, and did not alter the peripheral κ:λ ratio. The significance of these findings for murine κ locus rearrangement is unknown because at present it is unclear what role the "antisilencer" element plays in the murine κ locus.

10. Negative Regulation of κ Locus Rearrangement by Enhancers

Some data from transgenic mice carrying κ rearrangement constructs have suggested a negative role for the $3'E_\kappa$ in the regulation of κ locus rearrangement. Transgenic rearrangement constructs containing a V_κ segment, the J_κ region, iE_κ, and C_κ show a partial loss of lineage and stage specificity of rearrangement. These constructs undergo rearrangement in T cells, in which the κ locus does not normally rearrange, and they undergo abnormally early rearrangement in progenitor B cells (Goodhardt *et al.*, 1989; Hiramatsu *et al.*, 1995). A similar construct containing the $3'E_\kappa$ element rearranged with normal B/T specificity, but still underwent some abnormal rearrangement in late pro-B cells (Hiramatsu *et al.*, 1995). Whereas most $V_\kappa J_\kappa$ joins in normal B cells lack N regions due to their rearrangement after downregulation of TdT, $V_\kappa J_\kappa$ joins in the transgenic rearrangement construct lacking the $3'E_\kappa$ element showed frequent N regions, suggesting that rearrangement ocurred at an earlier stage than normal. Addition of the entire 10-kb $3'E_\kappa$-containing flanking region downstream of C_κ resulted in normal pro-B/pre-B-stage specificity of rearrangement, whereas addition of just the $3'E_\kappa$ decreased but did not eliminate rearrangement in pro-B cells. These results suggested that the $3'E_\kappa$ element may mediate negative control of $V_\kappa J_\kappa$ rearrangement in T lymphocytes and may, either alone or in conjunction with other sequences 3′ of C_κ, mediate the pro-B/pre-B specificity of $V_\kappa J_\kappa$ rearrangement (Hiramatsu *et al.*, 1995).

However, the PCR assay detects low levels of rearrangement, and nonphysiologic aspects of this κ minilocus rearrangement construct may alter regulation of the construct. The transgenes are integrated in multiple tandem repeats of 2–40 transgene copies, which could allow V_κ promoters or iE_κ elements of nearby transgenes to promote $V_\kappa J_\kappa$ rearrangement. In previous analysis of λ locus constructs, high copy number transgenes underwent rearrangement in thymus (Bucchini *et al.*, 1987). In the κ construct, V_κ placement near J_κ might alter topological constraints, eliminat-

ing the normal requirement for the $3'E_\kappa$ positive element (Hiramatsu *et al.*, 1995). Absence of the negative element in transgenic constructs lacking $3'E_\kappa$ might then allow construct rearrangement in thymocytes and pro-B cells. Preliminary analyses of $3'E_\kappa$ mutant mice do not show substantial κ rearrangement in thymocytes, although more detailed analyses will be required to assay for low levels of rearrangement in T cells (Gorman *et al.*, 1996). $3'E_\kappa$ mutant mice will provide a useful system to further assay whether $3'E_\kappa$ and/or other elements normally influence the lineage and stage specificity of κ locus rearrangement.

11. Similar and Distinct Activities of iE_κ/MAR and $3'E_\kappa$ Elements

The reason that the κ locus harbors two different transcriptional enhancers is still speculative. However, current data suggest that the iE_κ/MAR and $3'E_\kappa$ elements have partially overlapping activities, with each playing a relatively more important role in particular functions. Only $3'E_\kappa$ has been clearly shown to influence surface κ expression on resting B cells (Gorman *et al.*, 1996). Both enhancers affect $V_\kappa J_\kappa$ and RS/κde rearrangement, with the iE_κ playing a greater role (Gorman *et al.*, 1996; Xu *et al.*, 1996b; Zou *et al.*, 1993). Thus, the iE_κ/MAR region likely influences the κ:λ ratio primarily through effects on rearrangement, whereas the $3'E_\kappa$ likely influences this ratio through effects on both rearrangement and expression. Their respective contributions to the control of germline transcription are not known.

In this context, the IgH locus also contains several widely spaced enhancer elements, including $E\mu$ and $3'E_H$. It remains to be determined whether the IgH locus enhancers work together for some processes. However, $E\mu$ appears to be important for V(D)J recombination, whereas the $3'E_H$ and/or associated elements may regulate germline transcription of and switching to downstream C_H genes. The TCRβ locus, unlike IgH and IgL κ loci, does not undergo genetic modifications subsequent to rearrangement. Notably, specific deletion of the TCRβ enhancer completely inactivates this locus (Bories *et al.*, 1996; Bouvier, 1996).

Double mutation of the $3'E_\kappa$ and the iE_κ elements could further address the role of known versus unknown elements in κ locus rearrangement and expression. Analysis of rearrangement and expression in B cells of mutant mice lacking both enhancers would allow clearer assessment of the requirement for additional elements in κ locus rearrangement and expression. Clear identification of all required elements might help clarify the different regulation of κ locus expression in transgenic constructs compared to the endogenous locus. The role of enhancer elements and of κ locus expression in mediating allelic and isotype exclusion of the Ig LC loci is also incompletely understood. The extent of loss of allelic and isotype exclusion in κ

enhancer mutant B cells could be clarified by analysis of VJ joins in mutant κ-expressing B cells. Loss of allelic exclusion would be evidenced by an increased percentage of cells carrying two productively rearranged κ chromosomes, whereas loss of isotype exclusion would be evidenced by an increased percentage of κ cells carrying productively rearranged λ chromosomes.

12. Mode of Preferential κ versus λ Rearrangement Control

To explain preferential κ versus λ rearrangement and the observed κ:λ ratio in normal mice, we propose a model incorporating a higher intrinsic rate of rearrangement at the κ locus, as does the relative ordered model, (Chen *et al.*, 1993a), and also a tendency of κ chromosomes to rearrange multiple sequential times (Fig. 7). Because of the occurrence of inversional rearrangements and rearrangements from upstream V segments to downstream J segments, each κ locus can undergo sequential $V_\kappa J_\kappa$ rearrangement attempts until a productive and functional κ LC is produced (Alt *et al.*, 1980; Arakawa *et al.*, 1996; Feddersen *et al.*, 1990; Harada and Yamagishi, 1991; Lafaille *et al.*, 1989; ten Boekel *et al.*, 1995). In addition, the κ locus has been calculated to have an intrinsic tendency to rearrange that is 10–100 times higher than that of the murine λ locus (Langman and Cohn, 1995; Ramsden and We, 1991).

In this model, because each locus can undergo several rearrangement attempts, the proportion of κ loci accomplishing productive rearrangements greatly exceeds 30%. In fact, one report compared κ and λ rearrangement rates in normal and gene targeted mice containing two, one, or zero functional κ loci and found that the probability of productive rearrangement per κ chromosome is actually 60%, a finding consistent with multiple κ gene rearrangement attempts per κ chromosome (Arakawa *et al.*, 1996).

B. Receptor Editing

Productive LC gene rearrangement resulting in the expression of membrane Ig on the cell surface does not always result in cessation of LC gene rearrangement. Ongoing or reactivated LC gene rearrangement may occur, for example, when an antibody is autoreactive (Gay *et al.*, 1993; Radic *et al.*, 1993; Tiegs *et al.*, 1993) or when it fails to promote positive selection (Rolink *et al.*, 1993). Newly generated B cells carrying an autoreactive BCR can undergo ongoing LC gene rearrangement in the bone marrow to replace the autoreactive LC with a nonautoreactive κ or λ chain (C. Chen *et al.*, 1994; Prak *et al.*, 1994). Replacement of an autoreactive κ LC could be accomplished either by sequential κ locus rearrangement events (Levy *et al.*, 1989) or by activation of λ locus rearrangement. Autoreactive B cells unable to undergo receptor editing are deleted in the bone

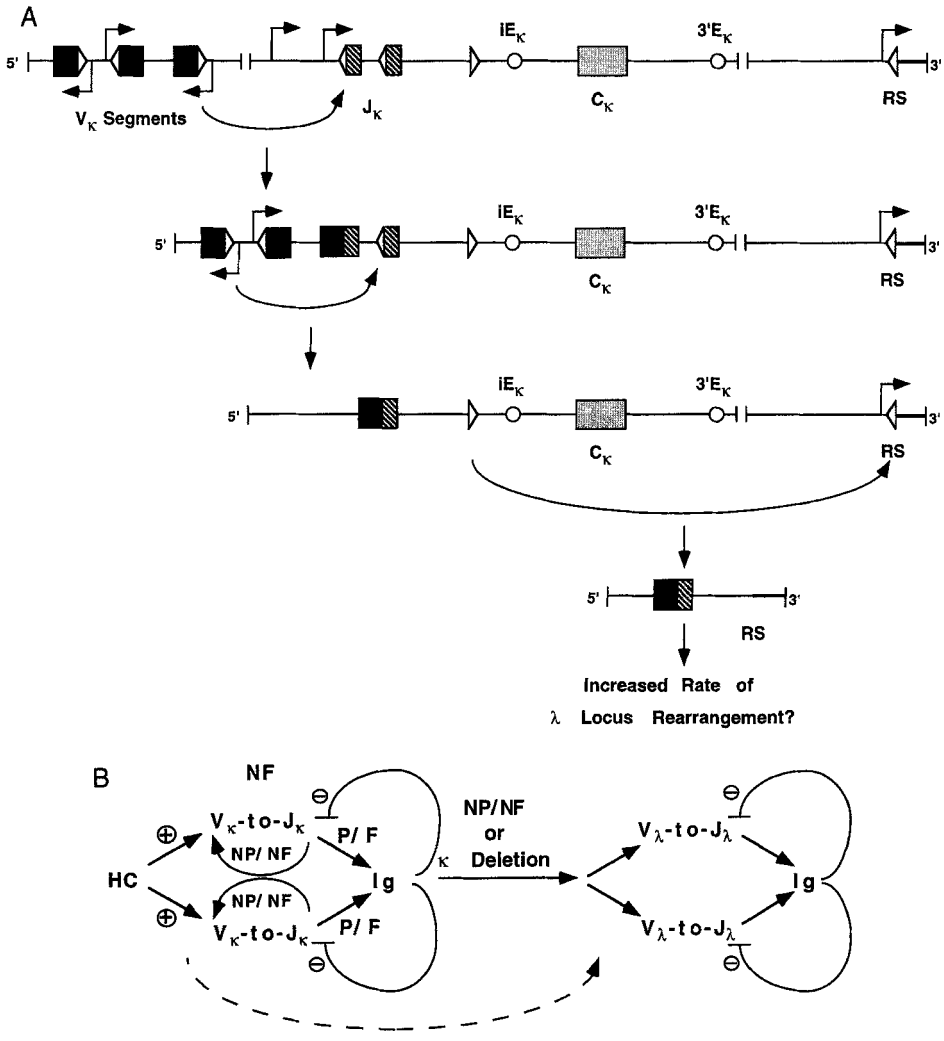


FIG. 7. Sequential Ig LC κ locus rearrangement. (A) Sequential rearrangement of the Ig LC κ locus. Promoters are shown as arrows indicating transcriptional orientation. Enhancers are shown as open circles. Representative V segments are shown as black boxes, J segments as cross-hatched boxes, and C segments as gray dots on a white background. Open triangles next to gene segments represent RSS. The open triangle in the 3′ end of the locus represents the RS sequence and in the JC intron represents the intronic heptamer target site for RS rearrangements (Selsing and Daitch, 1995). Curved arrows represent κ locus rearrangements. (B) Model of Ig LC gene rearrangement. +, activation of rearrangement; −, inhibition of rearrangement; NP, nonproductive rearrangement; NF, nonfunctional rearrangement; P, productive rearrangement; F, functional rearrangement. Solid arrows represent major physiologic pathways. Dotted arrow represents a minor physiologic pathway, enhanced by processes that inhibit or inactivate κ locus rearrangement.

marrow (Chen *et al.*, 1995). In addition, Rag-1 and Rag-2 expression has been shown to be reactivated in mature germinal center B cells (Han *et al.*, 1996; Hikida *et al.*, 1996), a possibility that is consistent with reactivation of LC gene rearrangement on exposure to antigen. Such processes could remove autoreactive B cells that arise as a result of somatic hypermutation, either by secondary LC gene rearrangements or by HC V_H replacement events (M. Reth *et al.*, 1986).

B. Role of RS/κde Rearrangement in Ig LC Gene Rearrangement

1. Discovery of RS Rearrangement

Southern blot analysis of DNA from human λ-expressing lymphoid cells revealed that they frequently lack a detectable C_κ sequence (Hieter *et al.*, 1981a). In human and murine λ- but not κ-expressing cells, C_κ or the entire $J_\kappa C_\kappa$ region is frequently deleted (Durdik *et al.*, 1984; Klobeck and Zachau, 1986; Korsmeyer *et al.*, 1981, 1982; Lafaille *et al.*, 1989; Moore *et al.*, 1985; Muller and Reth, 1988; Nadel *et al.*, 1990; Persiani *et al.*, 1987; Siminovich *et al.*, 1985). The highly conserved sequence elements mediating this deletion closely resemble the canonical RSS flanking the germline V gene segments (Durdik *et al.*, 1984; Siminovich *et al.*, 1985). These RSS-like elements are flanked downstream by a highly conserved 500-bp region with open reading frames of several hundred nucleotides (Durdik *et al.*, 1984; Siminovich *et al.*, 1985). The mouse recombining sequence (RS) and the human κ-deleting element (κde) will together be referred to as "RS/κde."

Located about 25 kb 3′ of the C_κ exon (Klobeck and Zachau, 1986; Siminovich *et al.*, 1985), the RS/κde can recombine with a V_κ region or with an isolated heptamer located in the $J_\kappa C_\kappa$ intron, just upstream from the iE_κ (Figs. 3, 7A, and 8). Rearrangements to the upstream V_κ gene segments result, depending on the orientation of the upstream V_κ gene

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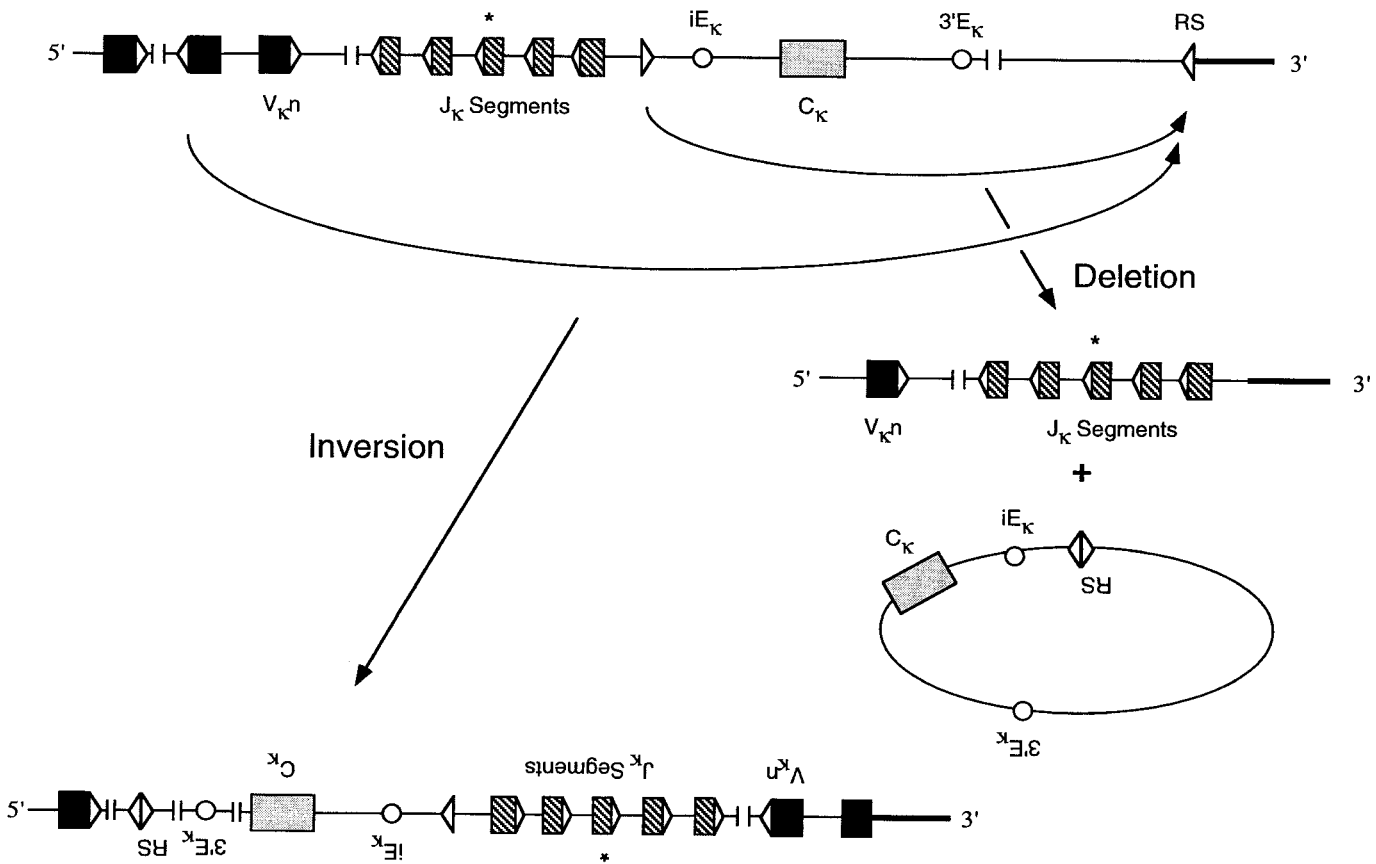
FIG. 8. RS rearrangements in the murine Ig LC κ locus. V, variable region gene; D, diversity segment; J, joining segment; C, constant region gene segment. Open triangles next to gene segments represent RSS. The open triangle in the 3′ end of the locus represents the RS sequence and in the JC intron represents the intronic heptamer target site for RS rearrangements (Selsing and Daitch, 1995). Asterisks indicate pseudogenes. Curved arrows represent inversional and deletional RS rearrangements. Rearrangements to V_κ genes with RSS facing the RS element (i.e., in opposite orientation to the RS element) result in deletion, with loss of the intervening sequences (shown on right). Rearrangements to V_κ genes with RSS in the same orientation as the RS element result in inversion, with retention of intervening sequences (shown on left).


5'
$V_\kappa n$
J_κ Segments
*
iE_κ
C_κ
$3'E_\kappa$
RS
3'
Deletion
Inversion
+
C_κ
iE_κ
RS
$3'E_\kappa$

segment, in either deletion of or inversion of the region containing J_κ, iE_κ, C_κ, and $3'E_\kappa$. Rearrangements to the intronic heptamer result in deletion of the region containing iE_κ, C_κ, and $3'E_\kappa$, with retention of the J_κ region (Figs. 7A and 8).

2. *Confirmation of RS/κde Rearrangement in Normal Cell Populations*

Studies in purified normal human and mouse λ-expressing B lymphocytes confirmed that κ-locus deletions are common in normal B cells (Korsmeyer *et al.*, 1982; Zou *et al.*, 1993). In long-term λ B-cell lines and leukemias, 95% of the C_κ alleles were deleted and the remaining 5% were rearranged (Hieter *et al.*, 1981a). In contrast, in normal λ cells purified from the peripheral blood of one individual or in several newly EBV-transformed human B cell lines, only 60% of the C_κ alleles were deleted, whereas about 35% were rearranged and 5% remained in the germline configuration (Korsmeyer *et al.*, 1982). These results suggest that in normal λ-expressing cells of both human and mouse, more than one-third of C_κ alleles are deleted, and most of the remainder are rearranged.

3. *Relationship between RS and λ Rearrangement*

The correlation between RS/κde-mediated κ locus deletion and λ gene rearrangement (Durdik *et al.*, 1984; Korsmeyer *et al.*, 1983; Muller and Reth, 1988; Nadler *et al.*, 1984; Persiani *et al.*, 1987; Siminovich *et al.*, 1985) suggested that κ locus deletions might promote λ rearrangements. RS/κde rearrangements might influence λ rearrangement by allowing expression of a regulatory transcript or protein, by activating an element that stimulates λ rearrangement, or by deleting an element that inhibits λ rearrangement (Durdik *et al.*, 1984; Moore *et al.*, 1985; Muller and Reth, 1988; Nadel *et al.*, 1990; Persiani *et al.*, 1987; Siminovich *et al.*, 1985; and reviewed by Selsing and Daitch, 1995).

Although iE_κ, $J_\kappa C_\kappa$, and $C_\kappa neo^r$ replacement mutant mice produce only λ-expressing B cells, iE_κ and $J_\kappa C_\kappa$ *neo*^{r} mice undergo no detectable RS rearrangement, and $C_\kappa neo^r$ mice undergo substantially reduced RS rearrangement. Thus, in mice, RS rearrangement is not absolutely required for the generation of λ-expressing B lymphocytes (Chen *et al.*, 1993a; Takeda *et al.*, 1993; Zou *et al.*, 1993). However, the effects of the element deletions and *neo*^{r} gene insertion might mimic the physiologic effect of an RS rearrangement. If deletion of the iE_κ or $3'E_\kappa$ by RS/κde rearrangement normally helps promote or permit λ rearrangmenet, then targeted replacement of the iE_κ or $3'E_\kappa$ element might allow λ rearrangement to efficiently proceed in the absence of RS/κde rearrangement. Indeed, as replacement of either the iE_κ or the $3'E_\kappa$ by a *loxP* sequence dramatically increases the percentage and number of λ-expressing B cells, these two enhancers likely

promote preferential κ versus λ rearrangement (Gorman *et al.*, 1996; Xu *et al.*, 1996b). Thus, RS-mediated deletion of the iE_κ and $3'E_\kappa$ elements probably represents a major mechanism by which RS rearrangement promotes or permits λ rearrangement, and targeted deletion of either enhancer element likely mimics this mechanism.

4. *Enhancers and Control of RS/κde Rearrangement*

The process of RS/κde rearrangement appears to be correlated with an open/accessible chromatin structure and with low level transcription of the RS/κde region (Daitch *et al.*, 1992). Accessibility of the RS/κde locus could be controlled by the same elements and signals that govern the accessibility of the rest of the κ locus, or RS/κde accessibility could undergo specific control wholly or partially independent of the rest of the κ locus. Retention of RS germline sequences in B cells lacking the iE_κ or the $3'E_\kappa$ suggests that both of these elements normally stimulate the frequency and/or the rate of RS rearrangement (Chen *et al.*, 1993a; Gorman *et al.*, 1996; Takeda *et al.*, 1993; Xu *et al.*, 1996a; Zou *et al.*, 1993). The Cre-deleted iE_κ/MAR mutant mice undergo little RS rearrangement, suggesting that the iE_κ element plays a primary role in promoting this process (Xu *et al.*, 1996a). The reduced RS rearrangement seen in Cre-deleted $3'E_\kappa$ mutant mice suggests that $3'E_\kappa$ further promotes efficient RS rearrangement (Gorman *et al.*, 1996).

Most RS/κde rearrangement likely occurs after $V_\kappa J_\kappa$ rearrangement, as RS/κde rearrangements are found frequently in λ-expressing cells but infrequently in κ-expressing cells (Durdik *et al.*, 1984; Nadel *et al.*, 1990; Persiani *et al.*, 1987; Siminovich *et al.*, 1985). Preferential κ versus RS/κde rearrangement could occur by a sequential mechanism in which RS/κde rearrangement is stimulated by specific signals that occur only after κ locus V gene rearrangement. Alternately, preferential κ versus RS/κde rearrangement could occur by a relative ordered mechanism in which the RS/κde sequences become accessible with the rest of the κ locus. $V_\kappa J_\kappa$ rearrangement might initially occur much more frequently than RS/κde rearrangement due to the noncanonical nature of the RS/κde recombination sequences or to topological constraints related to the position of the RS/κde in the 3′ end of mice and human κ loci. The rate of RS/κde rearrangements would increase as nonfunctional κ locus rearrangements accumulate.

The question of whether RS/κde rearrangement may play an important role in the process of receptor editing to eliminate autoreactive or otherwise nonfunctional productive κ rearrangements remains unresolved. Autoreactive surface Ig molecules might cause the entire κ locus to remain recombination competent, resulting in continuing LC gene rearrangement. A pro-

ductive and functional secondary κ locus rearrangement would terminate rearrangement, whereas a secondary nonproductive, nonfunctional, or autoreactive rearrangement would result in continuing competency, until eventually, the less-likely RS/κde rearrangement events would accumulate. Further understanding of these processes will require experiments designed to more specifically test the role of RS/κde rearrangement, the mechanisms of its control, and the phenotype of B cells in mice unable to undergo RS/κde rearrangement.

5. Testing Models of κ versus RS/κde and κ versus λ Rearrangement

A comprehensive relative ordered model of preferential κ versus RS/κde versus λ rearrangement might work as follows. Initially, the rate of $V_\kappa J_\kappa$ rearrangements might greatly exceed the rate of κ locus RS/κde and λ locus rearrangements. Accumulation of nonproductive $V_\kappa J_\kappa$ rearrangements might increase the rate of RS/κde rearrangements without significantly increasing the rate of λ locus rearrangements. RS/κde rearrangement, with concomitant deletion of the iE_κ and $3'E_\kappa$, might result in greatly increased probability of λ locus rearrangement. This sequence involves three separate changes at the κ loci: accumulation of nonproductive $V_\kappa J_\kappa$ rearrangements, rearrangement of RS/κde per se, and concomitant deletion of the enhancer elements and other intervening sequences.

Deletion of the $J_\kappa C_\kappa$ region, including the iE_κ element, results in an increased rate of λ-expressing B-cell generation, possibly due to deletion of the J_κ and/or deletion of the iE_κ. In this knockout, the effect of J_κ deletion cannot be assessed independently of iE_κ deletion. Specific deletion of the J_κ locus without deletion of the iE_κ could more directly test the relative ordered model of preferential κ versus RS/κde rearrangement. Such a deletion would mimic the physiologic effect of accumulated nonproductive κ locus rearrangements in the presence of both κ locus enhancers. If the rate of RS/κde rearrangement is increased, in contrast with the prediction of a purely probabilistic mechanism, a relative ordered model of preferential κ versus RS/κde rearrangement would be supported. The effect of specific J_κ locus deletion on preferential κ versus λ locus rearrangement and on the κ:λ ratio would also be of interest, since at present it is unclear whether the effect of the enhancer deletions derives solely from their effect on $V_\kappa J_\kappa$ rearrangement.

Whether RS/κde rearrangement normally increases the rate of λ cell generation, independently of the effect of nonproductive $V_\kappa J_\kappa$ rearrangement or of enhancer deletion, could be tested by specifically deleting the J_κ locus and the RS/κde element, leaving the enhancers and other intervening sequences intact. Deletion of J_κ would mimic the effect of sequential nonproductive rearrangement at the κ locus, with retained iE_κ and $3'E_\kappa$

elements, whereas deletion of the RS/κde element would block the cell from deleting the enhancers and intervening sequences by RS/κde rearrangement. Whether deletion of the enhancers per se, aside from an effect on decreased $V_\kappa J_\kappa$ rearrangement, is necessary for increased λ generation would help to further clarify the role of these elements in preferential κ versus λ locus rearrangement.

C. Control and Function of κ:λ Ratios in Mice and Humans

1. κ:λ Ratios in Different Species Vary Widely

In LC loci, relative usage of the κ and λ isotypes varies widely between species (Hood *et al.*, 1967). Some species apparently have only λ LCs, many species use both LC isotypes, and some species have predominantly κ LCs. How such different κ:λ ratios are generated in different species remains controversial, especially given the degree of preferential κ versus λ rearrangement in the species for which the most data is available, including mice and humans (Alt *et al.*, 1980; Coleclough *et al.*, 1981; Korsmeyer *et al.*, 1982).

2. κ:λ Ratios in Mice and Humans

Generation of the murine κ:λ ratio has received substantial attention. Although a few early hybridoma studies found κ : λ ratios of 2 : 1 in neonatal mouse spleen and bone marrow (Golding *et al.*, 1980; Haughton *et al.*, 1978; Weiss *et al.*, 1981), most murine studies, and virtually all murine studies in the past decade, have found κ : λ ratios of 10 : 1 or greater in the periphery, in the bone marrow, and even in sorted newly generated B cells in the bone marrow (LeJeune *et al.*, 1981; McGuire and Vitetta, 1981; McIntire and Rouse, 1970; Takemori and Rajewsky, 1981; Zou *et al.*, 1993). IgL κ gene rearrangements similarly exceed IgL λ gene rearrangements by a ratio of 10 : 1 or more in fetal liver (Paige *et al.*, 1992), in single cells from bone marrow (ten Boekel *et al.*, 1995), and in pre-B-cell culture systems (Rolink *et al.*, 1993; Rolink and Melchers, 1991).

The newly generated and peripheral κ : λ ratio of approximately 10:1 or greater in mice differs from the 2 : 1 ratio observed in humans (Hood *et al.*, 1967; Kubagawa *et al.*, 1982). These κ:λ ratios in mice and humans correlate with the relative complexity of the V_κ to V_λ loci in those species. Mice have 200 or more V_κ and 3 V_λ segements, whereas humans probably have more than 100 V_λ gene segments and 76 V_κ gene segments. The relative number of V genes in each locus could influence the observed κ:λ ratios by influencing the probability of κ versus λ locus rearrangement due to differing numbers of sites for recombination (Alt *et al.*, 1980). Alternately, the relative number of V genes in each locus could affect the κ:λ ratio by antigenic selection operating on the V regions of expressed

LC proteins (Cohn and Langman, 1990; Langman and Cohn, 1992, 1995). In both humans and mice, κ genes are rearranged or deleted in λ cells, but not vice versa (Alt *et al.*, 1980; Coleclough *et al.*, 1981; Korsmeyer *et al.*, 1982), an observation that cannot be explained by the relative number of unique V_κ to V_λ genes alone.

3. *Normalized κ:λ Ratio in 3′E_κ Mutant Mice*

Both the iE_κ/MAR region and the 3′E_κ normally influence the κ:λ ratio, as a κ:λ ratio of about 1 : 1 was found in Cre-deleted iE_κ mutant mice (Xu *et al.*, 1996a), whereas a ratio of about 2 : 1 was found in Cre-deleted 3′E_κ mutant mice (Gorman *et al.*, 1996). The effect of these enhancers on the κ:λ ratio could occur through an effect on κ versus λ rearrangement, through altered postrearrangement selection of κ versus λ populations, or both. The iE_κ principally affects κ versus λ rearrangement, whereas the 3′E_κ affects both κ versus λ rearrangement, and also expression of the rearranged κ locus. Altered postrearrangement selection mechanisms that might also contribute to the altered κ:λ ratio of 3′E_κ mutant mice will be considered here and later.

Decreased surface κ expression in the BCR due to the absence of the 3′E_κ could alter positive selection or expansion of κ-expressing cells in the bone marrow, migration of these cells from the bone marrow to the periphery, or selection by antigen in the periphery. The more dramatic alteration of the κ:λ ratio in the peripheral versus the newly generated bone marrow lymphocyte compartments suggests that some degree of selective expansion of λ-expressing cells is occurring in the periphery.

4. *Nature of the Normal Unselected Murine κ:λ Ratio*

As discussed earlier, although most studies have found a κ:λ ratio of about 10 : 1 or greater for newly generated and peripheral B cells of normal mice (Paige *et al.*, 1992; Rolink *et al.*, 1993; Rolink and Melchers, 1991; ten Boekel *et al.*, 1995; Zou *et al.*, 1993), a few early mouse hybridoma studies found κ:λ ratios of 2 : 1 in neonatal spleen and bone marrow (Golding *et al.*, 1980; Haughton *et al.*, 1978; Weiss *et al.*, 1981). Based on these early studies, a κ:λ ratio of 2 : 1 in the antigen-unselected murine B-cell compartment has been proposed (Claverie and Langman, 1984; Cohn and Langman, 1990; Langman and Cohn, 1987, 1992, 1995). This model argues that the newly generated κ:λ ratio cannot exceed 2 : 1 and that the higher κ:λ ratio observed in the mouse periphery, in the bone marrow, and in culture systems must result from antigenic selection. Although current approaches cannot exclude the possibility that newly generated B cells in normal mouse bone marrow have been exposed to prior selection during generation in the bone marrow, the conclusion that the theoretically pre-

dicted κ:λ ratio cannot exceed 2:1 without reflecting the consequences of antigenic selection does not appear inevitable. The κ:λ ratio of 10:1 or greater in normal mice can be readily explained without invoking selection. If each κ locus undergoes an average of three rearrangement attempts prior to a λ rearrangement, then a ratio greatly exceeding 2:1 would be predicted. It is therefore suggested that, while selection may influence the κ:λ ratio of mice or humans, a 20:1 κ:λ ratio in the unselected B-cell population of mice could also result from intrinsic, known features of the rearrangement process.

5. *The Problem of Human Preferential κ versus λ Rearrangement and the Human κ:λ Ratio*

In humans the normal κ:λ ratio is about 2:1. At the same time, κ loci are rearranged or deleted in human λ cells, whereas λ loci are not rearranged in human κ cells (Hieter *et al.*, 1981a; Korsmeyer *et al.*, 1981, 1982). This observation could be explained by a sequential rearrangement mechanism operating first at κ loci and then at λ loci (sequential model) (Alt *et al.*, 1980), by λ rearrangement occurring at a constant rate more slowly than the rate of κ locus rearrangement (stochastic model) (Alt *et al.*, 1980), or by a λ rearrangement rate that is initially low but that increases with accumulation of nonproductive κ rearrangements (relative ordered model) (Chen *et al.*, 1993a). Highly preferential κ versus λ rearrangement achieved by any of these mechanisms would have two clear effects. First, most κ chromosomes would be rearranged in λ cells, whereas most λ loci would remain unrearranged in κ cells, as is observed. Second, a κ:λ ratio greater than 2:1, potentially significantly greater, would be generated in the unselected B-cell population, as is observed in the mouse.

Why the observed peripheral κ:λ ratio in humans is 2:1, less than would be expected given a preferential κ versus λ rearrangement mechanism, is unclear. One previous study found that the κ:λ ratio of B cells in human fetal bone marrow was 1.5, whereas that of B cells in adult bone marrow was 1.4 (Kubagawa *et al.*, 1982). However, we are aware of no study measuring the κ:λ ratio among newly generated human B cells. In the absence of data to resolve this issue, the κ:λ ratio in the primary human repertoire could initially exceed 2:1 and could then undergo selection operating on the expressed V_L regions, favoring an expansion of the λ population to more closely reflect the approximately 2:1 ratio of human κ to λ V gene segments. Such selection would not occur in the mouse because the primary κ:λ ratio of 20:1 closely resembles the approximately 20:1 ratio of mouse κ to λ V gene segments. Thus, selection might explain why the observed peripheral human κ:λ ratio does not exceed 2:1, in contrast to theories suggesting that a proposed primary mouse κ:λ ratio

of 2:1 is altered by selection to the observed 10:1 ratio (Langman and Cohn, 1995). These alternate models could be tested most directly by measuring the κ:λ ratio in purified newly generated B cells in the bone marrow of humans and other species with similar κ:λ ratios to clarify whether, as hypothesized here, the newly generated human κ:λ ratio is greater than 2:1.

Selective forces acting to alter the Ig repertoire have been described previously in normal mice and humans (Malynn *et al.*, 1990; Yancopoulos *et al.*, 1988; and reviewed by Pascual *et al.*, 1992; Schwartz and Stollar, 1994; Stewart *et al.*, 1992) and in transgenic mice (Neuberger *et al.*, 1989). Although most mouse V_H genes appear to undergo some level of rearrangement, the most J_H-proximal V_H genes preferentially rearrange in developing B lymphocytes (reviewed by Alt *et al.*, 1987). A substantial bias of V_H gene usage is therefore seen in the primary mouse repertoire, in which J_H-proximal V_H gene products are overrepresented, as opposed to the adult repertoire, in which the products of all V_H genes are more equally represented (Malynn *et al.*, 1990; Yancopoulos *et al.*, 1988). In humans, the repertoire also differs between neonatal and adult individuals, although preferential usage of J_H-proximal V_H genes has not been reported (reviewed by Pascual *et al.*, 1992; Schwartz and Stollar, 1994; Stewart *et al.*, 1992).

These repertoire shifts in mice and humans most likely occur by postrearrangement selection mechanisms operating on the V_H regions of the BCR to expand B-cell clones bearing less frequently expressed V gene segments (Pascual *et al.*, 1992; Schwartz and Stollar, 1994; Stewart *et al.*, 1992; Yancopoulos *et al.*, 1988). Thus, in humans and in mice, selective processes appear capable of altering the B-cell repertoire during ontogeny. By analogy, the newly generated κ:λ ratio in humans could be greater than 2:1 and could be altered by selection to a ratio of 2:1.

6. *Different κ:λ Ratios in 3′$E_κ$, i$E_κ$, and KI/KII Mutant Mice*

In KI/KII mutant mice, rearrangement was reduced by 80% on the targeted allele, and yet the κ:λ ratio was not altered (Ferradini *et al.*, 1996). Thus, in otherwise normal mice, a level of κ locus rearrangement 20% of normal appears sufficient for the generation of a normal κ:λ ratio. The KI/KII mutation probably does not affect expression of the rearranged locus because the KI/KII sequences, which lie upstream of the $J_κ$ region, are excised by deletional κ locus rearrangement. The i$E_κ$ appears to have a more pronounced role in promoting $V_κJ_κ$ rearrangement than does the 3′$E_κ$ (Gorman *et al.*, 1996; Takeda *et al.*, 1993; Xu *et al.*, 1996b), suggesting that accessibility for $V_κJ_κ$ rearrangement depends primarily on the i$E_κ$ element and secondarily on the 3′$E_κ$ element. However, the κ:λ ratio was altered dramatically in 3′$E_κ$ and i$E_κ$ mutant mice. In i$E_κ$ mutant mice, this

altered ratio may result from a more severe block of κ versus λ rearrangement than that found in KI/KII mutant mice. Although a subtle effect on expression of the rearranged κ locus of iE_κ mutant mice cannot be excluded, κ expression in resting cells examined by flow cytometry did not appear to be affected (Xu *et al.*, 1996a). In $3'E_\kappa$ mutant mice, the altered κ:λ ratio might result from an inhibition of κ versus λ rearrangement and from altered κ versus λ selection due to reduced surface expression of the κ chain. For example, if decreased κ expression on mutant B cells causes preferential expansion of λ-expressing $3'E_\kappa$-mutant B cells, the κ:λ ratio in mutant mice but not in KI/KII mutant mice would be altered.

7. *Functions of κ and λ*

To date, no specific functions of κ versus λ B lymphocytes are known. Mice lacking functional κ loci have not been found to have immune defects so far (Chen *et al.*, 1993a; Pricop *et al.*, 1994; Takeda *et al.*, 1993; Zou *et al.*, 1993). Similarly, no striking immune deficiency has been identified in humans with κ or λ deficiencies of varying degrees (Barandun *et al.*, 1976; Bernier *et al.*, 1972; Kelus and Weiss, 1977; Stavnezer-Nordgren *et al.*, 1985; Zegers *et al.*, 1976; and reviewed by Rosen *et al.*, 1984). In the mouse, substantial alteration of the κ:λ ratio under carefully controlled housing conditions does not interfere with viability and survival, suggesting grossly normal immune function (Gorman *et al.*, 1996; Takeda *et al.*, 1993; Xu *et al.*, 1996b). Finally, some species have apparently lost one or the other locus in the course of evolution (Hood *et al.*, 1967), but have no obvious defect in immune function.

Although most animals have κ and λ isotypes, the κ:λ ratio varies widely between species (Hood *et al.*, 1967). Some species have 90–100% λ LCs (horse, sheep, cow, cat, dog, and mink), some species have more than 90% κ LCs (mice, rats, rabbits, and ducks), and some have roughly equal amounts of κ and λ LCs (pig, guinea pig, baboon, and human). Although the preservation of κ:λ expression ratios appears quite stable within each species, the wide variation among κ:λ ratios between species suggests that specific ratios may not hold specific functional implications. The preservation of two isotypes in most species may confer an evolutionary advantage, such as increased LC diversity or increased efficiency of B-cell development due to the increased probability of a given pre-B cell producing a functional LC gene rearrangement.

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Role of Immunoreceptor Tyrosine-Based Activation Motif in Signal Transduction from Antigen and Fc Receptors

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I. Introduction

One of the most remarkable features of the immune system is the ability of immunocytes to recognize an exceedingly wide range of specific antigens in different structural contexts and respond with vastly divergent biological responses leading to cell death, survival, multiplication, or differentiation. External factors determining the behavior of cells include soluble and cell-associated physiological ligands that interact with a large number of cell surface receptors. However, cell fate decisions in lymphocytes are primarily based on signals provided by engagement of the antigen receptors.

Antigen receptors are multisubunit structures composed of highly polymorphic membrane-anchored, antigen-binding polypeptides in noncovalent association with several invariant chains that function as signal transducing elements (Figs. 1 and 2). These receptors are devoid of catalytic activity and their function is strictly dependent on cytosolic protein tyrosine kinases (PTKs). This is in contrast to the single chain growth factor or cytokine receptors in which a membrane-spanning hydrophobic region links the extracellular ligand-binding "sensory" domain to an intracellular signaling region with a functional kinase domain (Fig. 1) (Yarden and Ullrich, 1988).

Lymphocyte antigen-receptor ligation results in a rapid process of tyrosine phosphorylation of receptor subunits followed by the assembly of signaling complexes at the receptor site. The cell activation process is strictly dependent on a conserved module in the receptor subunit cytoplasmic tails that functions as a scaffold for downstream effector molecules. This module, termed immunoreceptor tyrosine-based activation motif (ITAM) (Cambier, 1995b; Reth, 1989), exists in one or more copies in each of the receptor-associated signal-transducing molecules and possesses two repeats of the consensus sequence Tyr-X-X-Leu/Ile spaced by six to

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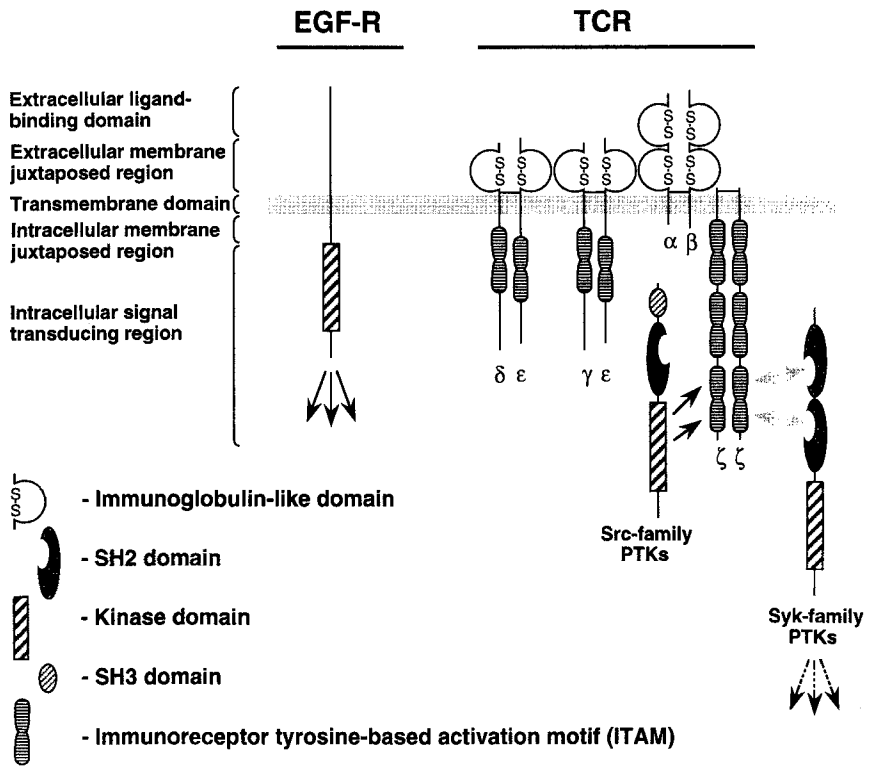
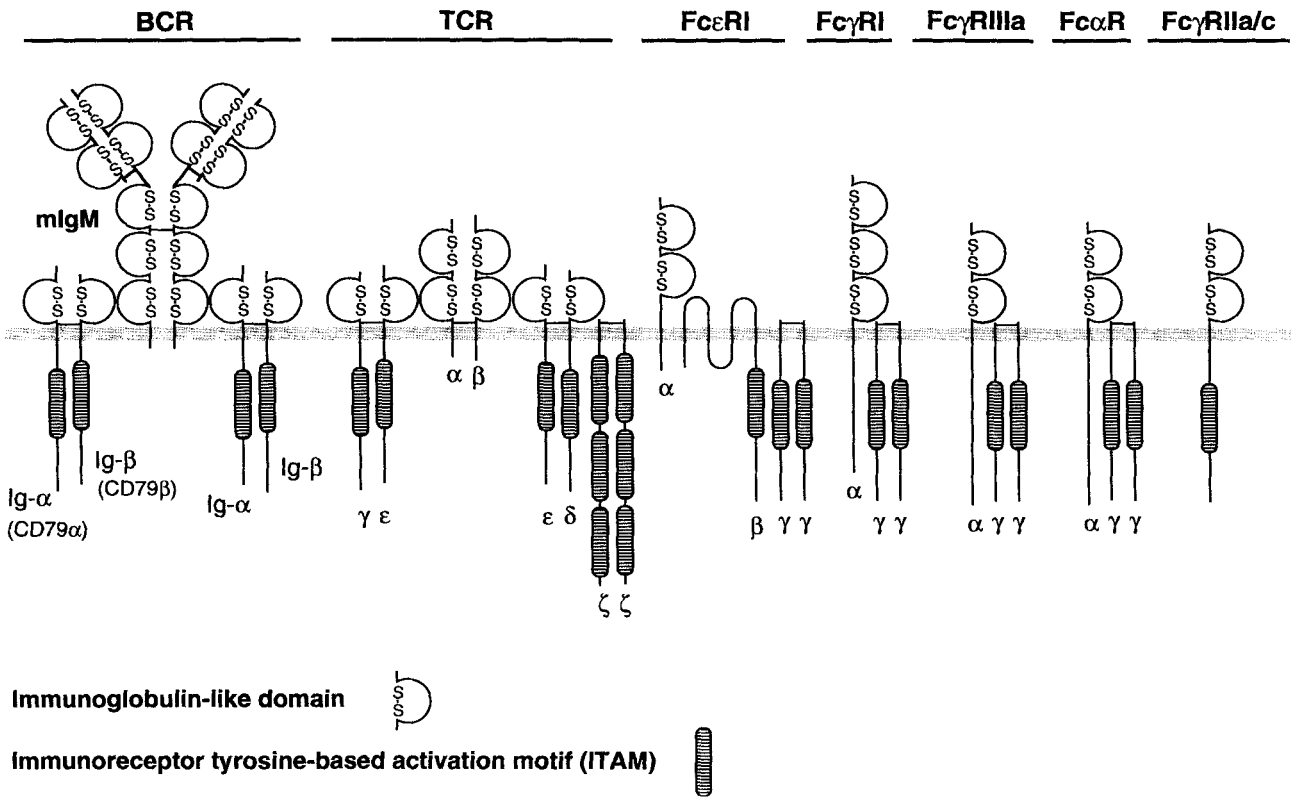


FIG. 1. Functional regions in the epidermal growth factor receptor and the T-cell antigen receptor. A wide range of growth factor and cytokine receptors share structural and functional homology resembling that of the epidermal growth factor receptor (EGF-R). They consist of a single-chain polypeptide in which an extracellular ligand-binding domain and a catalytically active intracellular signal-transducing domain are linked by a short hydrophobic transmembrane region. In contrast, antigen receptors on T cells, B cells (not shown), and many Fc receptors are composed of multiheteromeric structures in which the extracellular antigen-binding domains and the intracellular signal-transducing domains reside in different polypeptides. Antigen receptors are devoid of catalytic activity, and their signal transducing capability is dependent on cytosolic PTKs that either constitutively or transiently associate with individual signal-transducing receptor subunits.

FIG. 2. Schematic structure and subunit composition of antigen and Fc receptors. ITAM and immunoglobulin-like domains and intradomain disulfide bonds are indicated. Intersubunits disulfide bonds are indicated by a straight line. In a small proportion of TCRs the α–β antigen recognition unit is replaced by a γ–δ heterodimer. In addition, the ζ–ζ homodimer is replaced in some receptors by heterodimers of ζ and either the Fc receptor γ chain or the alternatively spliced form of ζ, the η chain. Homodimers of Fcγ chains in Fc receptors can be replaced by either γ–ζ heterodimers or ζ–ζ homodimer. FcγRII represents the a and c forms; the FcγRIIb is ITAM-less and possesses instead a negatively regulating module ((ITIM). An additional isoform of FcγRIII that lacks ITAM (FcγRIIIb) possesses only the extracellular portion of the molecule that is embedded in the cell membrane via a glycophosphatidylinositol moiety. Other Fc receptors are involved in immunoglobulin transcytosis and are devoid of ITAMs and ITIMs.


BCR
TCR
FcεRI
FcγRI
FcγRIIIa
FcαR
FcγRIIa/c
mIgM
Ig-α
(CD79α)
Ig-β
(CD79β)
Ig-α
Ig-β
γ ε
α β
ε δ
ζ ζ
α
β γ γ
α
γ γ
α γ γ
α γ γ
Immunoglobulin-like domain
Immunoreceptor tyrosine-based activation motif (ITAM)

eight amino acids (Fig. 3). Receptor engagement is followed by a rapid and transient phosphorylation of tyrosine residues within their ITAMs, thereby creating temporary binding sites for Src homology 2 (SH2)-containing signaling molecules operating downstream of the activated receptor. Apart from the nonpolymorphic subunits of the T-cell antigen receptor (TCR) and B-cell antigen receptor (BCR), ITAMs are also found in Fc receptors for various immunoglogubin (Ig) subclasses (Pfefferkorn and Yeaman, 1994; Ravetch and Kinet, 1991) and in cytoplasmic regions of several distinct membrane viral proteins (Alber *et al.*, 1993; Beaufils *et al.*, 1993).

This review focuses on the progress in our understanding of the functional role of ITAMs in the antigen and Fc receptor-mediated signal transduction pathways. In addition, it emphasizes critical PTKs operating upstream and downstream of the ITAMs and discusses the potential involvement of ITAMs in tolerance induction, immunosuppression, and viral immunopathology.

II. Structure and Function of Immunoreceptor Tyrosine-Based Activation Motifs (ITAMs)

A. The Modular Architecture of Immunoreceptors

The antigen receptors and most of the Fc receptors are composed of a multisubunit protein complex that includes a ligand-binding extracellular portion and a signal-transducing intracellular structure. These two functional moieties are compartmentalized in the TCR, BCR, and most of the Fc receptors, in distinct receptor subunits (see Fig. 2).

The BCR is composed of a membrane-anchored, antigen-binding immunoglobulin molecule that is noncovalently associated with two pairs of disulfide-linked Ig-α (CD79a; MB-1) and Ig-β (CD79b; B29) heterodimers (Campbell and Cambier, 1990; Hombach *et al.*, 1990; Reth, 1992; Reth *et al.*, 1991). A third chain, termed Ig-γ, is a truncated form of Ig-β that dimerizes with Ig-α in some BCR complexes in bone marrow or other types of activated B cells (Friedrich *et al.*, 1993). All classes of surface immunoglobulins associate with apparently identical Ig-α and Ig-β heterodimers. Although the ratio between the Ig-α/Ig-β heterodimers and the surface immunoglobulins within a single receptor is unknown, the requirement for signal-transducing elements indicates a minimal ratio of 1:1, whereas the bilateral symmetry of the receptor suggests a ratio of 2:1.

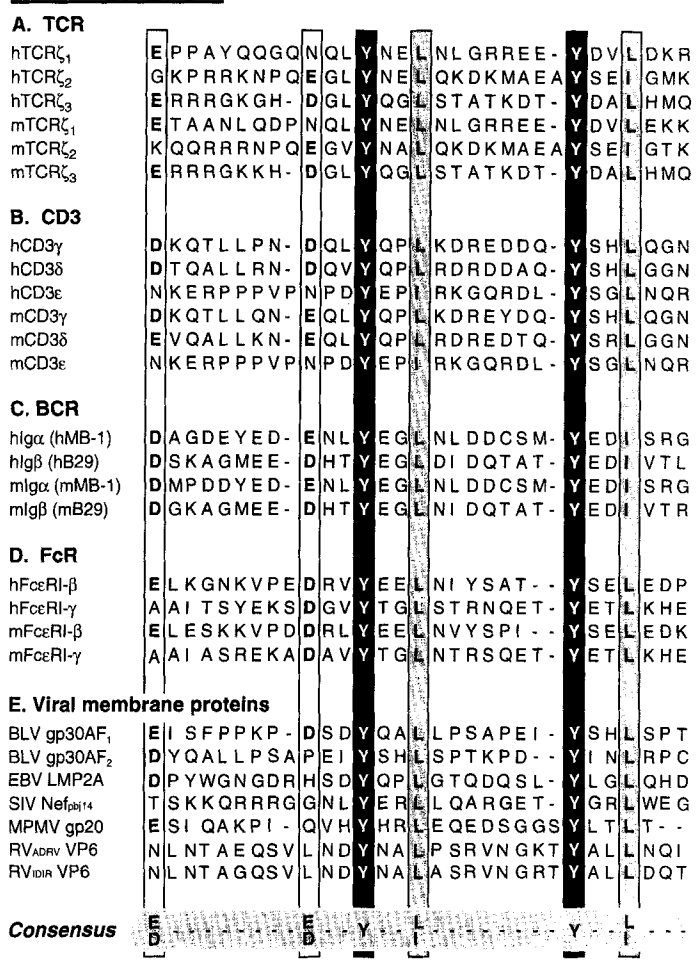


FIG. 3. Amino acid sequence alignments of immunoreceptor tyrosine-based activation motifs (ITAMs) found in the cytoplasmic regions of human and murine antigen and Fc receptors and in various viral proteins. The minimal consensus ITAM sequence consists of two YxxL/I repeats (one-letter code of amino acids, where "x" stands for any residue), 6–8 residues apart. The biological role of the partially conserved E/D residues located at positions -3 and -11/12 amino-terminal to the first ITAM's tyrosine is not clear. Sequences are aligned according to the conserved tyrosines (Y) and leucine (L)/isoleucine (I) residues and the partially conserved glutamic acid (E)/aspartic acid (D) residues. Gaps are indicated by a hyphen. BLV, bovine leukemia virus; gp, glycoprotein; EBV, Epstein–Barr virus; LMP2A, latent membrane protein 2A; SIV, simian immunodeficiency virus (isolate pbj14); Nef, negative factor; MPMV, Mason–Pfizer monkey virus; RT, rotavirus; VP, inner capsid viral protein; ADRV, adult diarrhea rotavirus; IDIR, infectious diarrhea or infant rats.

The BCR recognizes, and directly interacts, with soluble unprocessed antigens.

The TCR is more complexed and consists of highly polymorphic antigen-binding α–β or γ–δ heterodimers that mediate the specific recognition of antigen peptides in the context of the major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Each of these four polypeptides contains a single transmembrane domain and a short cytoplasmic tail (up to 12 amino acids) that is insufficient to play a direct role in signal transduction. The signal-transducing moiety in the TCR consists of three pairs of disulfide-linked molecules, collectively termed the CD3 complex (Blumberg *et al.*, 1990; Clevers *et al.*, 1988; Koning *et al.*, 1990), that are noncovalently associated with the clonotypic α–β or γ–δ chains.

The CD3 includes the γ–ε and δ–ε heterodimers (Blumberg *et al.*, 1990; Koning *et al.*, 1990) and a disulfide-linked homodimer of ζ–ζ or ζ linked to its alternatively spliced form, the η chain (Baniyash *et al.*, 1988a; Mercep *et al.*, 1988; Weissman *et al.*, 1988). Under certain conditions a larger heterogeneity in receptor subunits may possibly occur by FcεRI γ chain heterodimerization with the TCRζ or η chains (Orloff *et al.*, 1990). The γ, δ, and ε chains are homologous with each other and with several other members of the immunoglobulin supergene family (Gold *et al.*, 1987; Krissansen *et al.*, 1986). Their corresponding genes are located within a 75-kb region of the human chromosome 11 (Tunnacliffe *et al.*, 1988), suggesting a common origin by gene duplication. In contrast, the ζ chain is distinct in structure and possesses a much shorter extracellular region (9 vs 79–104 amino acids) and a longer cytoplasmic tail (112–113 vs 44–79 amino acids) with a consensus ATP-binding domain.

The antigen recognition subunits in both BCR and TCR possess only a short cytoplasmic tail that is devoid of catalytic activity. In contrast, the accessory subunits possess a long cytoplasmic tail that includes a unique module capable of autonomously triggering cell activation. This conserved activation domain, termed ITAM (Cambier, 1995b), was initially described by Reth (1989) and was designated as the tyrosine-based activation motif (TAM; Samelson and Klausner, 1992), antigen recognition activation motif (ARAM; Weiss, 1993), antigen receptor homology 1 motif (ARH1; Clark *et al.*, 1992), YxxL-motif (Wegener *et al.*, 1992), or the Reth motif (Exley *et al.*, 1994). ITAMs are duplicate of the sequence YxxL/I (where Y is tyrosine, L is leucine, I is isoleucine, and X is any amino acid; see also Fig. 3), with six to eight intervening residues. Presence of the conserved

tyrosine and leucine/isoleucine residues and the spacing between them are critical for the biological function of ITAMs (Shaw *et al.*, 1995). Their conserved physical structure is mandatory for their function as temporal scaffolds and enables them to assemble multiple critical signaling molecules following receptor ligation and phosphorylation of their tyrosine residues.

Fc receptors are members of a large family of transmembrane proteins that recognize the Fc portion of immunoglobulins (Fig. 2). By themselves they are devoid of antigen-binding capacity, but when complexed with antibodies they can interact with soluble antigens. Each of the five classes of immunoglobulins has one type or more of corresponding Fc receptors, including Fcγ, Fcα, Fcε, Fcμ, and Fcδ receptors (reviewed in Daëron, 1997a; Hulett and Hogarth, 1994; Ravetch and Kinet, 1991). Members of the largest subgroup of Fc receptors are composed of a ligand-binding α subunit in association with one or more ITAM-containing signaling subunits. On receptor aggregation they trigger cell responses using signal transduction pathways similar to those of the BCR and TCR.

A different group of Fc receptors constitutes a family of a single chain IgG receptors (FcγRIIb) whose intracytoplasmic region possesses the inhibitory motif, ITIM, instead of ITAM. The functional relevance of these receptors to the regulation of responses induced by co-ligation of the BCR is discussed in section VI. Other Fc receptors that are devoid of signal-transducing capacity include the FcγRIIIb, which is embedded in the cell membrane via a glycophosphatidylinositol moiety and possesses only the extracellular portion of the molecule, and a group of Fc receptors that are involved in immunoglobulin transcytosis.

B. Functional Role of ITAMs in Immunoreceptor-Linked Signaling Pathways

Several observations have initially led to the hypothesis that ITAMs may play a role in mediating receptor interaction with cytoplasmic-signaling effector molecules. These include the predominant occurrence of ITAMs in receptor subunits that are devoid of both ligand-binding capacity and catalytic activity, and the restricted location of ITAMs in cytoplasmic regions of transmembrane molecules. More recent studies provided substantial data to support this hypothesis and established the critical role of ITAMs in linking antigen receptors to specific signaling pathways.

The functional importance of ITAMs as signaling modules was first revealed in experiments in which "inert" receptor molecules were modified by means of genetic engineering techniques creating chimeric receptor

molecules with different ITAM-containing cytoplasmic tails. In one such experiment, the extracellular and transmembrane domains of CD8 were linked to the cytoplasmic tail of the TCRζ chain, which possesses three consecutive ITAM sequences (Irving *et al.*, 1993; Irving and Weiss, 1991). Transient expression of the chimeric receptor in a variety of T-cell lines followed by cross-linking with bivalent antibodies induced both proximal and distal activation events that are normally associated with the stimulation of intact TCR. Similar results were obtained following expression of other chimeric receptor constructs that possess the TCRζ cytoplasmic tail and the extracellular and transmembrane domains of other receptor molecules, such as the CD4, CD16, or IL2-Rβ chain (Tac receptor; CD25) (Letourneur and Klausner, 1991; Romeo and Seed, 1991). A single TCRζ ITAM (the membrane juxtaposed, or amino-terminal ITAM, which will be referred to as ITAMζ_1), which was linked to the extracellular and transmembrane domains of CD8, was also capable of transducing a wide variety of activation signals. These were sufficient for the induction of early T-cell activation events, including a rise in the intracellular concentration of free Ca^{2+} ions ($[Ca^{2+}]_i$), and increased tyrosine phosphorylation of various protein substrates. In addition, ligation of this chimeric receptor induced late activation events, such as the induction of NF-AT transcription factor activity and the expression of the CD69 cell surface activation marker (Irving and Weiss, 1991). Introduction of three copies of the ITAMζ_1 sequence resulted in quantitatively augmented signaling events, suggesting that ITAM multiplication functions as a signal amplifying mechanism (Irving and Weiss, 1991).

ITAM sequences derived from the CD3ε or the FcεRIγ chains have also been manipulated in genetic studies and were linked to the extracellular and transmembrane regions of the CD4, Fc, or IL2-Rβ chain receptors. Expression of the chimeric receptors in various cell types enabled them to transduce signals leading to degranulation of basophils and differentiation of T lymphocytes into cytotoxic effector cells (Letourneur and Klausner, 1992; Romeo and Seed, 1991; Wegener *et al.*, 1992). Additional ITAM sequences were also functional in coupling chimeric receptor molecules to their cytoplasmic signaling pathways, including ITAMs from the cytoplasmic region of the CD3γ and CD3δ chains, the BCR Ig-α and Ig-β chains, the FcγRIIa (Flaswinkel and Reth, 1994; Gauen *et al.*, 1994; Jouvin *et al.*, 1994; Kim *et al.*, 1993; Romeo *et al.*, 1992a,b; Sanchez *et al.*, 1993), and various viral membrane glycoproteins, such as the bovine leukemia virus (BLV) glycoprotein (gp) 30 and the Epstein–Barr virus (EBV) latent membrane protein 2A (LMP2A) (Alber *et al.*, 1993; Beaufils *et al.*, 1993).

In order to define a minimal signaling unit in the cytoplasmic tail of the receptors, transient transfection studies were performed utilizing chimeric receptors possessing cytoplasmic tails of varied lengths. Studies by Letourneur and Klausner (1992) demonstrated that the ITAM-containing minimal signaling unit in CD3ε possesses ≤22 residues. Romeo *et al.* (1992a) and Koyasu *et al.* (1994) narrowed this region by demonstrating that an ITAMζ_1-containing sequence of 18 residues was able to confer to the chimeric receptors the ability to transduce signals leading to Ca^{2+} mobilization, IL-2 production, tyrosine phosphorylation, and induction of cytotoxic activity. Both pairs of conserved tyrosine and leucine/isoleucine residues within the ITAM appeared to be essential for the ability to transduce activation signals; mutation of any of these residues completely abrogated the signaling capacity (Koyasu *et al.*, 1994; Letourneur and Klausner, 1992; Romeo *et al.*, 1992a).

C. Antigen and Fc Receptor Cross-Linking Results in Tyrosine Phosphorylation of the Receptors ITAMs

Tyrosine phosphorylation of receptor subunits is among the first detectable biochemical events that follow receptor engagement. This was observed in the cytoplasmic regions of TCR and BCR subunits, as well as the Fcε, Fcγ and Fcα, receptor subunits (Baniyash *et al.*, 1988b; Gold *et al.*, 1991; Paolini *et al.*, 1992; Pfefferkorn and Yeaman, 1994; Samelson *et al.*, 1986, 1987). Mutational analysis of chimeric receptors demonstrated that the tyrosine residues within the ITAMs are critical for receptor coupling to cytoplasmic signaling pathways (Koyasu *et al.*, 1994; Letourneur and Klausner, 1992; Romeo *et al.*, 1992a). These findings were substantiated by *in vitro* studies demonstrating that phosphorylation of the two conserved tyrosine residues is essential for ITAM interaction with downstream signaling molecules (Bu *et al.*, 1995; Isakov *et al.*, 1995; Iwashima *et al.*, 1994).

In an attempt to test more precisely the biological importance of the phosphorylated tyrosine residues within the ITAMs, an *in vivo* experiment was designed in which synthetic peptides that correspond in sequence to individual ITAMs and possess phosphorylated tyrosine residues were introduced into T lymphocytes and their effects on cell activation responses were monitored. Because phosphopeptides are extremely vulnerable to the activity of protein tyrosine phosphatases (PTPases), which are abundant within cells, a nonhydrolyzable phosphotyrosyl mimetic compound was used for the preparation of PTPase-resistant phospho-ITAM peptidomimetics (Wange *et al.*, 1995a). In these 17-mer peptides, a group of difluorophosphonomethyl phenylalanyl (F_2Pmp) was used to replace the amino- and/or carboxy-terminal tyrosine residues in a sequence corresponding to

ITAMζ_3. Incubation of permeabilized T cells with a peptide, in which both tyrosine residues were replaced with F_2Pmp [$(F_2Pmp)_2$-ITAMζ_3], competitively blocked ZAP-70 binding to activated TCR. Peptides possessing only one F_2Pmp group, replacing either the amino- or the carboxy-terminal tyrosine, were nonfunctional as competitive inhibitors. These results were in accordance with *in vitro* binding studies showing low binding affinities of monophosphorylated peptides to the ZAP-70 tandem SH2 domains (Isakov *et al.*, 1996). Furthermore, introduction of $(F_2Pmp)_2$-ITAMζ_3 into T cells inhibited both TCR activation-induced phosphorylation of ZAP-70 and activation of its catalytic domain. This coincided with reduced tyrosine phosphorylation of a number of substrate proteins, suggesting that $(F_2Pmp)_2$-ITAMζ_3 interacts directly with the ZAP-70 tandem SH2 domains (Fig. 4), thereby blocking the association of ZAP-70 with the TCR.

It is worth noting that tyrosine phosphorylation of the TCRζ and CD3ε receptor chains was also decreased in $(F_2Pmp)_2$-ITAMζ_3-treated T cells. However, the reduced tyrosine phosphorylation of TCR subunits was not due to reduced tyrosine kinase activity, as ZAP-70 exhibits no activity for TCR subunits (Isakov *et al.*, 1996), and $(F_2Pmp)_2$-ITAMζ_3 (or its homologous peptide $pTyr_2$-ITAMζ_3) failed to block Lck activity (Isakov *et al.*, 1996; Iwashima *et al.*, 1994; Wange *et al.*, 1995a). The decreased TCR tyrosine phosphorylation may have reflected blocking of all available ZAP-70 SH2 domains by excess $(F_2Pmp)_2$-ITAMζ_3 peptide, thereby preventing ZAP-70 from binding to the endogenous TCR phospho-ITAMs and protecting them from dephosphorylation (Iwashima *et al.*, 1994). A similar mechanism has been demonstrated for phospholipase Cγ1 (PLCγ1) on its binding to the epidermal growth factor (EGF) receptor (Rotin *et al.*, 1992a,b).

Results obtained with the phospho-ITAM peptidomimetics support the model in which the two phosphotyrosine residues within the TCR ITAMs regulate the early TCR-mediated signaling events through a ZAP-70-dependent mechanism.

III. ITAMs Interaction with Protein Tyrosine Kinases (PTKs)

A. Src Family PTKs Are the Putative ITAM Kinases

A variety of studies with the Src family PTKs established their critical role in tyrosine phosphorylation of ITAM sequences. The Src family of genes consists of at least eight mammalian members (Src, Lck, Fyn, Yes, Lyn, Hck, Fgr, Blk) whose protein products exhibit an overall structure homology with similar mechanisms of regulation and function (Brown and Cooper, 1996; Cooper and Howell, 1993). However, individual Src kinases may differ in tissue distribution, subcellular compartmentalization, and

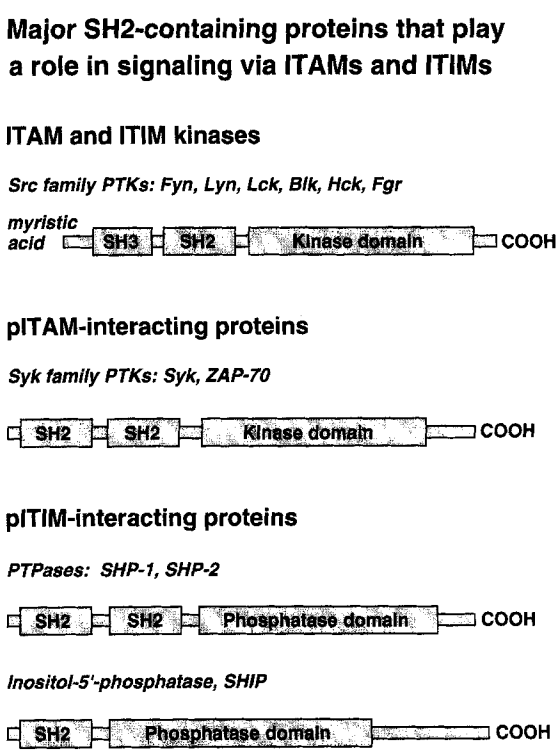


FIG. 4. ITAM and ITIM interacting protein tyrosine kinases and phosphatases. Members of the Src family of PTKs are the putative ITAM and ITIM phosphorylating enzymes. Ligation of an ITAM-containing receptor is followed by phosphorylation of tyrosine residues within the ITAM and recruitment of Syk family PTKs to the activated receptor. Binding occurs by interaction of the tandem SH2 domains of PTK with the biphosphorylated ITAM. This enables further phosphorylation and activation of Syk and/or ZAP-70, which in turn activates downstream signaling events. Coligation of ITIM-containing receptors and the BCR results in tyrosine phosphorylation of the ITIM followed by recruitment of SH2-containing effector proteins. Among the pITIM-binding proteins are the SHP-1 and SHP-2 PTPases, which can then dephosphorylate positively regulating effector molecules and turn off the activation signals. The inositol-5′-phosphatase, SHIP, can interact with pITIM and inhibits B-cell activation by interfering with the formation of inositol phosphate second messengers and the Ca^{+2}-dependent activation signals.

substrate specificity (Cooper and Howell, 1993; Hanks and Hunter, 1995; Mustelin and Burn, 1993).

Six of these gene products (Lck, Fyn, Lyn, Hck, Fgr, Blk; see Fig. 4) are expressed in hematopoietic cells and are involved in receptor-linked signal transduction pathways. Among them, Lck and Fyn are involved in T-cell signaling and they both respond by a rapid and transient increase

in activity following engagement of the TCR (Burkhardt *et al.*, 1994; da Silva *et al.*, 1992; Tsygankov *et al.*, 1992). The three predominant Src family members expressed in B cells are Lyn, Fyn, and Blk, and on ligation of the BCR they all undergo a rapid process of activation (Burkhardt *et al.*, 1991; Saouaf *et al.*, 1994; Yamanashi *et al.*, 1992). Transient activation of Lyn was also demonstrated following engagement of the FcεRI in mast cells (Penhallow *et al.*, 1995). These results suggested a role for the Src family members in the very early steps that follow immunoreceptor ligation, perhaps in the phosphorylation of the antigen receptor ITAMs (Burkhardt *et al.*, 1994).

Src kinases possess a conserved tyrosine residue at their carboxy terminus (e.g., Y505 and Y528, in Lck and Fyn, respectively) that, when phosphorylated [apparently, by Csk (Bergman *et al.*, 1992; Okada *et al.*, 1991)], interacts directly with the autologus SH2 domain (Amrein and Sefton, 1988; Cooper and MacAuley, 1988; MacAuley and Cooper, 1989; Veillette *et al.*, 1992). This results in folding of the molecule and acquisition of a conformation in which the catalytic domain is not accessible for its substrates. Thus, phosphorylation of the carboxy-terminal tyrosine residue is considered to downregulate Src kinases activity.

Replacement of the carboxy-terminal tyrosine by phenylalanine gives rise to a constitutively active kinase that is refractory to phosphorylation and downregulation by Csk. Overexpression of a mutated constitutively active Lck (Y505F) or Fyn (Y528F) in T cells resulted in augmented TCR-induced tyrosine phosphorylation of both TCRζ and CD3ε chains (Weil *et al.*, 1995). In contrast, phosphorylation of TCRζ was found to be impaired in activated J.CaM1 T cells that are devoid of a functional Lck (Straus and Weiss, 1992). Studies in thymocytes from Lck- and/or Fyn-deficient mice demonstrated that Lck, but not Fyn, is required for regulating the constitutive phosphorylation of the TCRζ chain (van Oers *et al.*, 1996a). A low level of phosphorylation of the TCRζ was observed in peripheral T cells isolated from Lck-deficient mice (Molina *et al.*, 1992). These results implied that Lck is essential for the regulation of TCR signaling in thymocytes. However, in peripheral blood T cells, other Src PTKs, such as Fyn, could partially compensate for the absence of Lck (Groves *et al.*, 1996; van Oers *et al.*, 1996b).

In some studies, knockout of the Lyn kinase by targeted gene disruption resulted in a lack of phosphorylation of BCR ITAMs and impaired BCR signaling (Nishizumi *et al.*, 1995; Takata *et al.*, 1994). In a different study, it was found to induce greater activation of the MAPK cascade and enhanced proliferative response to anti-IgM treatment (Chan *et al.*, 1997). The enhanced B-cell proliferation was suggested to reflect a defective FcγRIIb-mediated downregulation, implying Lyn involvement in both the initiation

of activation of BCR-induced activation signals and the FcγRIIb-induced negative regulation of the response.

A different approach for analysis of the potential role of Src kinases in the regulation of immunoreceptor ITAMs was attempted by Songyang *et al.* (1994a) who screened a random synthetic peptide library for potential Src substrates. Their results indicated that YxxL/I, the consensus ITAM sequence, is the preferred phosphorylation sequence for Src kinases (Songyang *et al.*, 1994a). In addition, Lck immunoprecipitated from T cells and recombinant purified Lck were found to efficiently phosphorylate synthetic peptides derived from TCRζ and CD3ε ITAMs (Affolter *et al.*, 1994; Isakov *et al.*, 1996).

Differences in the primary structure of individual Src PTKs are likely to be reflected by variable affinities of the enzymes to specific protein substrates. As a result, Src PTKs may differ in their ability to phosphorylate specific ITAMs or even specific tyrosine residues within a single ITAM. For example, TCR stimulation of Lck^- or Fyn^- T cells has demonstrated that, besides their ability to phosphorylate shared substrates, these two PTKs also regulate tyrosine phosphorylation of unique protein substrates (Qian *et al.*, 1997). *In vitro* kinase assays revealed that Lyn incubation with the Ig-α ITAM resulted in preferred phosphorylation of the conserved amino-terminal tyrosine residue (Y182) (Flaswinkel and Reth, 1994), with little or no phosphorylation of the carboxy-terminal tyrosine (Y193). In contrast, Blk exhibited a high level of phosphorylation of both Ig-α ITAM tyrosine residues *in vitro,* although it was much less active toward the same substrate *in vivo* (Flaswinkel *et al.*, 1995). Thus, expression of a unique set of Src kinases within an individual cell type may provide an additional level of specificity that contributes to the biologic outcome of activation of an ITAM-containing receptor.

A small fraction of the Src family kinases is associated directly with immunoreceptor subunits in resting cells. This includes the Fyn that is bound directly to the CD3ε chain (Samelson *et al.*, 1990; Timson Gauen *et al.*, 1992) and Fyn, Lyn, and Blk that associate with the Ig-α and Ig-β in B cells (Clark *et al.*, 1992). However, receptor engagement with its cognitive ligand is rapidly followed by recruitment of additional kinases to the receptor site. It is assumed that these PTKs phosphorylate the ITAM tyrosine residues, thereby enabling the recruitment of additional SH2-containing signaling molecules to the activated receptors.

Although TCR engagement is sufficient for the induction of certain activation events in T cells (such as IL-2 receptor expression), full activation and induction of proliferation and IL-2 production require simultaneous stimulation of the CD4 or CD8 coreceptor molecules (Swain, 1983). The CD4 and CD8 are expressed on T helper and cytotoxic cells, respectively,

and their cytoplasmic tails associate with a large proportion of the cellular Lck (Rudd *et al.*, 1988; Veillette *et al.*, 1988). This specific association is mediated via the amino terminus of Lck that includes a double cysteine-containing motif, unique among the Src PTKs (Shaw *et al.*, 1989), that enables a noncovalent association with the cysteine-containing motif in the cytoplasmic tail of CD4 and CD8 (Shaw *et al.*, 1990). Following interaction between a TCR and an MHC-bound antigenic peptide, the CD4 or CD8 coreceptors interact with nonpolymorphic regions on class II or class I MHC molecules, respectively, thereby recruiting the associated Lck into close proximity with the cytoplasmic regions of the TCR polypeptides (Rojo *et al.*, 1989; Saizawa *et al.*, 1987). The formation of these multimolecular complexes may be followed by further activation of Lck and/or association with and phosphorylation of substrates within the TCR. Studies with N-terminal truncated CD4 or CD8 constructs, which cannot associate with Lck, further demonstrated that the biological function of the coreceptor molecules is dependent on their ability to bind Lck (Glaichenhaus *et al.*, 1991; Miceli *et al.*, 1991).

Using mild detergent for the preparation of cell lysates, a single study demonstrated that Lck can coimmunoprecipitate with the TCR (Díez-Orejas *et al.*, 1994). However, coimmunoprecipitation required the presence of CD4 on the cells, suggesting that a certain proportion of the CD4-bound Lck associated with the TCR indirectly.

B. Syk-Family PTKs Are Regulated by Phospho-ITAMs and Are Downstream Effectors of the Activated Receptors

Members of the Syk family of PTKs that are actively involved in signaling via ITAM-containing receptors include Syk and the 70-kDa ζ chain associated protein, ZAP-70. The two enzymes possess two adjacent SH2 domains at their amino terminus and have a high level of overall structural homology (Fig. 4). Their tandem SH2 domains are critical for their function and serve to couple the enzymes to doubly tyrosine phosphorylated ITAMs. However, sequence heterogeneity between Syk and ZAP-70 at the carboxy terminus and the region that links the carboxy-terminal SH2 and the kinase domain may account for the differences in their regulation and substrate specificity.

The independent ability of the two enzymes to transduce signals from immunoreceptors revealed different patterns of regulation. For example, chimeric receptors possessing Syk as their cytoplasmic tail were able to transfer signals leading to activation of T cells and induction of cytotoxic activity (Kolanus *et al.*, 1993). In contrast, chimeric receptors possessing ZAP-70 as their cytoplasmic tail were unable to transduce activation signals in T cells. Co-cross-linking of the ZAP-70 receptor with an additional

chimeric receptor possessing Fyn in its cytoplasmic tail was essential for the induction of cell activation. These results suggested that ZAP-70 may have more stringent requirements for its *in vivo* activation and that ZAP-70 but not Syk activation may require prephosphorylation by a Src kinase. This model is supported by findings in transiently transfected Cos cells where overexpressed Syk induced strong phosphorylation activity whereas ZAP-70 required coexpression of either Lck or Fyn in order to achieve similar cellular effects (Iwashima *et al.*, 1994). The *in vivo* differential activation dependency of Syk and ZAP-70 on Src kinases is in correlation with the *in vitro* findings demonstrating that binding of Syk (Kimura *et al.*, 1996b; Rowley *et al.*, 1995; Shiue *et al.*, 1995), but not ZAP-70 (Isakov *et al.*, 1996; Saxton *et al.*, 1994), to tyrosine-phosphorylated ITAM peptides upregulates the catalytic activity of the PTK, and that activation of ZAP-70 required prephosphorylation by a Src kinase (Chan *et al.*, 1995; Wange *et al.*, 1995b; Zoller *et al.*, 1997).

1. ZAP-70

ZAP-70 is restricted to T lymphocytes and natural killer cells and is implicated in signaling via the TCR (Chan *et al.*, 1991, 1992; Negishi *et al.*, 1995). It associates with tyrosine-phosphorylated ITAMs on TCR subunits, but is unable to phosphorylate tyrosine residues within TCRζ or CD3ε chain ITAMs (Isakov *et al.*, 1996). Receptor engagement is followed by tyrosine phosphorylation and activation of ZAP-70 (Chan *et al.*, 1995; Wange *et al.*, 1995b). ZAP-70 is constitutively associated with the TCRζ chain in thymocytes and lymph node T cells (van Oers *et al.*, 1993, 1994), but in T-cell lines it associates with the TCR only following receptor engagement (Chan *et al.*, 1991).

The information that led to the understanding of the critical role of ZAP-70 in T-cell activation and differentiation was obtained from the analysis of T cells from a naturally occurring human severe combined immunodeficiency disease (SCID), characterized by either complete lack or reduced levels of the ZAP-70 protein (Arpaia *et al.*, 1994; Chan *et al.*, 1994a; Elder *et al.*, 1994). Patients lacking ZAP-70 are devoid of mature $CD8^+$ T cells whereas their $CD4^+$ T cells exhibit reduced tyrosine phosphorylation of substrate proteins and fail to produce IL-2 or to proliferate in response to antigens or mitogens. ZAP-70-deficient T cells respond normally to pharmacological agents that bypass the early TCR-linked activation events supporting the assumption that nonresponsiveness of these T cells is due to a block at a proximal step downstream to the activated receptor. Further analysis of these patients demonstrated that ZAP-70 is indispensable for the development of mature $CD8^+$ T cells and for TCR-induced signaling and activation of $CD4^+$ T cells. The essential role of

ZAP-70 in T-cell differentiation was further demonstrated in mice in which the entire gene segment encoding the ZAP-70 protein has been deleted (Negishi *et al.*, 1995). The number of thymocytes in these mice is comparable to that of wild-type mice, but they lacked both CD4 and CD8 single-positive T cells. Furthermore, lack of ZAP-70 prevented not only positive selection of thymocytes and their differentiation into single-positive mature T cells, but also the negative selection and elimination of autoreactive T cells by a process of apoptosis. In contrast, natural cytotoxicity of NK-sensitive target tumor cells and antibody-dependent cell-mediated cytotoxicity (ADCC) of spleen-derived NK cells was not impaired in ZAP-70-deficient mice. Thus, despite the fact that ADCC is mediated by the FcγRIIIa low-affinity IgG receptor (CD16), which can associate with either ITAM-containing TCRζ chain or FcεRIγ chain (Ravetch, 1994), ZAP-70 appears to be dispensable for either NK cell development or function.

2. *Syk*

Syk is more ubiquitous then ZAP-70 and is expressed in most hematopoietic cell types (Taniguchi *et al.*, 1991). It has been implicated in signaling through a wide range of ITAM-containing receptors, including the BCR (Hutchcroft *et al.*, 1991; Law *et al.*, 1993; Yamada *et al.*, 1993), TCR (Chan *et al.*, 1994b; Chu *et al.*, 1996; Latour *et al.*, 1997), FcεRI (Benhamou *et al.*, 1993; Hutchcroft *et al.*, 1992; Minoguchi *et al.*, 1994), and Fcγ receptors (Agarwal *et al.*, 1993; Chacko *et al.*, 1994; Corey *et al.*, 1994; Durden and Liu, 1994), as well as non-ITAM-containing receptors (Clark *et al.*, 1994; Minami *et al.*, 1995; Rezaul *et al.*, 1994).

On engagement of an immunoreceptor, Syk is recruited and directly associates with tyrosine-phosphorylated ITAMs (Hutchcroft *et al.*, 1992). High-affinity interaction of Syk SH2 domains with the consensus phosphorylated ITAM (pITAM) sequence, pYxxL, has also been demonstrated *in vitro* using the Syk SH2 domains for screening of random peptide libraries (Songyang *et al.*, 1994b).

In contrast to ZAP-70, Syk is able to phosphorylate *in vitro* ITAM-containing synthetic peptides such as ITAMζ_1 (Latour *et al.*, 1997). Furthermore, Syk could also stimulate tyrosine phosphorylation of a ζ-bearing chimera in transiently transfected Cos-1 cells. These data raised the possibility that Syk, similar to Lck, can regulate initiation of TCR signaling by promoting phosphorylation of the TCR ITAMs (Latour *et al.*, 1997). Nevertheless, both T-cell differentiation and activation in either human or mice are absolutely dependent on the presence of intact ZAP-70. Therefore, some of the data obtained by Latour *et al.*, (1997) may be attributed to the fact that they used the transient expression of genetically engineered proteins and either T-cell hybridoma (BI-141) or transfected Cos-1

cells. This artificial system may permit the occurrence of biochemical events that normally do not take place in normal T cells or alternatively, it is still possible that mature T cells express very little or no Syk (Chan *et al.*, 1994b; Law *et al.*, 1994).

Inactivation of the Syk gene in a chicken B-cell line using a gene targeting technique completely abolished the ability of the BCR to transduce activation signals leading to tyrosine phosphorylation of substrate proteins, production of inositol phosphates, and induction of $[Ca^{2+}]_i$ rise (Takata *et al.*, 1994). A selective inhibitor of Syk, piceatanol, also blocked FcεRI downstream signaling events in activated mast cells (Oliver *et al.*, 1994).

The wide expression of Syk in hematopoietic cells and its potential role in signaling via numerous receptors suggested the involvement of this enzyme in a wide variety of biological functions. Support for this assumption was obtained in genetic studies in which a mutated *syk* gene was introduced into embryonic stem cells by homologous recombination and by the use of targeted clones for transmitting the mutation into the mouse germline (Cheng *et al.*, 1995; Turner *et al.*, 1995). Mice homozygous for the *syk* mutation ($syk^{-/-}$) suffered severe systemic hemorrhages and died shortly after birth. The number of progenitor B cells in $syk^{-/-}$ embryos was unaltered, but lymphoid organs from either $syk^{-/-}$ embryos or radiation chimeras that were reconstituted with Syk-deficient fetal liver cells showed no detectable IgM-bearing mature B cells. Further analyses of lymphoid cells from Syk-deficient mice have indicated that the differentiation of B cells was blocked at the pro-B- to pre-B-cell transition, apparently as a result of disrupting signaling from the pre-B cell receptor. In contrast, lymphoid organs of Syk-deficient chimeras showed normal proportions of mature single-positive CD4 and CD8 T cells, normal cell surface expression levels of CD3, αβ TCR, and CD69, and normal proliferative responses following TCR or IL-2 receptor engagement. Thus, data obtained from analysis of the Syk-deficient mice have indicated a critical role for Syk in B-cell development, but not in the development and responsiveness of T lymphocytes. Other studies confirmed that *Syk* disruption did not affect the maturation of T cells expressing the αβ TCR, but also demonstrated that the development of epithelial T cells expressing the γδ TCR was abolished (Mallick-Wood *et al.*, 1996).

IV. ITAMs as Scaffolds for Multiple Effector Molecules

A. Tyrosine-Phosphorylated ITAMs Function as Temporary Scaffolds for Effector Molecules

As mentioned earlier, only a relatively small number of molecules associate with nonphosphorylated antigen and Fc receptors. However, ligand–

receptor engagement or receptor cross-linking with antibodies is followed by tyrosine phosphorylation of ITAMs and binding of additional signaling molecules to the cytoplasmic tails of the receptor subunits. The recruitment and tethering of molecules to the activated receptors represent steps in the signal transduction pathway that are critical for the regulation of downstream biochemical events.

In general, two types of molecules were found to associate with tyrosine-phosphorylated ITAMs. The first group includes adapter molecules that are devoid of catalytic activity but can recruit additional molecules via their multiple binding sites. The second group includes molecules with a catalytic domain(s) that are involved in signal transmission by regulating more distal effector molecules.

The Shc adapter molecule associates with both TCRζ and Ig-α/Ig-β chain tyrosine-phosphorylated ITAMs (Lankester *et al.*, 1994; Osman *et al.*, 1995; Ravichandran *et al.*, 1993; Saxton *et al.*, 1994) and was suggested to couple the antigen receptor to the Ras pathway by recruiting GRB2-Sos into the receptor complex (Ravichandran *et al.*, 1993). The Shc molecule possesses a single SH2 domain with which it interacts with tyrosine-phosphorylated receptors, such as insulin or EGF receptors (Batzer *et al.*, 1994; Pronk *et al.*, 1994; Sasaoka *et al.*, 1994). As a result, Shc undergoes phosphorylation on tyrosine residues that function as additional binding sites for SH2-containing molecules. A second adapter molecule, GRB2, can then interact via its own SH2 domain with the Shc phosphotyrosyl groups. Because GRB2 is constitutively associated, via its SH3 domain, with the GDP/GTP exchange protein, Sos, it recruits the GDP/GTP exchange factor for Ras to the receptor site and by coupling Ras to the activated receptor it may initiate the activation of one or more of the MAPK signaling cascades (Buday and Downward, 1993; Rozakis-Adcock *et al.*, 1992, 1993; Skolnik *et al.*, 1993).

According to one model, binding of Shc to an activated TCR is followed by the Src family PTK-induced tyrosine phosphorylation of Shc that creates new docking sites for the SH2 of GRB2. The subsequent interaction of GRB2 with the receptor results in T-cell activation via a Ras-dependent mechanism (Ravichandran *et al.*, 1993). Additional studies supported this initial finding and further demonstrated that phosphorylation of a single carboxy-terminal tyrosine within the TCRζ is optimal for binding of Shc (Osman *et al.*, 1995). However, in this case, the TCRζ-associated Shc was not complexed to GRB2-SOS, suggesting that Shc binding to the TCR may function by recruiting other SH2-containing molecules.

Syk and ZAP-70 are among the very early molecules that associate with tyrosine-phosphorylated ITAMs following receptor ligation. ZAP-70 interaction with the TCR requires binding of its tandem SH2 domains to

the doubly tyrosine-phosphorylated ITAM sequences (Irving *et al.*, 1993; Isakov *et al.*, 1995; Iwashima *et al.*, 1994; Koyasu *et al.*, 1994; Timson Gauen *et al.*, 1994; Wange *et al.*, 1993). Mutations in either of the SH2 domains of ZAP-70 abolish binding to the TCRζ chain (Iwashima *et al.*, 1994).

In order to study binding requirements for the ZAP-70 tandem SH2 domains, a recombinant protein that is fused to glutathione *S*-transferase [GST-ZAP$(SH2)_2$] has been prepared. When this fusion protein was immobilized to beads, it could precipitate tyrosine-phosphorylated TCR subunits, as well as associated endogenous ZAP-70 kinase from lysates of activated T cells (Wange *et al.*, 1993). In addition, an immobilized doubly tyrosine-phosphorylated ITAMζ_1 peptide, but not singly tyrosine-phosphorylated peptides, was able to precipitate ZAP-70 from a lysate of activated T cells (Iwashima *et al.*, 1994). However, these experiments did not necessarily measure direct binding of the GST-ZAP$(SH2)_2$ or native ZAP-70 to distinct phospho-ITAMs because interaction could be mediated via adapter molecules.

In order to define the structural requirements for the direct interaction of TCR ITAMs with ZAP-70, an *in vitro* binding assay was performed utilizing immobilized GST fusion proteins containing amino- and/or carboxy-terminal SH2 domains of ZAP-70 and soluble synthetic peptides with the sequence of the cytoplasmic region of the TCRζ chain (TCRζ_{cyt}) or individual TCRζ and CD3ε ITAM sequences (Isakov *et al.*, 1995). Direct binding studies demonstrated that the tandem ZAP-70 SH2 domains bind phosphorylated, but not nonphosphorylated, TCRζ_{cyt}. Similar studies demonstrated that the ZAP-70 tandem SH2 domain binding to a TCRζ_3 ITAM peptide (ITAMζ_3) required the phosphorylation of both tyrosine residues within the ITAM.

Hatada *et al.*, (1995) solved the crystal structure of the human ZAP-70 tandem SH2 domains bound to a doubly tyrosine-phosphorylated peptide derived from the ITAMζ_1. Conformation analysis revealed the structure of a coiled-coil loop between the SH2 domains and formation of one of the two critical phosphotyrosine-binding pockets at the interface of the two adjacent SH2 domains. This unique structure provided the molecular basis for the observed cooperativity between the two SH2 domains on their binding of doubly tyrosine-phosphorylated ITAM and for the high selective association of ZAP-70 with the activated TCR.

The high degree of sequence homology between ZAP-70 and Syk and their overall structure similarity suggested that binding of ZAP-70 and Syk to pITAMs of activated receptors is mediated by a similar mechanism (Benhamou *et al.*, 1993; Chacko *et al.*, 1996a; Johnson *et al.*, 1995; Ting *et al.*, 1995). However, Syk binding to phospho-ITAMs, in contrast to ZAP-

70, has a dual function that includes enzyme accumulation at the receptor site and upregulation of its enzymatic activity. Thus Syk binding to tyrosine-phosphorylated FcεRI γ chain ITAM peptide (Shiue *et al.*, 1995) or tyrosine-phosphorylated peptides derived from Ig-α or Ig-β ITAMs (Rowley *et al.*, 1995) resulted in over a 10-fold stimulation of the specific activity of Syk. In contrast, incubation of immunoprecipitated or purified recombinant ZAP-70 with tyrosine-phosphorylated ITAM peptides did not affect the catalytic activity of the enzyme (Isakov *et al.*, 1996; Neumeister *et al.*, 1995). Furthermore, under certain activation conditions, ZAP-70 can associate and coimmunoprecipitate with the TCR without undergoing phosphorylation or activation (Madrenas *et al.*, 1995; van Oers *et al.*, 1994). Thus, while Syk binding to phospho-ITAMs can augment its catalytic activity, activation of ZAP-70 is strictly dependent on its tyrosine phosphorylation (Chan *et al.*, 1995; Wange *et al.*, 1995b), apparently by a Src family kinase such as Lck or Fyn (Chan *et al.*, 1992; Hall *et al.*, 1993; Iwashima *et al.*, 1994).

A different hypothesis regarding the role of the tyrosine-phosphorylated ZAP-70 *in vivo* suggested that receptor-associated ZAP-70 functions as a secondary scaffold protein by virtue of the fact that it undergoes tyrosine phosphorylation at multiple sites and that it associates with additional signaling molecules that do not bind directly to the TCR (Neumeister *et al.*, 1995). The fact that no endogenous ZAP-70 substrates were identified, despite the fact that its absence results in complete T-cell anergy, has led to the assumption that a major function of ZAP-70 in activated T cells is to recruit additional molecules to the TCR instead of phosphorylating downstream substrates (see Fig. 5).

Additional tandem SH2 domain-containing signaling molecules that were shown to associate with tyrosine-phosphorylated ITAMs include PI-3K (Chacko *et al.*, 1996b; Exley *et al.*, 1994) and PLCγ1 (Kimura *et al.*, 1996a). Single SH2 domain-containing molecules that bind tyrosine-phosphorylated ITAMs include the Src kinases, Fyn (Pleiman *et al.*, 1994), and Lyn (Johnson *et al.*, 1995), that are involved in the early stages of B-cell activation.

B. Different ITAMs Link Immunoreceptors to Distinct Signaling Pathways

1. Studies in T Cells

The biological importance of the existence of multiple distinct ITAMs in antigen receptors is not completely understood. Initially it was suggested that the distinct ITAMs are functionally redundant and that their multiplication within a single receptor serves to amplify rare activation events that

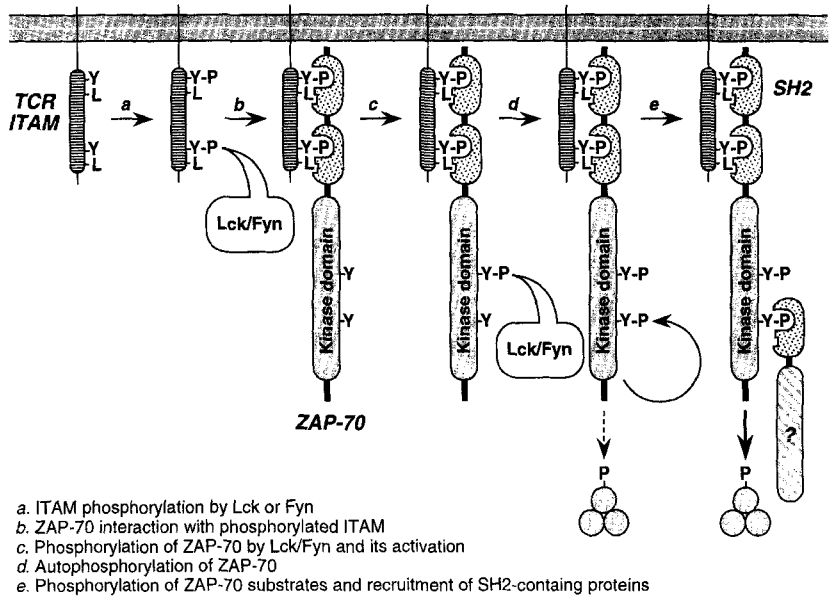


FIG. 5. Sequential early activation events that follow TCR engagement. High-affinity interaction of an MHC-bound peptide with the TCR induces a stable interaction between the MHC and the CD4/CD8 coreceptor, which brings the coreceptor-associated Lck PTK into close proximity with the TCR, enabling tyrosine phosphorylation on the TCR/CD3 ITAMs. ZAP-70 is then recruited to biphosphorylated ITAMs and is further phosphorylated (on Tyr^{493}) by Lck or possibly another Src kinase, such as Fyn. This leads to the activation of ZAP-70, which can then autophosphorylate (on Tyr^{492} and possibly additional sites) and either create additional binding sites for SH2-containing signaling molecules and/or phosphorylate and activate proximal protein substrates.

are induced by low levels of antigens (Irving and Weiss, 1991; Weiss and Littman, 1994). This model is supported by studies with chimeric receptors that encode one, two, or three TCRζ ITAMs, showing that activation of T cells via the three different chimeric receptors delivered activation signals and induced similar patterns of tyrosine-phosphorylated substrate proteins (Irving *et al.*, 1993). In these experiments, ITAM multimerization resulted in augmented responses but showed no qualitative differences. Furthermore, each of the three distinct TCRζ ITAMs was functional and able to induce similar biochemical events (Irving and Weiss, 1991; Romeo *et al.*, 1992a).

In vivo studies by Shores *et al.* (1997) provided further support for this model. They tested the role of ζ chain in T-cell development and function and the potential ability of FcεRIγ to replace it, utilizing ζ chain-deficient

mice that were reconstituted with an FcεRIγ-encoding transgene. Thymocyte development analysis revealed that FcεRIγ overexpression was able to support maturation of both αβ TCR$^+$ and γδ TCR$^+$ T cells. The peripheral mature T cells from these mice were functional and responded to TCR-specific stimuli, suggesting that FcεRIγ and ζ are interchangeable in their ability to mediate T-cell development and function. However, positive selection of αβ TCR$^+$ thymocytes in ζ-deficient mice was more efficient in ζ chain-reconstituted mice than in FcεRIγ-reconstituted mice. This difference may represent a distinct quantitative rather than qualitative properties of the ITAMs in the two immunoreceptor chains because ζ contains three ITAMs versus a single ITAM in the FcεRIγ chain.

A second model suggested that distinct ITAMs may serve unique functions by coupling specific SH2-containing signaling molecules to the receptor or by mediating some other unknown activation events. This model is in agreement with studies that utilized the chimeric subunit approach in order to compare the activation capacity of distinct ITAM regions. Stimulation of a transfected BW5147 T-cell hybridoma by means of the cytoplasmic tails of either TCRζ or CD3ε resulted in equivalent amounts of IL-2 production, but the two ITAMs induced a reproducibly distinct pattern of tyrosine phosphoproteins (Letourneur and Klausner, 1992). A different study employing the same hybridoma T cells demonstrated that chimeric receptors possessing the cytoplasmic tail of CD3ε, but not of TCRζ, were capable of signal transduction leading to Ca^{2+} mobilization (Cambier, 1995a).

Although these studies support the assumption that distinct ITAMs play unique roles in the activation of different signal transduction pathways, additional experiments are required in order to rule out a role for flanking sequences in the regulation of different biochemical events.

Differential binding affinities of pITAMs to proximal SH2-containing effector molecules were demonstrated *in vitro* using short synthetic peptides that correspond to different tyrosine-phosphorylated ITAMs. These include Shc, which was found to associate preferentially with the distal phosphotyrosyl of the ITAMζ_3 (Osman *et al.*, 1995), PI-3K which interacted preferentially with pITAMζ_1 (Exley *et al.*, 1994), and ZAP-70 tandem SH2 domains which bound pITAMζ_1 with a 30-fold higher affinity than pITAMζ_3 (Isakov *et al.*, 1995).

An additional indirect support for the potential role of individual ITAMs in linking the TCR to different signaling pathway was obtained by Qian *et al.* (1997), who compared TCR-induced activation responses in T cells from lck$^{-/-}$ or fyn$^{-/-}$ mice to those of T cells from wild-type mice. They found that the new member of the focal adhesion kinase (Fak) family, Pyk2 (Lev *et al.*, 1995), undergoes tyrosine phosphorylation in wild-type

as well as Lck-negative thymocytes, but not in Fyn-negative thymocytes. Tyrosine phosphorylation of Pyk2 in heterologous Cos-7 cells could also be observed, but only on Pyk2 coexpression with Fyn, but not Lck. Selectivity in phosphorylation of Pyk2 was also demonstrated *in vitro* when purified recombinant Fyn, but not Lck, was able to tyrosine phosphorylate a kinase-inactive Pyk2 protein. These results imply that Fyn regulates specific functions during TCR signaling that are not shared by Lck.

Studies by Wegener *et al.* (1992) supported the view that the TCR is composed of two autonomous transduction modules. Based on their findings, Qian *et al.* (1997) suggested that the two distinct signaling pathways, which are triggered following receptor engagement, operate via TCR → Fyn → Pyk2 and TCR → Lck → ZAP-70. These two signaling pathways may regulate either different cellular activation events or function as complementary upstream pathways that are required for full activation of a single critical downstream signaling event (Qian *et al.*, 1997).

Fyn and Lck were also shown to reside in different subcellular compartments: whereas Fyn was found to associate preferentially with cytoskeletal microtubules, Lck was localized predominantly at the plasma membrane (Ley *et al.*, 1994a). In addition, Fyn, but not Lck, associated with a fraction of α-tubulin in unstimulated Jurkat T cells (Marie-Cardine *et al.*, 1995). Several additional studies supported the model in which Fyn and Lck regulate specific activation events that may possibly be linked to distinct receptor subunits (Hurley *et al.*, 1993; Page *et al.*, 1997; Samelson *et al.*, 1990; Susa *et al.*, 1996; van Oers *et al.*, 1992). Although not directly proven, these studies imply that the TCR → Fyn → Pyk2 pathway is likely to be dependent on tyrosine-phosphorylated CD3ε-ITAMs, whereas the TCR → Lck → ZAP-70 pathway is preferentially dependent on tyrosine-phosphorylated TCRζ-ITAMs.

2. *Studies in B Cells*

The BCR Ig-α and Ig-β chains are critical for B-cell development in general and antigen-induced B-cell activation responses in particular. The cytoplasmic tail of both Ig-α and Ig-β possesses a partially conserved ITAM sequence that is essential for their signal transduction capacity. However, nonconserved sequences within the ITAM, as well as flanking sequences, may differ in their ability to recruit downstream signaling proteins and, as a result, link the receptor to distinct signaling pathways.

In an attempt to discriminate between the potential biological functions of Ig-α and Ig-β cytoplasmic tails, Teh and Neuberger (1997) prepared transgenic mouse lines expressing chimeric IgM receptors in which the extracellular IgM was fused, via a hydrophobic transmembrane domain, to the cytoplasmic domains of either Ig-α or Ig-β. They found that each

of the IgM/α and IgM/β chimeras was functional *in vivo* and independently sufficient to mediate allelic exclusion, rescue B-cell development in gene-targeted Igμ^- mice that lack endogenous antigen receptors, and signal for B7 upregulation. All these functions were dependent on the presence of an intact ITAM in the cytoplasmic tail of the chimeric receptor and were abolished in transgenic mice in which tyrosine residues within the IgM/β ITAM were substituted with leucines. Although allelic exclusion in a (IgM/α) x (IgM/β) double-transgenic mouse was slightly more efficient, these data suggest that each of the BCR Ig-α and Ig-β ITAMs is sufficient for mediating many essential BCR functions.

Studies by others confirmed that both Ig-α and Ig-β ITAMs are efficient in triggering protein tyrosine kinase activation and are capable of inducing an increase in intracellular Ca^{2+} concentration (Kim *et al.*, 1993; Sanchez *et al.*, 1993; Taddie *et al.*, 1994).

A different approach to discriminate between the potential functions of Ig-α and Ig-β ITAMs was adopted by Clark *et al.*, (1992), who attempted to identify and characterize effector molecules from a B-cell extract that can associate with immobilized GST-Ig-α or Ig-β ITAMs. The Ig-α cytoplasmic tail precipitated the Src family kinases Lyn and Fyn, phosphatidylinositol-3 kinase (PI-3 kinase), and a 38-kDa phosphoprotein. In contrast, the Ig-β cytoplasmic tail precipitated the PI-3 kinase and two additional unidentified 40- and 42-kDa phosphoproteins. These data indicate that Ig-α and Ig-β can interact with both shared and unique protein substrates and suggest that Ig-α and Ig-β might mediate coupling of the BCR to both shared and unique signaling pathways. Similarly, chimeric receptors possessing the FcγRII extracellular region and either the Ig-α or the Ig-β cytoplasmic tail were found to induce distinct activation signals. Ig-α-containing chimeric receptors were able to trigger an efficient signal transduction, leading to an extracellular calcium influx and IL-2 production (Choquet *et al.*, 1994), whereas Ig-β triggered only oscillatory release of Ca^{2+} from intracellular stores and no IL-2 production.

Ig-α and Ig-β chain ITAMs differ by four amino acids located before the second conserved tyrosine (DCSM in Ig-α and QTAT in Ig-β; see Fig. 3). These distinct sequences, as well as immediate flanking regions outside of the ITAMs, appear to provide Ig-α and Ig-β with different binding specificities that may enable them linking the BCR to divergent signaling pathways (Cassard *et al.*, 1996).

3. *Studies in Mast Cells*

Use of the yeast tribrid system in order to identify proteins that interact with tyrosine-phosphorylated FcεRIγ ITAM revealed a protein that was characterized as the inositol polyphosphate 5′-phosphatase, SHIP

(Osborne *et al.*, 1996). SHIP interacted equally with wild-type FcεRIγ and β-chain pITAMs, but not with mutated versions of these ITAMs in which the conserved tyrosine or leucine residues were mutated. Although it is yet unclear whether such association occurs *in vivo, in vitro* data suggest that SHIP interacts with FcεRIγ and β chains with similar binding affinities. Phosphorylation measurements in mast cells indicated that tyrosine residues in FcεRIγ and β-chain ITAMs underwent phosphorylation *in vivo* to a similar extent (Pribluda *et al.*, 1997).

Other studies suggested the existence of substrate specificity for FcεRIγ and β chains, as FcεRIγ, but not the β chain, was able to couple the receptor to signaling pathways involving protein tyrosine phosphorylation and activation of MAPK and phospholipase A2 (Hirasawa *et al.*, 1995; Wilson *et al.*, 1995). In addition, the PLCγ1 and SHIP proteins interacted *in vitro* with immobilized phosphorylated FcεRIβ, but not γ chain (Kimura *et al.*, 1996a, 1997).

Further investigations are required in order to verify the existence of different binding proteins for FcεRIγ and β chains in mast cells. Previous observations relevant to the TCR and BCR pITAMs specificities strongly support this prediction.

C. Involvement of ITAMs in Linking Immunoreceptors to Cytoskeletal Elements

Lymphocytes activation induces reorganization of multiple cytoskeletal elements and temporal morphological changes in the shape of the cell (Lee *et al.*, 1988; Parsey and Lewis, 1993). The early activation events include tyrosine phosphorylation of cytoskeletal proteins [e.g., tubulin (Ley *et al.*, 1994b; Marie-Cardine *et al.*, 1995; Peters *et al.*, 1996) and ezrin (Egerton *et al.*, 1992)] and physical interaction of cell surface receptors [e.g., CD2 (Offringa and Bierer, 1993), LFA-1 (Pardi *et al.*, 1992) and CD44 (Geppert and Lipsky, 1991)] with cytoskeletal elements. In addition, treatment of cells with drugs that disrupt the actin cytoskeleton block Ca^{2+} mobilization and the cyclic changes in the cell shape and inhibit cell activation (Maness and Walsh, 1982; Valitutti *et al.*, 1995). These results suggest that the actin cytoskeleton may be involved in the regulation of signal transduction pathways leading to cell activation.

To test the possibility that antigen receptor chains, and more specifically ITAM regions, are involved in linking the receptors to the cytoskeleton, T-cell lysates were fractionated according to solubility in a detergent-containing buffer and the presence of TCR chains was monitored. A significant portion (10–40%) of the cell surface-expressed TCRζ chains was found to reside in the cytoskeleton-containing insoluble fraction (Caplan and Baniyash, 1996; Caplan *et al.*, 1995). Anti-TCR/CD3-induced cross-

linking increased the proportions of the detergent-insoluble fraction-residing ζ chain (Caplan *et al.*, 1995; Marano *et al.*, 1989; Rozdzial *et al.*, 1995). The two types of receptors differed also with respect to their ζ chain SDS-gel mobility rate. Although the detergent-soluble, phosphorylated TCRζ possessed a molecular mass of 21 kDa, the phosphorylated, cytoskeleton-associated ζ chain appeared as a 16-kDa protein (Caplan and Baniyash, 1996). Despite the fact that the distinct biological functions of the two types of TCRs are unclear, their inclusion of two different forms of phosphorylated TCRζ, which is comparable to the TCRζ chains observed in activated versus anergized T cells (Madrenas *et al.*, 1995; Sloan-Lancaster *et al.*, 1994), suggests that the two receptors may be linked to distinct intracellular signaling pathways that regulate opposing modes of T-cell differentiation or activation.

In order to define the region within the TCRζ chain-cytoplasmic tail that mediates anchoring to the cytoskeleton, a transient transfection system of Cos cells was used. Expression of different truncation mutants of TCRζ revealed that a stretch of 42 amino acids within the cytoplasmic tail (amino acids 109–150) is required for cytoskeletal association (Caplan *et al.*, 1995). This region includes the ITAMζ_2, suggesting that a minimum of one intact ITAM is required for the association (Caplan *et al.*, 1995). Whether ITAMζ_2 is unique in this respect or whether other TCR ITAMs can also mediate TCR-cytoskeleton association remains to be tested.

An independent study demonstrated that TCR chains containing at least one ITAM are both sufficient and necessary for association with the microfilament cytoskeleton following antigen-receptor ligation (Rozdzial *et al.*, 1995). Actin, but not tubulin, coimmunoprecipitated with TCRζ from T cell lysates, and, under conditions favoring cytoskeleton depolymerization, binding was enhanced after receptor ligation. Interaction of TCRζ with actin may be direct, although indirect association via adapter proteins (Pawson, 1995) or other cytoskeleton-interacting molecules that function as bifunctional scaffolds (Coghlan *et al.*, 1995) may also occur. Based on their findings, Rozdzial *et al.* (1995) speculated that the cytoskeleton may function as a signaling intermediary in activated T cells, either by colocalizing enzymes (e.g., kinases and phosphatases) and their substrates or by transmitting signals to the nucleus.

Studies performed in B lymphocytes demonstrated that BCR engagement results in association of surface immunoglobulin with the detergent-insoluble cytoskeletal matrix (Braun *et al.*, 1982). Furthermore, Fc receptors coclustering in DT40 B cells triggered the submembranous actin assembly that was dependent on the presence of a functional ITAM-containing Fcγ receptor chain (Cox *et al.*, 1996). This activity required an intact ITAM and was absent in Syk-deficient B cells.

Potential involvement of Syk and ZAP-70 in the regulation of ITAM-dependent cytoskeletal rearrangement is supported indirectly by studies demonstrating interaction of these PTKs with cytoskeletal elements. *In vitro* studies showed that both Syk (Peters *et al.*, 1996) and ZAP-70 (Isakov *et al.*, 1996) can efficiently interact with tyrosine phosphorylate α-tubulin; that is, among the very early cellular substrates that undergo tyrosine phosphorylation in activated lymphocytes (Ley *et al.*, 1994b; Marie-Cardine *et al.*, 1995; Peters *et al.*, 1996). In addition, both enzymes were found to physically interact with α-tubulin (Huby *et al.*, 1995; Peters *et al.*, 1996).

Further studies are required in order to determine the precise role of the interaction of immunoreceptor ITAMs with the cytoskeletal elements in transmembrane signal transduction and in regulation of receptor internalization and/or recycling.

V. Involvement of ITAMs in Tolerance Induction and Immunopathology

A. Potential Role of ITAMs in Induction of Lymphocyte Anergy and Desensitization of Immunoreceptors

1. Role of ITAMs in Altered Peptide-Induced T-Cell Anergy

Many of the cell surface receptors for exogenous ligands function as "on and off" switches of transmembrane signal transduction pathways. Occupation of such receptors by their cognitive ligands activates a preprogrammed pathway of sequential biochemical events leading to activation of an appropriate set of genes and induction of specific cellular functions. Unlike other receptors, the TCR regulates not only intensity and duration of responses, but also redirects T cells into one of several differentiation pathways. For example, peptide antigen interaction with a TCR may induce either maturation or apoptosis of thymic T cells (Kappler *et al.*, 1988; MacDonald *et al.*, 1988) and either activation or anergy of peripheral blood T cells (Rammensee *et al.*, 1989; Ramsdell and Fowlkes, 1992).

Studies have demonstrated that a single amino acid substitution in agonistic peptides may convert them to antagonists or partial agonists that, when presented to T cells, induce a state of proliferative unresponsiveness (Sloan-Lancaster *et al.*, 1993). Tolerance induction could also be induced by agonistic peptides when presented by MHC molecules in which amino acid substitution at their hypervariable region occurs (Madrenas *et al.*, 1995). These studies and others demonstrated flexibility in the TCR recognition step, indicating that interaction of structurally related peptide antigens with a single type of TCR can induce distinct T-cell differentiation pathways (Kersh and Allen, 1996; Schwartz, 1996).

Activation of T cells with a classical peptide agonist followed by Western blotting analysis of receptor subunits demonstrated a dose-dependent ap-

pearance of three tyrosine-phosphorylated receptor chains of 21, 23, and 27 kDa. These protein bands correspond to two phosphorylated isoforms of the TCRζ chain (pp21 and pp23) and a single CD3ε chain (pp27) (Qian *et al.*, 1993). Antagonistic peptides, which can induce a limited number of T-cell-specific functions, including lymphokine production, could also induce tyrosine phosphorylation of TCR chains, although with different relative intensities (Madrenas *et al.*, 1995). Although agonists induced the formation of similar levels of both forms of the tyrosine-phosphorylated ζ chain, antagonists induced fair amounts of tyrosine-phosphorylated pp21, but relatively low levels of tyrosine-phosphorylated pp23. In addition, the antagonist-induced increase in pp21 TCRζ was not accompanied by a significant increase in tyrosine phosphorylation of CD3ε (Madrenas *et al.*, 1995; Sloan-Lancaster *et al.*, 1994). The results also demonstrated that formation of tyrosine-phosphorylated pp21, by either agonistic or antagonistic peptide antigens, was sufficient to support ZAP-70 association with the TCR. Nevertheless, tyrosine phosphorylation and activation of ZAP-70 occurred only in agonist-activated T cells (Madrenas *et al.*, 1995). These results suggest that the antagonist-induced altered phosphorylation of ζ is sufficient for allowing ZAP-70 interaction with the TCR, but is insufficient to support additional biochemical events that regulate the activation of ZAP-70. They also provide a biochemical explanation for the different biological responses that accompany engagement of the TCR by structurally related peptide–MHC molecules.

Activation-induced tyrosine phosphorylation of TCR chains is mediated by the Src family PTKs, Lck or Fyn. Antagonistic peptide antigens are likely to induce only low levels of activation of these enzymes and/or partial recruitment to the vicinity of the engaged receptor. Alternatively, activation of a single kinase, such as Lck, may lead to phosphorylation of selected tyrosine residues and generation of the pp21 ζ chain, whereas a different kinase, such as Fyn, may be required for phosphorylation of additional sites that give rise to the pp23 ζ chain. It is also possible that TCR engagement by antagonists or partial agonists, but not by agonistic peptide antigens, may induce an early activation response of PTPases that prevents the formation of pp23.

The cytoplasmic tail of the human TCRζ possesses three consecutive ITAMs and six tyrosine residues, and therefore the differences between pp23 and pp21 may reflect quantitative and/or qualitative differences in tyrosine residues that undergo phosphorylation. Although complete phosphorylation of TCR ITAMs may be essential for the full activation of TCR-coupled signaling pathways that regulate cell activation and proliferation, partial phosphorylation may create only a limited number of binding sites for particular SH2-containing proteins that initiate selected downstream

signaling events, leading to partial or incomplete activation of T cells and induction of a state of anergy. Additional studies are required in order to verify whether different Src kinases operating in a hierarchial manner are responsible for the phosphorylation of the different tyrosine residues within the TCR ITAMs and whether tyrosine phosphorylation occurs in a predetermined sequential order.

The physical properties of the variant TCR ligands and the biochemical changes that they induce are of critical importance for a wide range of biological processes. These include positive and negative selection of thymocytes (Hogquist *et al.*, 1994; Page *et al.*, 1994), tolerance induction of mature peripheral T cells (Sloan-Lancaster *et al.*, 1993), qualitative and quantitative regulation of cytokine production by T cells (Evavold and Allen, 1991; Racioppi *et al.*, 1993; Windhagen *et al.*, 1995), modulation of autoimmune conditions (Miller *et al.*, 1993), and viral and tumor cell escape from effector T cells (Bertoletti *et al.*, 1994; Klenerman *et al.*, 1994). Some of these processes may possibly be regulated by non-TCR, accessory receptors. Nevertheless, the indirect demonstration of the involvement of different phosphorylation patterns of TCRζ ITAMs in induction of T-cell anergy suggests that different combinations of phosphorylation of tyrosine residues within the TCRζ and CD3ε subunit ITAMs may dictate specific downstream events that contribute to the induction of specific T-cell functions. In this respect it should be mentioned that the Syk and ZAP-70 possess similar tandem SH2 domains that, *in vitro,* mediate binding to each of the three biphosphorylated TCRζ ITAMs. However, activation of these two PTKs *in vivo* is regulated by distinct mechanisms (Zoller *et al.*, 1997), which may reflect their *in vivo* preferential binding to distinct biphosphorylated ITAMs.

2. *Role of ITAMs in BCR Desensitization and Induction of B-Cell Unresponsiveness*

Antigen binding to a small fraction of the BCRs (as low as 5%) results in B-cell desensitization and a state of refractoriness of the entire antigen-free receptors to antigenic challenge (Cambier *et al.*, 1988; Goodnow *et al.*, 1989). On reexposure to the same type of antigen, B cells do not respond by Ca^{2+} mobilization or activation of PKC (Cambier *et al.*, 1990; Lazarus *et al.*, 1990). Studies of nitrophenol (NP)-specific lymphoma B cells and splenic B cells from 3-83 $\mu\delta$ Ig transgenic mice have shown that exposure of B cells to a high-dose antigen subsequent to desensitization by a low dose of the same antigen failed to induce protein tyrosine phosphorylation or an increase in activity of cellular PTKs (Vilen *et al.*, 1997). Furthermore, Lyn and Syk did not undergo tyrosine phosphorylation nor did the Ig-α and Ig-β ITAMs. These results indicate that despite the ability of desensitized

receptors to bind antigens, the initial receptor ligation triggers an inhibitory mechanism that affects both antigen-occupied as well as antigen-free receptors and prevents tyrosine phosphorylation of BCR ITAMs. Failure to phosphorylate the BCR ITAMs may reflect a defect in recruitment and/or activation of Src family PTKs or an equilibrium shift in receptor-associated PTKs and PTPases that result in dephosphorylation and inactivation of the ITAM-specific PTKs.

B. ITAMs as Targets for Mimicry by Viral Proteins

Persistent and latent infections have evolved during coevolution of viruses and their hosts and led viruses to develop efficient strategies that enable them to circumvent antiviral immune responses. Different viruses use divert strategies to escape immune surveillance, and many of them, via molecular mimicry of cellular epitopes, acquired a variety of immunomodulatory molecules that can interact with signaling proteins and intervene with critical cellular functions, such as activation, proliferation, differentiation, or transformation. These virus-encoded proteins may interfere with the process of antigen presentation by host cells (Jennings *et al.*, 1985; Maudsley and Pound, 1991; McFadden and Kane, 1994), inhibit specific steps in the complement cascade (Albrecht and Fleckenstein, 1992; Harris *et al.*, 1990; Kotwal *et al.*, 1990), and modulate cytokine or growth factor responses by producing molecules with agonistic activities (Lyttle *et al.*, 1994; Ryon *et al.*, 1993; Stroobant *et al.*, 1985) or soluble receptor-like molecules that function as antagonists (Alcami and Smith, 1992; Spriggs *et al.*, 1992; Upton *et al.*, 1992).

The molecular mimicry of cellular epitopes by viruses is of critical importance in viral pathogenesis because it may lead to the generation of autoantibodies, thereby contributing to the development of autoimmune diseases (reviewed in Barnett and Fujinami, 1992; Gianani and Sarvetnick, 1996). Alternatively, viral proteins may interact with various components of the cellular signaling machinery, thereby interfering with the normal functions of the cell (reviewed in Collette and Olive, 1997; Gooding, 1992; Spriggs, 1996).

The critical role of ITAMs in signaling via immunoreceptors makes them excellent candidates for mimicry by viral proteins. Acquisition of such sequences may enable viruses to intervene with the normal immunoreceptor-linked signaling machinery and thereby affect cell proliferation. Computer search of a protein data bank (Reth, 1989) initially revealed that an ITAM sequence exists in the envelope glycoprotein of the bovine leukemia virus (Rice *et al.*, 1984). Further searches indicated that this example is not unique and that additional viruses encode proteins with ITAM sequences. Among these are the Epstein–Barr virus (EBV)

latent membrane protein 2A (LMP2A) (Laux *et al.*, 1988), the negative factor (Nef) protein of the simian immunodeficiency virus (SIV) pbj14 substrain (Dewhurst *et al.*, 1990), the envelope glycoprotein (gp20) of the Mason–Pfizer monkey virus (MPMV) (Sonigo *et al.*, 1986), and the major inner capsid protein (VP6) of either the infectious diarrhea of infant rats (IDIR) strain (Eiden *et al.*, 1992) or the adult diarrhea rotavirus (ADRV) strain of group B rotavirus (Chen *et al.*, 1991) (see Fig. 3). Support for the assumption that these viral-encoded ITAM sequences may indeed interfere with normal cell growth processes is provided by the observations that lymphocytes infected with such viruses exhibit either enhanced proliferation and/or immunosuppression. For example, EBV is a herpesvirus that infects human B lymphocytes and induces either lymphoproliferative disorders, such as mononucleosis, or transformation and immortalization, resulting in African Burkitt's lymphoma or nasopharyngeal carcinoma (Sugden, 1982). However, the SIVpbj14 that belongs to the lentiviruses is a primate pathogen that exhibits cytopathic effects on $CD4^+$ T cells and causes an acute fatal disease in pig-tailed macaques (Fultz *et al.*, 1989; Israel *et al.*, 1993).

In order to determine whether virus-encoded ITAM sequences are endowed with signaling functions, chimeric receptors were constructed in which the intracellular portions of inert cell surface receptors were replaced by the relevant viral ITAM-containing sequences. In two such chimeric receptors, the extracellular and transmembrane domains of CD8α were linked to the cytoplasmic tail of either BLV gp30 or EBV LMP2A, followed by overexpression in T or B lymphocytes. Stimulation of the chimeric receptors by cross-linking with anti-CD8 antibodies activated cellular PTKs, induced phosphorylation of multiple cellular substrates, and triggered calcium mobilization and production of cytokines (Alber *et al.*, 1993; Beaufils *et al.*, 1993). The patterns of tyrosine-phosphorylated protein bands obtained following stimulation of gp30-containing CD8α or LMP2A-containing CD8α were almost indistinguishable from those obtained following stimulation of Ig-α-containing CD8α, suggesting that the two distinct viral-derived ITAMs and the Ig-α ITAM trigger identical or very similar biochemical activation events (Alber *et al.*, 1993). Tyrosine residues within the LMP2A ITAM underwent phosphorylation *in vitro* by isolated Fyn PTK (Alber *et al.*, 1993). In addition, replacement of the tyrosine residues in LMP2A and gp30 by phenylalanine completely abolished the ability of these proteins to function as signaling molecules, indicating that this property is the result of functional ITAM sequences (Beaufils *et al.*, 1993). Although BLV and EBV possess very different genomic organizations, the two types of viruses induce strong proliferation of B cells in their hosts. Despite the fact that viral nuclear factors may contribute to this

effect (Willems *et al.*, 1992; Rowe *et al.*, 1992), it is also possible that their ITAM-containing proteins mimic the functions of Ig-α- and Ig-β-ITAMs and signal B cells to proliferate, even in the absence of extracellular triggers.

The LMP2A protein may have a significant contribution to the chronic state of EBV infection in long-lived memory B cells. Under physiological conditions, activation of the BCR triggers B cells to differentiate into memory cells. However, the cytoplasmic ITAM-containing tail of LMP2A in EBV-infected cells may possibly mimic this effect and allow infected cells to differentiate into long-lived memory cells in the absence of extracellular antigenic triggers. This suggestion is supported by findings showing that LMP2A is the only EBV protein found in latently infected B cells (Gregory *et al.*, 1991; Qu and Rowe, 1992). A LMP2A-negative EBV has been produced by targeted mutation of the LMP2A gene (Longnecker *et al.*, 1992, 1993), and the resulting virus was still able to infect human B cells *in vitro* and to transform them. Thus, the LMP2A is not essential for viral growth and induction of transformation under *in vitro* conditions, but it does play an *in vivo* role in viral propagation.

Support for the potential *in vivo* role of the ITAM of SIVpbj14-derived Nef protein in cell growth regulation and viral pathogenesis was obtained in experiments in which the structure and function of this protein were compared with those of the SIVmac239-derived Nef protein. Many of the SIV strains, like their human immunodeficiency virus (HIV) counterparts, use the cell surface CD4 molecule as the initial receptor for entry into cells (Dalgleish *et al.*, 1984). Optimal replication of these viruses occurs in activated $CD4^+$ lymphocytes that represent a very small fraction of cells in the peripheral blood (McDougal *et al.*, 1985). An exception to this is the SIVpbj14, which is able to replicate in resting peripheral blood mononuclear cell (PBMC) cultures and may do so by preactivation of its host target cells (Fultz, 1991). This virus is also exceptional in pathogenicity in pig-tailed macaques, where it induces an acute and fatal disease that is characterized by extensive proliferation of lymphocytes, especially in gut-associated lymphoid tissues (Fultz *et al.*, 1989; Israel *et al.*, 1993). The SIVpbj14-derived Nef protein possesses a complete ITAM sequence (**Y^{17}ERL** LRARGET **YGRL**), while the SIVmac239-derived Nef protein possesses only a partial ITAM sequence (R^{17}**QRL** LRARGET **YGRL**), that has been experimentally mutated ($R^{17}Q \rightarrow$ YE) to express a full ITAM (Du *et al.*, 1995). Change of these two amino acids resulted in an altered virus that is able to replicate *in vitro* in resting PBMC, induce lymphocyte activation, and infect and transform NIH 3T3 cells (Du *et al.*, 1995). Furthermore, the mutated virus produced an acute clinical disease in both pig-tailed and rhesus monkeys and induced extensive proliferation of lymphocytes, preferentially in the gastrointestinal tract. These results

imply that the presence of an ITAM sequence in the Nef protein plays a critical role in the induction of lymphocyte activation and proliferation and suggest that the ITAM-containing Nef protein interacts with, and affects, components of the normal cellular signaling machinery that regulate cell activation and growth.

C. Potential Role of ITAMs in Cancer

Numerous reports established that patients with advanced cancer are immunocompromised (Broder and Waldmann, 1978), and experimental models in tumor-bearing mice indicated that the impaired immune response becomes more apparent as the tumor mass increases (Deckers *et al.*, 1973). Although the reasons for this phenomenon may differ from one disease to another (Bodmer *et al.*, 1993; Browning and Bodmer, 1992; Sulitzeanu, 1993; Townsend and Allison, 1993), studies have shown that certain types of malignancies are characterized by decreased T-cell functions. Additional reports indicated that these T cells possess altered signal transduction machinery that is implicated in the immune suppression.

Analysis of splenic T cells from mice bearing the MCA-38 colon carcinoma revealed an altered pattern of tyrosine-phosphoryated proteins and a significant loss of expression of the Src family PTKs, Lck and Fyn (Mizoguchi *et al.*, 1992). Furthermore, TCRs of these T cells were almost completely devoid of the ζ chain, but possessed a homodimer of the FcεRIγ chain. Because of the fundamental role of TCRζ in signaling via the TCR and the quantitative and qualitative differences between TCRζ ITAMs and those of the FcεRIγ chain, it was assumed that this structural difference is the basis for the altered TCR-linked signaling and the functional impairment of the cells.

Studies of other tumor models in experimental animals and human patients have demonstrated that modified patterns of tyrosine phosphorylation can be observed in tumor-infiltrating lymphocytes (TILs), splenic T cells, and peripheral blood T cells (Finke *et al.*, 1993; Matsuda *et al.*, 1995; Nakagomi *et al.*, 1993; Salvadori *et al.*, 1994; Zier *et al.*, 1996). Alterations varied from one model to another where some were characterized by reduced levels of expression of Lck (Finke *et al.*, 1993; Zier *et al.*, 1996) and/or Fyn (Mizoguchi *et al.*, 1992; Zier *et al.*, 1996) and others exhibited reduced levels of the TCRζ chain (Farace *et al.*, 1994; Finke *et al.*, 1993; Matsuda *et al.*, 1995; Nakagomi *et al.*, 1993; Tartour *et al.*, 1995; Zier *et al.*, 1996). Reduced levels of the ζ chain were also observed in the CD16 receptor on natural killer cells from a human colorectal cancer patient (Finke *et al.*, 1993; Matsuda *et al.*, 1995; Nakagomi *et al.*, 1993).

Alterations in levels of expression of PTKs and/or components of the TCR were not ubiquitous. Analysis of T cells in other tumor systems

showed that none of these proteins was impaired, despite of the fact that these T cells were immunosuppressed (Levey and Srivastava, 1995; Wang *et al.*, 1995). Thus, T-cell immune impairment in tumor bearers is not necessarily due to changes in components of the TCR and/or its signaling machinery. Additional studies of T cells from tumor bearers that exhibit abnormal TCR or TCR-linked signaling pathway are necessary in order to verify whether the immune status of the cells is directly the result of the observed molecular changes.

In a comparative study of T cells from mice that were inoculated with a progressive or a regressive tumor cell line, Salvadori and Zier (1996) observed that T cells from mice bearing the progressive tumor exhibited (a) decreased *in vitro* kinase activity associated with the TCR, (b) reduced activation-dependent tyrosine phosphorylation of the TCRζ chain, and (c) lack of activation-dependent functionally active TCR-associated ZAP-70. The patterns of tyrosine-phosphorylated TCRζ chains and the absence of activity of the TCR-associated ZAP-70 in activated T cells from mice bearing the progressive tumor are similar to those observed in T cells that were made anergic by exposure to an antagonistic peptide (Madrenas *et al.*, 1995; Sloan-Lancaster *et al.*, 1994). Thus, because incomplete phosphorylation of TCRζ reflects partial phosphorylation of tyrosine residues within its ITAMs, data suggest that the T-cell impairment in tumor-bearing mice correlates with the activation-dependent partial phosphorylation of TCRζ ITAMs and lack of activation of the TCR-associated ZAP-70.

Although the role of ITAMs in the regulation of T-cell suppression in cancer patients is only suggestive and may play a role in selected malignancies, this possibility should be analyzed further because reversion of the tumor-induced immune suppression may result in spontaneous tumor rejection or contribute to the improvement and efficacy of immunotherapy.

VI. Temporal and Spatial Determinants That Coordinate Downregulation of ITAM-Induced Activation Signals

The central role played by PTKs in the early stages of agonist-induced signal transduction pathways, in general, and ITAM-coupled signals, in particular, suggests the coexistence in the cells of PTPase-mediated signal termination mechanisms that operate by dephosphorylation of critical activating molecules. Furthermore, the observations that antigen-receptor triggering may result in different consequences, including tolerance induction and receptor desensitization, suggest that cell surface receptors, other than the BCR and TCR, may play a role in the antigen-triggered cellular response and provide negative regulatory signals. Studies using a combination of genetic, molecular, and biochemical approaches supported this assump-

tion by demonstrating that additional cell surface receptors, such as FcγRIIb, CD22, and CTLA-4, are engaged in concert with the antigen receptors and provide inhibitory signals (reviewed in Daëron, 1997b). Activation of these receptors results in tyrosine phosphorylation of their cytoplasmic tails and recruitment of signaling molecules, predominantly protein phosphatases, that are inhibitory in function. These include SHP-1 [previously known as HCP, SHPTP1, PTP1C, or SHP (D'Ambrosio *et al.*, 1995; Doody *et al.*, 1995)] and SHP-2 [previously known as HSH-PTP2, PTP1D, or Syp (Olcese *et al.*, 1996)] PTPases and SHIP [SH2-containing inositol phosphatase (Damen *et al.*, 1996; Lioubin *et al.*, 1996; Ono *et al.*, 1996)]. The SHP-1, SHP-2, and SHIP phosphatases act to inhibit signaling cascades initiated by ligation of ITAM-containing receptors, but their relative importance and substrate specificity in the signaling pathways linked to each of the activator immunoreceptors are yet unknown.

An additional element that is relevant to the inhibitory signals in activated lymphocytes is a 13 amino acid sequence motif present in the cytoplasmic tail of some of the inhibitory cell surface receptors (Amigorena *et al.*, 1992; Daëron *et al.*, 1995a; Muta *et al.*, 1994). The sequence of this motif, I/VxYxxL (Daëron *et al.*, 1995a; Muta *et al.*, 1994 see Fig. 6), is both necessary and sufficient for conveying inhibitory activity to the FcγRIIb. It undergoes tyrosine phosphorylation on receptor ligation and functions

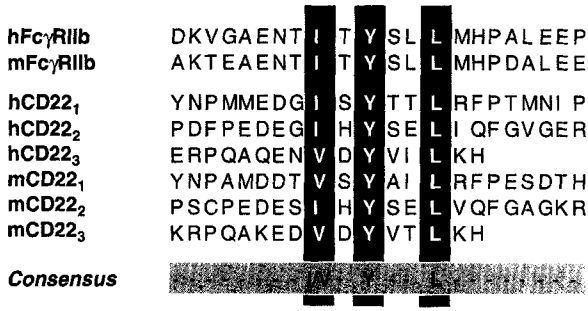


FIG. 6. Amino acid sequence alignments of immunoreceptor tyrosine-based inhibition motifs (ITIMs) from the cytoplasmic regions of human and murine FcγRIIb and CD22 receptors. The minimal consensus ITIM sequence in FcγRIIb and CD22 receptors consists of a I/VxYxxL motif. Another receptor that downregulates BCR-induced activation signals is the CTLA-4, which possesses a critical tyrosine residue, but different flanking sequences (GxYxxM). Other ITIM-containing receptors, such as p58 and p70 KIRs, PIR-B, SHPS, and SIRP, possess a classical ITIM sequence and were shown to exhibit inhibitory effects on various ligand-induced activation responses.

as a temporal scaffold for SH2-containing protein phosphatases (Burshtyn *et al.*, 1996; D'Ambrosio *et al.*, 1995; Daëron *et al.*, 1995a; Muta *et al.*, 1994). In analogy to ITAM, the I/VxYxxL motif was given the acronym ITIM, for immunoreceptor tyrosine-based inhibitory motif.

A. Mechanisms for Turning Off ITAM-Coupled Activation Signals in B Lymphocytes and Mast Cells

The first reported example of an inhibitory receptor in immunocytes is the low-affinity receptor for the Fc portion of IgG, FcγRIIb (Fig. 7). Although antibody-mediated Fc receptor ligation provides positive signals that induce B-cell activation, immune complexes consisting of antigen-bound IgG exhibit opposing effects and inhibit B-cell-dependent humoral immune responses. The inhibitory response was attributed to ligation of the BCR with FcγRIIb (Phillips and Parker, 1983), and the presence of the IgG Fc portion was essential for this negative response (Phillips and Parker, 1983; Sidman and Unanue, 1979). Furthermore, FcγRIIb coaggregation with the BCR inhibited phosphatidylinositol 4,5-bisphosphate hydrolysis (Bijsterbosch and Klaus, 1985) and Ca^{2+} mobilization (Choquet *et al.*, 1993; Muta *et al.*, 1994; Wilson *et al.*, 1987), but without altering the pattern of tyrosine-phosphorylated proteins (Muta *et al.*, 1994). The FcγRIIb is a single chain receptor expressed on the surface of a wide range of lymphoid and myeloid cells (Hulett and Hogarth, 1994; Ravetch and Kinet, 1991). Murine and human FcγRIIb are highly homologous and include two alternatively spliced isoforms, FcγRIIb1 and FcγRIIb2, which differ by a stretch of 47 (murine) or 19 (human) amino acids as a result of insertion in the cytoplasmic region of the molecule (Brooks *et al.*, 1989). In contrast to other Fc receptors, FcγRIIb has no ITAM in its cytoplasmic tail, and its cross-linking does not deliver activation signals (Daëron *et al.*, 1992; Latour *et al.*, 1992). Fc-dependent inhibition of B-cell activation was initially demonstrated in murine B cells that constitutively express the FcγRIIb1 isoform (Amigorena *et al.*, 1989). However, molecular analysis of the two FcγRIIb isoforms in both mouse and human established the presence of a conserved inhibitory motif within the cytoplasmic region of all FcγRIIb molecules. This implied that all isoforms are equally capable of delivering an inhibitory signal (Amigorena *et al.*, 1992; Muta *et al.*, 1994). This assumption was substantiated by further studies showing that the two murine FcγRIIb isoforms are equally inhibitory and that human FcγRIIb is as effective as its murine homolog in the inhibition of the BCR-induced activation of B cells (Amigorena *et al.*, 1992; Daëron *et al.*, 1995a; Muta *et al.*, 1994; Van den Herik-Oudijk *et al.*, 1994).

Studies with IgM chimeras possessing the cytoplasmic tails of Ig-α or Ig-β demonstrated that FcγRIIb prevents IgM-mediated B-cell activation by interfering with activation signals delivered from the Ig-α or Ig-β ITAM

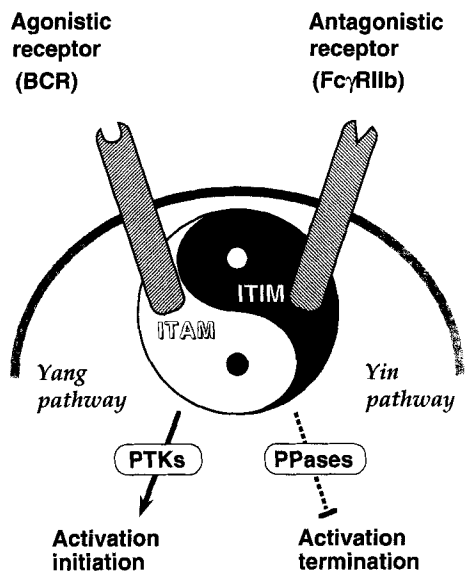


FIG. 7. Inhibition of B-cell activation by coligation of the BCR with FcγRIIb. Engagement of the antigen receptor on B cells by antigen or anti-Ig $F(ab')_2$ antibody induces early activation events, including increased formation of inositol 1,4,5-trisphosphate (IP_3) and elevation of cytoplasmic-free Ca^{2+} concentration. In contrast, co-cross-linking of the BCR and the FcγRIIb provides negative signals that abort B-cell activation. BCR ligation is characterized by a rapid phosphorylation of tyrosine residues within the BCR Ig-α and Ig-β ITAMS, which initiates signaling cascades. Simultaneous phosphorylation of tyrosine residues within the FcγRIIb ITIMs recruits a different set of SH2-containing effector molecules to the receptor site, including the SHP-1 (and possibly the SHP-2) PTPase and the inositol-5'-phosphatase, which dephosphorylate critical effector molecules, thereby negatively regulating B-cell activation. Inhibitory signals are provided by additional ITIM-containing cell surface receptors (e.g., killer cell inhibitory receptors, KIRs) or receptors possessing a tyrosine-based inhibitory domain, with distinct flanking sequences (e.g., mast cell-associated function antigen, MAFA, or CTLA-4 in T cells). In this oversimplified schematic model, BCR ITAMs and FcγRIIb ITIMs are presented as Yang and Yin entities, which symbolize opposing vital forces. A delicate balance between these two forces maintains optimal humoral immune responses.

sequences (Muta *et al.*, 1994). These findings led Muta *et al.* (1994) to suggest that FcγRIIb coaggregation with the BCR, which is followed by tyrosine phosphorylation of the ITIM region, recruits to the receptor SH2-containing proteins that interfere with the ITAM-derived activation signal.

In an attempt to test whether the negative effect of FcγRIIb is restricted to the BCR or whether other ITAM-containing receptors can also be affected, Daëron *et al.* (1995a,b) analyzed different cell types that express

FcγRIIb and either BCR, TCR, or other Fc receptors. They found that on receptor coaggregation, FcγRIIb inhibits BCR-, TCR-, FcεRI-, and FcγRIIa-dependent cell activation. Expression of a single chain chimeric receptor that possesses extracellular and transmembrane domains of the IL-2 receptor α chain (the Tac epitope) and the ITAM-containing cytoplasmic tail of FcRγ chain conferred on the RBL basophilic cell the ability to secrete serotonin on ligation of the Tac receptor. However, serotonin release was inhibited on coligation of Tac and the murine FcγRIIb. In addition, Daëron *et al.* (1995a,b) transfected RBL cells with a chimeric Tac receptor, which possesses the entire cytoplasmic tail of the TCRζ (with its three consecutive ITAMs), or a truncated TCRζ tail possessing a single ITAM (ITAMζ_1) and showed that on ligation, both receptors induced serotonin release. However, coaggregation of Tac with the transiently expressed murine FcγRIIb2 inhibited serotonin release. Coaggregation of Tac with a truncated murine FcγRIIb2, which lacks most of its cytoplasmic tail, including the conserved ITIM region, did not inhibit serotonin release, implying that the murine FcγRIIb2 inhibitory capability resides in its cytoplasmic tail. Their results indicated that a single ITAM-containing receptor subunit, which is sufficient for triggering cell activation on aggregation, is also a sufficient target for FcγRIIb-dependent inhibition.

1. Immunoreceptor Tyrosine-Based Inhibitory Motif

In order to define the polypeptide region(s) in FcγRIIb that mediates the inhibitory effect, a cDNA containing an internal deletion of 13 amino acids in the cytoplasmic domain of FcγRIIb was expressed in FcγRIIb-negative mutant B cells (Muta *et al.*, 1994). On cross-linking of the BCR with this truncated Fcγ receptor, no inhibition of B-cell activation was observed, suggesting that these 13 residues (AENTITYSLLKHP) are essential for the inhibitory function of FcγRIIb (Muta *et al.*, 1994). An FcγRIIb chimeric receptor, in which the cytoplasmic tail was replaced by the first 18 amino acids of the TCRζ attached to the 13 amino acids of FcγRIIb, was able to deliver inhibitory signals on its cross-linking with the BCR. This indicated that the 13 amino acid stretch is not only essential, but is also sufficient for mediating this inhibitory effect. Furthermore, these studies demonstrated that the tyrosine residue within the ITIM sequence is critical for the inhibitory effect because replacement of the tyrosine residue by phenylalanine markedly reduced the ITIM activity.

To identify critical regions within murine and human FcγRIIb2 that are potentially negative regulators of different ITAM-containing receptors, Daëron *et al.*, (1995a) further utilized the chimeric subunit approach. They expressed truncated or mutated murine FcγRIIb2 in RBL cells and found that the same stretch of 13 amino acids, within the cytoplasmic tail of

murine FcγRIIb2, is required for inhibiting serotonin release from mast cells activated via either FcεRI or TCRζ. The single tyrosine within this 13 residue motif was essential for inhibition. Truncation of the homologous ITIM-containing region from the cytoplasmic tail of human FcγRIIb2 also removed its inhibitory effect on IgE-induced serotonin release. These results demonstrated that the ITIM-containing regions within murine and human FcγRIIb account for the negative regulation of different ITAM-containing receptor subunits.

Another ITIM-containing B-cell surface molecule is CD22, a member of the sialoadhesion subclass of the immunoglobulin superfamily (Stamenkovic and Seed, 1990; Wilson *et al.*, 1991). CD22 possesses three copies of the V/IxYxxL motif in its cytoplasmic tail (see Fig. 6) that, on phosphorylation, are able to bind and activate SHP-1 (Doody *et al.*, 1995). The physiological ligand of CD22 is unknown and initial studies suggested that CD22 is a positive regulator of the antigen receptor signaling in B cells (Pezzutto *et al.*, 1987; Tuscano *et al.*, 1996). However, downregulation of CD22 with bead-immobilized anti-CD22 antibodies markedly enhanced BCR-induced activation responses (Doody *et al.*, 1995). Furthermore, B cells from CD22-deficient mice exhibited exaggerated Ca^{2+} responses following antigen receptor cross-linking and spontaneous modulation of surface IgM on peripheral B cells (O'Keefe *et al.*, 1996; Otipoby *et al.*, 1996; Sato *et al.*, 1996; Nitschke *et al.*, 1997). The B-cell phenotype in the CD22-negative mice was similar to that of the B cells from SHP-1-deficient motheaten mice (Pani *et al.*, 1995), supporting the view that CD22 functions as a scaffold for SHP-1 and negatively regulates antigen receptor signaling (Cyster and Goodnow, 1997).

ITIM-containing receptors also include the killer cell inhibitory receptors (KIRs) that are expressed on NK cells and a subset of T cells. KIRs recognize MHC class I molecules and are rapidly phosphorylated upon aggregation. This is followed by SHP-1 binding and activation and delivery of negative signals that inhibit a variety of cell-mediated cytotoxic functions (reviewed in Long *et al.*, 1997).

2. *Role of SHP-1*

Immune complex-mediated coligation of the BCR and FcγRIIb provided a negative signal by inducing tyrosine phosphorylation of ITIM in the FcγRIIb cytoplasmic tail followed by recruitment and activation of the hematopoietic cell-specific PTPase, SHP-1 (D'Ambrosio *et al.*, 1995). The activated SHP-1 apparently dephosphorylates tyrosine residues within the adjacent ITAMs and/or their associated PTKs, resulting in inhibition of the BCR-mediated activation signals.

SHP-1 is a member of a family of PTPases that possess two amino-terminal SH2 domains, a conserved catalytic domain, and a carboxy-terminal regulatory domain (Matthews *et al.*, 1992; Plutzky *et al.*, 1992; Shen *et al.*, 1991). It recruits to additional ITIM-containing activated receptors, including CD22 (Doody *et al.*, 1995) and the NK cell inhibitory receptor, p58 (Burshtyn *et al.*, 1996; Olcese *et al.*, 1996).

Support for the potential role of SHP-1 in the negative regulation of receptor-linked signal transduction pathways in hematopoietic cells was obtained from studies of motheaten (*me*) and motheaten viable (me^v) mice (Shultz *et al.*, 1984; Shultz and Green, 1976). These two inbred mouse strains carry an inherited congenital deficiency of SHP-1 (Shultz *et al.*, 1993; Tsui *et al.*, 1993), leading to either hyperproliferation and activation of a subtype of peripheral B lymphocytes or breakdown of self-tolerance and production of autoantibodies (Davidson *et al.*, 1979; Shultz and Green, 1976; Sidman *et al.*, 1978a,b). The two *motheaten* allelic mutations (Shultz *et al.*, 1993; Tsui *et al.*, 1993) result in either complete lack of expression of a functional SHP-1 (*me*) or expression of an aberrant phosphatase that exhibits a marked reduction in catalytic activity (me^v) (Kozlowski *et al.*, 1993; Shultz *et al.*, 1993; Tsui *et al.*, 1993).

The extensive impairment of *motheaten* mice in both B-cell differentiation and function raised the possibility that SHP-1 may be involved in the regulation of BCR-linked signal transduction pathways. Analysis of $CD5^+$ B cells from *motheaten* mice have shown hyperresponsiveness to suboptimal concentrations of $F(ab')_2$ anti-Ig antibodies and reduced susceptibility to the inhibitory effect of cross-linking of the FcγRIIb with the BCR (Pani *et al.*, 1995). An enzymatically active SHP-1 was found to constitutively associate with the BCR of resting B cells, and $F(ab')_2$ anti-Ig treatment of B cells resulted in a rapid tyrosine phosphorylation of SHP-1, followed by its complete dissociation from the BCR (Pani *et al.*, 1995). These results support the involvement of SHP-1 in the regulation of basal tyrosine phosphorylation levels of BCR subunits. Thus, association of active SHP-1 with BCR in resting B cells may result in dephosphorylation of critical tyrosine residues within the receptor or receptor-associated molecules, thereby keeping potential activation signals below a minimal threshold. Receptor engagement leads to tyrosine phosphorylation and dissociation of SHP-1 from the BCR (thereby removing the putative negative regulator) and to an increase in tyrosine phosphorylation of receptor subunits, leading to activation of receptor-linked signal transduction pathways. This hypothesis is further supported by the observation that SHP-1 can dephosphorylate *in vitro* a BCR-associated protein, with an apparent molecular mass of 35 kDa, that may possibly represent the Ig-α subunit (Pani *et al.*, 1995).

Cyster and Goodnow (1995) tested the potential role of SHP-1 in B-cell signaling by using transgenic mice that carry hen egg lysozyme-specific immunoglobulin genes and are homozygous for the *me*^{v} gene. B cells from SHP-1-deficient transgenics exhibited increased sensitivity to the hen egg lysozyme antigen, as demonstrated by their exaggerated intracellular Ca^{2+} response. In addition, lack of SHP-1 resulted in a reduced threshold for arrested development and deletion of bone marrow B cells. These findings indicated that SHP-1 is a negative regulator of the BCR and is involved in setting signaling thresholds for negative selection.

Although it remains to be verified whether SHP-1 can dephosphorylate BCR Ig-α and/or Ig-β pITAMs directly, specificity of this phosphatase to the phosphotyrosyl residue indicates that it downregulates cell activation by intervening with the activity of PTKs and/or their substrates.

3. *Role of SHIP*

A distinct mechanism for downregulation of B-cell activation involves the activity of another phosphatase that appears to intervene with different receptor-induced activation events. The basis for this discovery were the results by Ono *et al.* (1996), who analyzed mast cell degranulation in response to FcεRI stimulation with biotinylated IgE in the presence or absence of FcγRIIb co-cross-linking with streptavidin. They found that increasing FcγRIIb cross-linking to FcεRI–IgE complexes resulted in increasing inhibition of degranulation in mast cells from wild-type as well as *me/me* or *me*^{v}/*me*^{v} mice. The ability of FcγRIIb to inhibit IgE-triggered degranulation in mast cells from the mutant SHP-1-deficient mice was indistinguishable from that of mast cells from wild-type mice and was abolished only in mast cells from FcγRIIb knockout mice. These results were in contrast to previously obtained data showing that SHP-1-deficient B cells are unable to mediate FcγRIIb inhibition of BCR-induced cell activation (D'Ambrosio *et al.*, 1995; Pani *et al.*, 1995).

Because FcγRIIb, but not SHP-1, was essential for the induction of the negative signals, Ono *et al.* (1996) assumed that a protein which is distinct from SHP-1 provides the negative signal by recruiting to the tyrosine-phosphorylated cytoplasmic tail of activated FcγRIIb. They purified this protein by affinity chromatography using, as a bait, a tyrosine-phosphorylated synthetic peptide that possesses the 13 amino acid sequence derived from the FcγRIIb cytoplasmic tail (ITIM; Amigorena *et al.*, 1992). One major tyrosine-phosphorylated protein band that was isolated from mast cell extract turned out to be the inositol phosphatase, SHIP (Ono *et al.*, 1996). A second tyrosine-phosphorylated protein band was found in B cell, but not mast cell, extract and was identified as SHP-1. To verify whether SHIP associates with FcγRIIb in mast cells and

B cells, the FcγRIIb was immunoprecipitated after its coligation to FcεRI or BCR and was then immunoblotted with anti-SHIP antibodies (D'Ambrosio *et al.*, 1996; Ono *et al.*, 1996). SHIP was found to associate with FcγRIIb in both B cells and mast cells. However, the possibility that SHIP associates with tyrosine-phosphorylated FcεRI or BCR ITAMs, which might coimmunoprecipitate with the cross-linked FcγRIIb, has not been completely ruled out (Ono *et al.*, 1996). Direct binding of SHIP to FcγRIIb has also been demonstrated *in vitro* using immobilized synthetic peptides that correspond to the phosphorylated FcγRIIb ITIM sequence (Fong *et al.*, 1996; Sarmay *et al.*, 1997; Tridandapani *et al.*, 1997a,b).

The observation that the FcγRIIb-mediated inhibitory signals in mast cells do not require SHP-1 has led to suggest that SHIP mediates the FcγRIIb inhibitory effect by removing the 5′ phosphate from phosphatidylinositol 3,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate, thereby terminating Ca^{2+} fluxes and Ca^{2+}-dependent activation signals. A similar mechanism has been proposed for SHIP in the negative signaling in B cells (Chacko *et al.*, 1996b). Ono *et al.* (1996) further speculated that SHIP recruitment to activated FcγRIIb functions to downregulate early activation events, whereas SHP-1 recruitment inhibits more distal events that regulate cell proliferation.

Support for the involvement of SHIP in the regulation of mast cell activation was obtained by Osborne *et al.* (1996), who used a novel yeast tribrid system in order to identify FcεRI downstream effectors. Quite surprisingly, they found that the yeast-expressed SHIP interacts with tyrosine-phosphorylated ITAMs that are derived from either the FcεRI β chain or the γ chain. A GST-SHIP-SH2 interacted *in vitro* with biphosphorylated ITAM peptides derived from CD3γ, δ, or ε chains and each of the three TCRζ ITAMs, as well as a monophosphorylated ITAM ζ_3 peptide. In an independent study, SHIP from RBL-2H3 basophilic cells coprecipitated with immobilized phosphopeptides possessing a sequence of FcεRI β chain, but not γ chain (Kimura *et al.*, 1997). Nevertheless, *in vivo* association of SHIP with FcεRI β or γ chains in activated mast cells could not be confirmed by coimmunoprecipitation experiments, leaving open the question whether SHIP recruitment to cross-linked FcεRI/FcγRIIb is mediated by SHIP binding to tyrosine-phosphorylated FcεRI-ITAM or to FcγRIIb-ITIM. One possible explanation for the observation that SHIP binds phospho-ITAM *in vitro*, but not *in vivo*, stems from the fact that, unlike ZAP-70 and Syk, it possesses a single SH2 domain. Thus, under conditions of excess peptide, SHIP may interact *in vitro* with the shared core sequence pYxxL present in pITAMs and pITIMs, whereas *in vivo* constrains may permit only high-affinity binding with its appropriate ligand.

B. Mechanisms for Turning Off ITAM-Coupled Activation Signals in T Lymphocytes

Studies of the negative regulatory mechanisms that determine the intensity and duration of the T-cell responses are still in their infancy, and no clear physiological mechanism has yet been described to indicate how signal termination by the TCR may take place. Because TCR-ITAMs can mediate effective activation signals in T cells only following their tyrosine phosphorylation, it is assumed that PTPases that can dephosphorylate pITAMs will play a critical role in downregulating activation signals. Recent discoveries have begun to shed light on the molecular mechanisms underlying TCR-mediated inhibitory signals.

The TCR and CD4 or CD8 coreceptors are expressed on the surface of resting T cells and, on interaction with MHC-bound peptide antigen, they cocluster and initiate early activation events. Among them is the induction of increased surface expression of CTLA-4 (Brunet *et al.*, 1987), which can than interact with its countereceptors, B7-1 (CD80) or B7-2 (CD86), on the surface of antigen-presenting cells (Green *et al.*, 1994; Krummel and Allison, 1995; Walunas *et al.*, 1994). Several lines of studies support the negative role of CTLA-4 during the activation response of T cells: (a) Mice deficient in CTLA-4 have hyperactive T cells and are prone to lymphoproliferative disorders (Tivol *et al.*, 1995; Waterhouse *et al.*, 1995), (b) CTLA-4 cross-linking with antibodies inhibits anti-CD3-induced T-cell proliferation and IL-2 production *in vitro* (Kearney *et al.*, 1995; Krummel and Allison, 1996), and (c) soluble intact or Fab fragments of anti-CTLA-4 increased T-cell responses both *in vitro* and *in vivo* (Kearney *et al.*, 1995; Krummel *et al.*, 1996; Leach *et al.*, 1996).

In an attempt to dissect the molecular mechanism by which CTLA-4 transduces inhibitory signals, Marengère *et al.* (1996) have tested T cells from *Ctla-4*$^{-/-}$ mice. They found constitutive activation of Lck, Fyn, and ZAP-70 and high levels of tyrosine phosphorylation of the TCRζ. In addition, the Ras-MAPK pathway was also constitutively activated. Further analysis showed that CTLA-4 in activated T cells from wild-type mice interact with the SHP-2 PTPase, which may downregulate signals delivered through the TCR. This interaction is mediated by the tyrosine-phosphorylated YVKM motif in the cytoplasmic tail of CTLA-4 and an SH2 domain of SHP-2. Marengère *et al.* (1996) suggested that in analogy to the role of SHP-1 in FcγRIIb- and CD22-mediated downregulation of B-cell responses, the function of CTLA-4 in T cells is to recruit SHP-2 to the receptor site where dephosphorylation of critical tyrosine residues downregulates T-cell activation. It is not yet known which of the molecules that undergo tyrosine phosphorylation following TCR engagement are the

actual substrates for SHP-2. However, the observation that the TCRζ chain is constitutively tyrosine phosphorylated in CTLA-4-negative T cells suggests that SHP-2 is directly or indirectly involved in the dephosphorylation of tyrosine residues within ζ chain ITAMs.

SHP-1 has also been implicated in downregulation or termination of TCR-induced activation signals (Plas *et al.*, 1996). However, in contrast to B cells, where binding of SHP-1 to pITIM serves to inhibit signals derived from a different receptor (i.e., the BCR), SHP-1 in T lymphocytes interacts with the activated TCR. This is mediated by SHP-1 SH2 binding to phosphotyrosine residues on ZAP-70 that, in activated T cells, is associated with TCR pITAMs. The inhibitory role of SHP-1 in T-cell activation was supported by findings showing that overexpression of a dominant negative SHP-1 increases the sensitivity of the TCR to suboptimal concentrations of stimulators (Plas *et al.*, 1996).

VII. Perspectives and Concluding Remarks

The antigen and Fc receptors are key regulators of the immune system and determine the nature, intensity, and duration of the cell-mediated and humoral immune responses. Studies performed during the past several years contributed significantly to our understanding of the molecular and biochemical mechanisms used by these receptors to transduce extracellular signals into the cell. Despite the fact that these receptors are composed of protein subunits encoded by different genes that vary in structure and complexity, it has become clear that all of their signal-transducing subunits share a common cytoplasmic tail motif, termed ITAM, which link them to their signal-transduction machinery. Engagement of these receptors is followed by a rapid and transient phosphorylation of the ITAMs tyrosine residues that create temporary binding sites for SH2-containing signaling proteins. Although the presence of ITAMs enables recruitment of many shared effector molecules, ITAM multiplication in receptor subunits and amino acid heterogeneity within the ITAMs and flanking regions result in recruitment of unique molecules to distinct ITAMs. This enable ITAMs to link receptors to multiple shared and unique signal transduction pathways. As a result, ligation of antigen receptors does not always provoke identical responses and can direct cells to undergo activation, proliferation, differentiation, transformation, or self-suicide. It has been suggested that the physical nature of the ligand/receptor interaction determines the initial activation events and the consequent recruitment of specific effector molecules that define the cell fate and the nature of the resulting response. Although many of the polypeptides involved in the immunoreceptor downstream signal transduction pathways have been defined molecularly, the

fine-tuning and regulation of the mechanisms that direct the cell into one of several differentiation pathways are not clearly understood.

The discovery that viruses possess ITAM sequences in their envelope glycoproteins may clarify additional novel strategies of molecular mimicry by pathogens. It may also define molecular mechanisms by which such pathogens interfere with intracellular regulatory processes that may alter basic cell functions and affect cell growth, differentiation, and transformation.

Further studies are required in order to better understand the structural and functional properties of ITAMs, their mechanism of interaction with distinct SH2-containing molecules, and their regulatory role in signal transduction. Identification of the molecular components involved in immunoreceptor activation-induced signal transduction pathways and elucidation of the sorting and regulatory mechanisms that define the ensuing responses are essential for enabling logical decisions when therapeutical intervention is desired. Such information may eventually enable the prevention of immune rejection of transplanted tissues or organs and efficient treatment of immune disorders, including autoimmunity, allergy, and cancer.

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Note added in proof. Since submission of this review, Lanier *et al.* (1998) have reported the existence of an additional ITAM region, containing the receptor DAP12, that transduces activation signals from the KIR family member, KIR2DS2.

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The Atypical Serine Proteases of the Complement System

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I. Serine Proteases in the Complement System

Complement is a major recognition and effector system in humoral immunity that provides mammals and other animal species with mechanisms designed to eliminate pathogenic microorganisms and other antigens from blood and tissues. It consists of more than 30 serum and cellular components, including recognition proteins, proteases, cell receptors, and regulatory proteins that act in concert to form an efficient, self-controlled, and independent defense system. There are three different pathways of complement activation (Fig. 1). Triggering of the alternative pathway does not involve a proper recognition step, but results from the spontaneous, low-level hydrolysis or reactivity of a thioester bond in protein C3, which leads to the formation of a stable and efficient protease (C3-convertase) at the surface of foreign particles (Lachmann and Nicol, 1973; Pangburn *et al.*, 1981). In contrast, initiation of the classical pathway requires binding of the multimolecular protease C1 to a microorganism, through its component C1q. This occurs either directly or after prior recognition of the microorganism by antibodies and leads to subsequent C1 activation (Cooper, 1985). The more recently discovered "lectin pathway" of complement activation is triggered by a multimolecular protease homologous to C1, in which the recognition function is mediated by mannose-binding lectin (MBL), a protein that specifically binds carbohydrates at the surface of microorganisms (Matsushita and Fujita, 1992). Activation of the three pathways leads to the formation of distinct but homologous C3-convertases that share the same ability to mediate specific cleavage of protein C3, generating fragments C3a and C3b. The latter plays a pivotal role in the complement system. First, as it is both a product and a subunit of the alternative pathway C3-convertase, its production generates an amplification loop. Second, C3b can bind covalently to microorganisms, thereby acting as a marker that targets them to complement receptor-bearing cells such as phagocytic cells. C3b can also bind to C3-convertases, endowing them with the ability to cleave C5 and generate fragment C5b, which then

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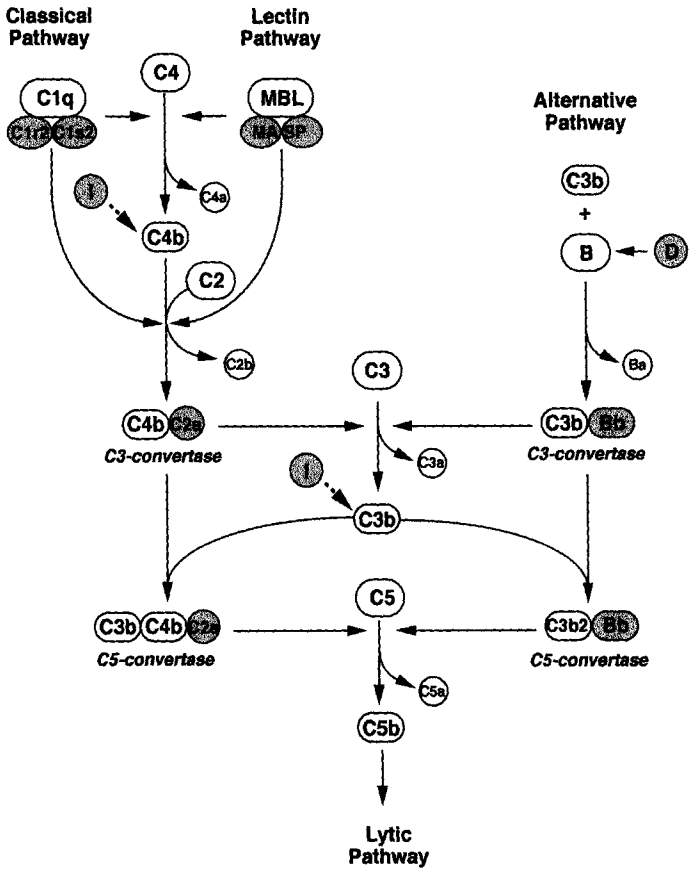


FIG. 1. The role of proteolytic enzymes in the three pathways of complement activation. Activation of the classical pathway is triggered by direct or antibody-dependent recognition of a microorganism by C1q, whereas that of the lectin pathway involves interaction of the mannose-binding lectin (MBL) with arrays of mannose or *N*-acetylglucosamine residues at the surface of the microorganism. Initiation of the alternative pathway requires formation of an initial pro-C3-convertase on an activator surface. Enzymatically active components are shaded. It is not fully established yet whether MBL is always associated with two functionally distinct MASPs that would form a complex equivalent to the $C1r_2C1s_2$ tetramer in C1. None of the inhibitors or regulators of the pathways are shown (modified from Reid, 1995).

initiates the assembly of the membrane attack complex, resulting in the lysis of susceptible microorganisms. These phagocytic and lytic effects, as well as the inflammatory and/or chemotactic activities expressed by small polypeptide fragments (C3a, C5a) released along the complement activation cascade, are designed to provide a first line of defense against microbial infection.

Proteolytic enzymes play essential roles in the generation of complement activities, both in the initial steps of the pathways and in the amplification and regulation of the cascade reactions (Fig. 1). Factor D (EC 3.4.21.46) is the enzyme responsible for cleavage of factor B (EC 3.4.21.47) in two fragments Ba and Bb, the latter of which becomes the enzymic component of the alternative pathway C3- and C5-convertases, respectively, C3bBb and $(C3b)_2Bb$. C1r (EC 3.4.21.41) mediates autolytic activation of the C1 complex and generates active C1s (EC 3.4.21.42), the protease responsible for C1-mediated cleavage of proteins C4 and C2. Although activation of the lectin pathway is not yet fully understood, it possibly involves two related but distinct proteases, the MBL-associated serine proteases (MASPs), which could fulfill functions equivalent to those of C1r and C1s. Both classical and lectin pathways result in the assembly of the convertases C4b2a and C3b4b2a, the enzymatic activity of which is carried out by the C2a fragment of C2. Another protease, factor I (EC 3.4.21.45), participates both in the regulation of complement activation and in the generation of biologically active fragments through its ability to cleave fragments C4b and C3b in cooperation with cofactors. These eight proteolytic enzymes exhibit very narrow trypsin-like specificities, restricted to the cleavage of a few specific arginyl bonds in their protein substrates. Most complement proteases do not function as free enzymes in solution, but within complex multimolecular assemblies, and/or with the help of cofactors. They all comprise serine protease domains which, on the basis of their primary structure, belong to the chymotrypsin family, but exhibit unique structural and functional features compared to typical pancreatic serine proteases. In addition, all complement proteases but factor D contain additional protein modules homologous to those found in other extracellular proteins. These modules likely endow complement enzymes with specific interaction properties that contribute for a large part to their highly specialized functions.

This review initially provides short overviews on the structural and functional features shared by serine proteases and by the different types of protein modules encountered in complement enzymes. It then summarizes current knowledge on the genomic organization, structure, and function of complement serine proteases, and provides insights into the complex mechanisms involved in their activation and activity in light of recent three-dimensional structural studies.

II. The Serine Protease Domain: Shared Features and Unique Structural Elements of Complement Proteases

Chymotrypsin-like serine proteases are widely distributed and are involved in many important biological processes, which in addition to comple-

ment activation include blood coagulation, kinin generation, fibrinolysis, fertilization, digestion, and cell differentiation. High-resolution structures have been produced for several chymotrypsin-like serine proteases by X-ray crystallography (Birktoft and Blow, 1972; Fehlhammer *et al.*, 1977; Sawyer *et al.*, 1978; Greer, 1990). All have very similar structural folds, consisting of two antiparallel β-barrel-type domains. Each barrel contains six β strands, which characteristically have the same topology in all enzymes. Because all chymotrypsin-like serine proteases, including the complement ones, have certain critical structural elements conserved in their primary structure, it is generally assumed that they all share the chymotrypsin structural fold.

Four structural elements necessary for substrate recognition and catalysis have been conserved in the structure of serine proteases: a catalytic triad, a primary specificity pocket, a nonspecific binding site for the P_1–P_3 residues of the substrate, and an oxyanion-binding hole (Perona and Craik, 1995). The essential role of the catalytic triad residues, Asp^{102}, His^{57}, and Ser^{195} (chymotrypsinogen numbering has been used for all serine protease domains throughout this review to facilitate comparisons), for efficient substrate hydrolysis was established many years ago (Dixon *et al.*, 1956; Shaw *et al.*, 1965; Blow *et al.*, 1969). However, the original proposal that the three residues participate in a charge-relay system (Blow *et al.*, 1969) has not been supported by more recent studies, which failed to demonstrate proton transfer between His^{57} and Asp^{102} (Bachovchin and Roberts, 1978; Kossiakoff and Spencer, 1981; Warshel *et al.*, 1989). It is currently held that substrate hydrolysis is mediated via nucleophilic attack on the carbonyl carbon of the scissile bond by the hydroxyl oxygen of Ser^{195}; His^{57} serves as a general base catalyst, increasing the nucleophilicity of Ser^{195}; and Asp^{102} is necessary for maintaining the proper tautomer of His^{57} in the ground state and for stabilizing the developing positive charge of His^{57} during the transition state (Craik *et al.*, 1987). The spatial relationships among the catalytic triad residues are crucial for their synergistic action and are invariable in all chymotrypsin-like serine proteases of known structure. All complement enzymes have conserved the three catalytic residues and the surrounding highly conserved regions (Fig. 2). Therefore, they should be expected to employ the same catalytic mechanism as all other serine proteases. Functional support for this proposal is provided by the fact that all complement enzymes are inhibited by diisopropylfluorophosphate. However, the catalytic triad of factor D, the only complement enzyme of known three-dimensional structure (Narayana *et al.*, 1994), displays considerable plasticity, such that in its "resting state" the enzyme has an apparently nonfunctional catalytic triad.

The three-dimensional structure of the substrate specificity pocket of chymotrypsin-like serine proteases is highly conserved, despite differences among them in substrate specificity. In γ-chymotrypsin the pocket has three walls formed by residues 189–195, 214–220, and 225–228 (Cohen *et al.*, 1981), which are similar to the corresponding residues of other serine proteases (Fig. 2). Differences in substrate specificity among groups of enzymes are attributed mainly to residue 189, which is located at the bottom of the specificity pocket. Thus, the presence of an Asp at this position endows trypsin-like proteases with specificity for positively charged Arg and Lys residues, whereas in chymotrypsin the preference for bulky aromatics is largely defined by Ser^{189}. With the exception of factor B and C2, complement serine proteases have an Asp at position 189, which is consistent with their specificity for arginyl peptide bonds (Table I). Unexpectedly, C2 has a Ser and factor B an Asn residue at position 189, although both cleave Arg bonds. Both enzymes have an Asp at position 187, which could conceivably be located within the pocket and serve to bind the guanidinium side chain of the P_1[1]–Arg residue of the substrate. However, site-directed mutagenesis experiments have indicated that at least in factor B Asp at position 187 is not functionally important (Y. Wang and Y. Xu, unpublished). Therefore, it appears that the side chain of the P_1 Arg of the substrate does not form ion bonds with the residue at the bottom of the primary specificity pocket of factor B or C2. Different interactions, perhaps H bonds, may be responsible for holding the P_1 residue in the correct orientation relative to the catalytic Ser^{195}.

Additional structural elements, particularly the conformation of the invariable Gly^{216}, as well as distant surface loops that contribute to the geometry of the specificity pocket without binding the substrate (Hedstrom *et al.*, 1992, 1994), play crucial roles in substrate specificity and catalysis. Comparative analyses of crystal structures of chymotrypsin, trypsin, and chymotrypsin-like trypsin mutants have indicated that the main chain conformation of Gly^{216} is a crucial structural determinant for the correct orientation of the scissile bond of the substrate (Perona *et al.*, 1995). In turn, accurate positioning of the scissile bond relative to the oxyanion hole and the Ser^{195}/His^{57} dyad is a major kinetic determinant of substrate specificity. In all serine proteases analyzed, Gly^{216} forms two antiparallel β-strand H bonds with the P_3 residue of the substrate. The H bonds formed in trypsin and chymotrypsin are different and are believed to act as specificity determinants (Perona *et al.*, 1995). It would be of interest to analyze the effect on substrate specificity of the presumed H bonds between Gly^{216} of complement serine proteases and the corresponding P_3 residues.

[1] The nomenclature for the individual amino acid residues (P_1, P_2, etc.) of a substrate and the corresponding subsites (S_1, S_2, etc.) of the enzyme is that of Schechter and Berger (1967).

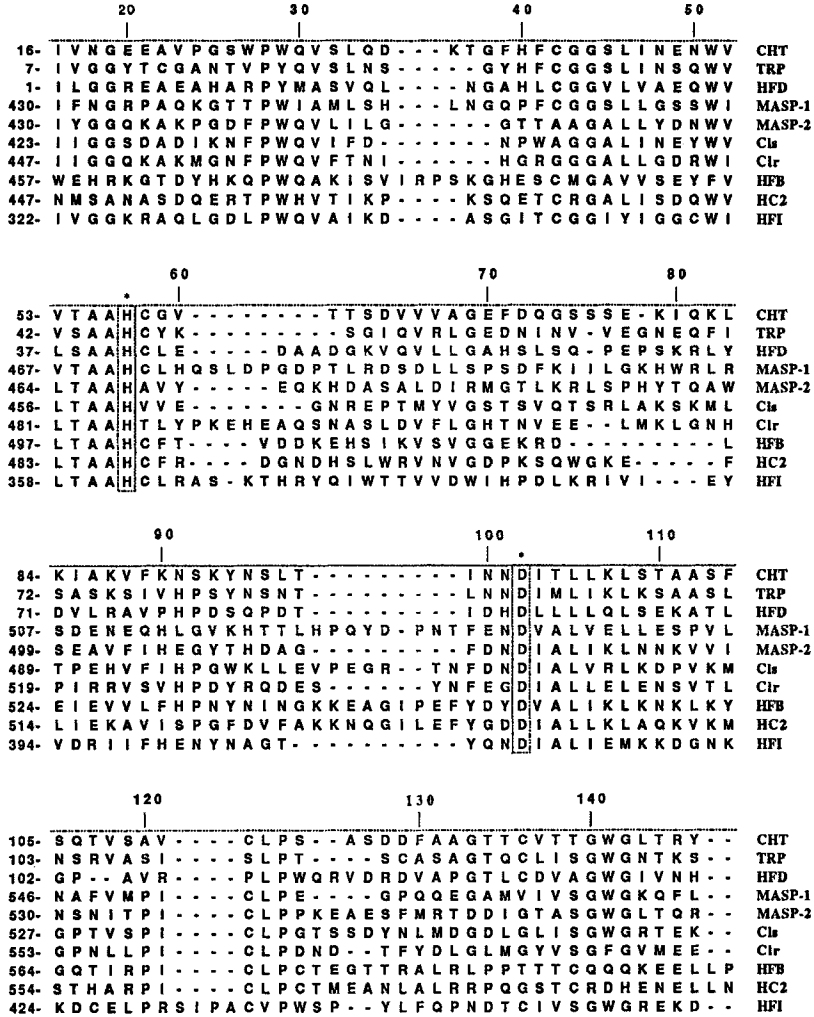


FIG. 2. Amino acid sequence alignment of typical chymotrypsin-like pancreatic serine proteases and complement serine protease domains. The three residues, Asp^{102}, His^{57}, and Ser^{195}, that form the catalytic triad are marked by asterisks and boxed. Residues that form the walls of the primary specificity pocket are shaded. Numbers at the top are for residues of the chymotrypsinogen sequence, and numbers on the left are for the amino acid sequence of individual proteases. CHT, bovine chymotrypsin; TRP, bovine trypsin; HFD, human factor D; MASP, mannose-binding lectin-associated serine protease; HFB, human factor B; HC2, human C2; HFI, human factor I.

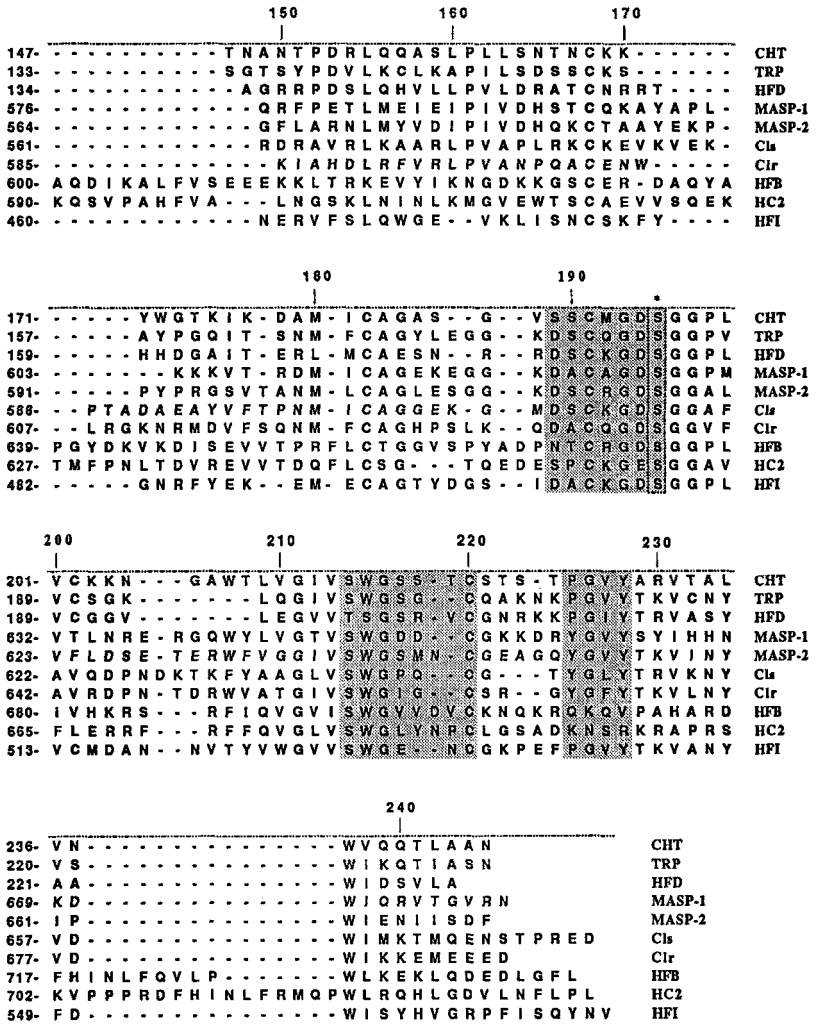


FIG. 2.—*Continued*

Despite their overall structural similarity to typical pancreatic serine proteases, complement enzymes have unique structural features that are probably responsible for their highly specialized functions. All complement enzymes, except for factor D, have additional domains that contain protein modules also present in functionally unrelated proteins. In MASPs, C1r, C1s, and factor I the serine protease domain occupies the smaller of two disulfide-linked polypeptide chains. In the former four enzymes the two-

TABLE I

COMPLEMENT ENZYMES: NATURAL SUBSTRATES AND CLEAVAGE SITES

Zymogen	Active Enzyme	Cofactor(s)	Substrate	Cleavage Sites[a] P_5 -P_4 -P_3 -P_2 -P_1 -P_1' -P_2' -P_3' -P_4'
C1r	C1r		C1r	^{442}Glu-Gln-Arg-Gln-Arg-Ile-Ile-Gly-Gly
C1r	$\overline{C1r}$		C1s	^{418}Glu-Glu-Lys-Gln-Arg-Ile-Ile-Gly-Gly
C1r	$\overline{C1r}$		$\overline{C1r}$	^{275}Ser-Gly-Asp-Ser-Arg-Gly-Trp-Lys-Leu
				^{207}Ile-Arg-Val-Glu-Arg-Gly-Leu-Thr-Leu
?	?		MASP[b]	^{425}Lys-Leu-Met-Ala-Arg-Ile-Phe-Asn-Gly
C1s	$\overline{C1s}$		[C4, C2][c]	^{733}Ala-Gly-Leu-Gln-Arg-Ala-Leu-Glu-Ile
MASP	$\overline{MASP}$			^{219}Glu-Ser-Leu-Gly-Arg-Lys-Ile-Gln-Ile
D	D	C3b	B	^{230}Glu-Gln-Gln-Lys-Arg-Lys-Ile-Val-Leu
B	$\overline{C3bBb}$		C3	^{722}Leu-Gly-Leu-Ala-Arg-Ser-Asn-Leu-Asp
C2	$\overline{C4b2a}$		C3	
B	$\overline{(C3b)_2Bb}$		C5	^{729}Met-Gln-Leu-Gly-Arg-Leu-His-Met-Lys
C2	$\overline{C4b3b2a}$		C5	
I	I	H, MCP, CR1	C3b	^{1772}Gln-Leu-Pro-Ser-Arg-Ser-Val-Lys-Ile
				^{1294}Ala-Ser-Leu-Leu-Arg-Ser-Glu-Glu-Thr
		CR1	iC3b	^{928}Glu-Arg-Leu-Gly-Arg-Glu-Gly-Val-Gln
		C4bp, MCP, CR1	C4b	^{933}Asp-His-Arg-Gly-Arg-Thr-Leu-Glu-Ile
				^{1313}Ser-Ser-Thr-Gly-Arg-Asn-Gly-Phe-Lys

[a] Nomenclature for the individual amino acid residues (P1, P2, . . . P1′, P2′, etc.) of the substrate is that introduced by Schechter and Berger (1967).

[b] The enzyme that cleaves and activates MASP has not been identified.

[c] C1s and MASP cleave identical peptide bonds in C4 and C2.

chain structure arises from cleavage of a peptide bond of the single polypeptide chain zymogen form during complement activation. Unlike all other chymotrypsin-like serine proteases, MASP-2, C1r, and C1s lack the "histidine loop" disulfide bridge. Factor I apparently has no circulating zymogen form, and conversion of its single-chain biosynthetic precursor to an active serine protease probably occurs intracellularly within the secretory pathway by enzymatic removal of a basic tetrapeptide. Conversion of pro-factor D to factor D also takes place intracellularly by cleavage of a single peptide bond, which results in the release of a small activation peptide. In the absence of structural zymogens in blood, the proteolytic activities of factors D and I are apparently regulated through other mechanisms, which probably depend on reversible conformational changes. Factor B and C2 lack the highly conserved and functionally important N-terminal sequence of serine proteases. Instead, following activation, their serine protease domains remain attached to a von Willebrand factor type A (VWFA) module. These and additional unique structural properties endow complement serine proteases with their extremely restricted substrate specificity, which is necessary for their function and which is reflected in their low reactivity with serine protease synthetic substrates and active site inhibitors.

III. Modular Structure of Complement Proteases

A schematic representation of the structure of complement proteases is shown in Fig. 3. Except for factor D, which only possesses a serine protease domain, complement proteases have additional polypeptide domains in their N-terminal regions, which are subdivided into a series of structural motifs or modules. Factor B and C2 on the one hand and C1r, C1s, and the MASPs on the other hand exhibit similar modular organizations, whereas factor I has a unique modular structure. The concept of protein modules has emerged in recent years with the determination of an increasing number of protein sequences and their comparative analysis, which revealed that many extracellular proteins, including the proteases of the blood coagulation, fibrinolysis, and complement systems, are made up from a limited repertoire of contiguous sequence motifs (Baron *et al.*, 1991; Patthy, 1993; Doolittle, 1995). More than 60 different types of protein modules have been identified thus far, which range in size from 30 to 350 amino acid residues (Bork and Bairoch, 1995). Each module type may be recognized at the sequence level by a characteristic consensus sequence, often restricted to a few amino acid residues that form the core of the module and determine its folding. Indeed, available three-dimensional structures of protein modules indicate that all members of a given family of modules exhibit similar architectures, but differ from each other by the

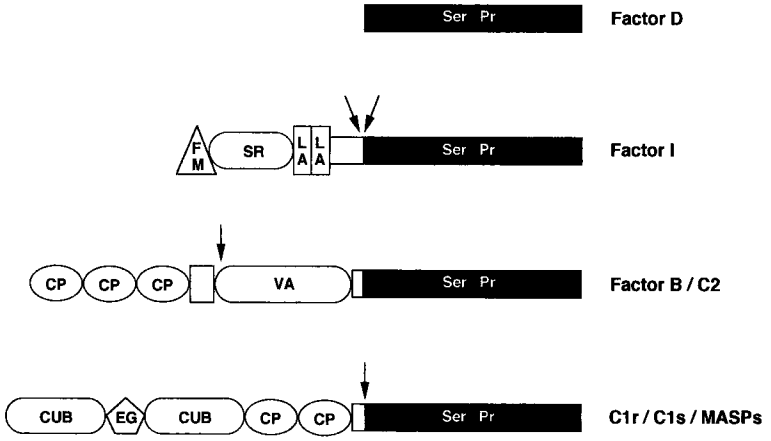


FIG. 3. Schematic representation of the modular structures of complement serine proteases. The two-letter nomenclature and symbols used for protein modules are those defined by Bork and Bairoch (1995). CP, CCP module; EG, EGF module; FM, FIMAC module; LA, LDLRA module; SR, SRCR module; VA, VWFA module. The relative sizes of the modules are approximate. Unlabeled portions of the proteins represent connecting segments. Arrows indicate peptide bonds cleaved on activation.

occurrence of surface loops of variable size and sequence, which confer a given module its particular function, often the ability to mediate specific protein–protein interactions (Bork *et al.*, 1996). It is currently hypothesized that modules of the same type have evolved from a common ancestor by gene duplication and spread through exon shuffling (Patthy, 1991, 1994).

Seven different types of modules are present in complement proteases (Fig. 3). The most commonly found are complement control protein (CCP) modules, which form contiguous double or triple arrays in C1r, C1s, the MASPs, C2, and factor B. CCP modules also occur, always in multiple copies, in various complement receptors and regulatory proteins, including receptors CR1 and CR2, factor H, C4b-binding protein, decay accelerating factor, and membrane cofactor protein, all known to interact with complement proteins C3b and/or C4b, as well as in proteins functionally unrelated to the complement system, such as β_2-glycoprotein I and blood clotting factor XIII (Reid *et al.*, 1986). CCP modules exhibit a consensus sequence comprising a few conserved hydrophobic and aromatic residues and four cysteine residues that form two disulfide bridges (Cys1–Cys3; Cys2–Cys4). The solution structure of the 5th, 15th, and 16th CCP modules of human factor H has been solved by nuclear magnetic resonance (NMR) spectroscopy, indicating that they fold autonomously and exhibit a common overall topology, characterized by a β-sandwich structure, with the N and C

termini located at opposite sides of the ellipsoid module (Norman *et al.*, 1991; Barlow *et al.*, 1992, 1993).

C1r, C1s, and the MASPs each contain a single epidermal growth factor (EGF)-like module. This small module (usually about 50 amino acids) is extremely widespread, with more than 600 different copies identified so far. It is found, often in multiple copies and associated with various other types of modules, in more than 70 different proteins, including extracellular or membrane-bound proteins involved in blood coagulation, fibrinolysis, neural development, or cell adhesion (Campbell and Bork, 1993). EGF modules are characterized at the sequence level by six conserved cysteine residues separated by segments of varying length and forming three disulfide bonds (Cys1–Cys3; Cys2–Cys4; Cys5–Cys6). A number of high-resolution structures of EGF modules have been produced by both NMR and crystallographic analyses, all indicating the presence of two antiparallel double-stranded β sheets. The overall shape is that of a small ellipsoid with its N and C termini located at opposite ends of the major axis (Bork *et al.*, 1996). The EGF modules of C1r, C1s, and the MASPs all belong to a particular subset of EGF-like modules shown to be involved in calcium binding and characterized by the particular consensus pattern Asp/Asn, Asp/Asn, Gln/Glu, Asp*/Asn*, Tyr/Phe (where asterisk indicates a β-hydroxylated residue). Similar EGF-like modules have been identified in various vitamin K-dependent plasma proteins and in fibrillin (Campbell and Bork, 1993), and details on the coordination of calcium have been obtained (Rao *et al.*, 1995).

As depicted in Fig. 3, the single EGF modules of C1r, C1s, and the MASPs are surrounded by two CUB modules. This type of module was first recognized in **C**1r/C1s, the sea urchin protein **U**egf, and the human **B**one morphogenetic protein-1, hence its name (Bork, 1991; Bork and Beckmann, 1993). It has now been identified in an increasing number of other extracellular proteins, mostly known to be involved in developmental processes. CUB modules contain approximately 110 residues and may be recognized by conserved hydrophobic and aromatic residues. Four cysteines are conserved in all CUB modules, except for the first ones in C1r, C1s, and the MASPs, and they likely form two disulfide bridges (Cys1–Cys2; Cys3–Cys4). Secondary structure predictions suggest that CUB modules have an antiparallel β-barrel fold comparable to that observed in the immunoglobulin module (Bork and Beckmann, 1993). This prediction will probably be tested experimentally shortly, as crystallisation and preliminary X-ray diffraction studies have been reported on bovine acidic seminal fluid protein, a spermadhesin consisting of a single CUB module (Dias *et al.*, 1997).

The von Willebrand factor type A (VWFA) module present in C2 and factor B is homologous to the one occurring in 3 copies in von Willebrand factor and in 1 to 12 copies in a wide range of other proteins. These include collagen types VI, VII, XII, and XIV, cartilage matrix protein and other extracellular matrix proteins, and the α subunits of certain heterodimeric integrins (LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), and CR4 (CD11c/CD18), VLA1 and VLA2) (Colombatti and Bonaldo, 1991; Perkins *et al.*, 1994). All of these proteins are involved in cell–cell, cell–matrix, or matrix–matrix interactions and their VWFA modules are believed to mediate ligand binding. Despite its large size (more than 200 residues), the VWFA module does not contain disulfide bridges, but single free Cys residues are present in C2 and factor B. The high-resolution structures of the VWFA modules from the α chains of CR3 (CD11b) and LFA-1 (CD11a) have been reported (Lee *et al.*, 1995a; Qu and Leahy, 1995). Both structures are essentially identical and conform to the classic α/β open sheet or "Rossmann fold," consisting of a central β-sheet core formed by five parallel and one short antiparallel β strands, surrounded on both faces by seven amphipathic α helices. Single Mg^{2+} or Mn^{2+} binding sites are located in CR3 and LFA-1, respectively, on the surface of the module at the top of the β sheet. Residues that coordinate through their side chains the divalent cation either directly or indirectly via H bonds to coordinating water molecules are completely conserved in all cation-binding VWFA modules. They have been called the MIDAS (**m**etal **i**on-**d**ependent **a**dhesion **s**ite) motif and include a Asp-Xxx-Ser-Xxx-Ser sequence, where Xxx can be any amino acid, and Thr and Asp residues from other parts of the polypeptide chain (Lee *et al.*, 1995a). The crystal structure reveals that the sixth coordinating site of the cation is "available," suggesting a direct role of this site in ligand binding.

Three other types of protein modules are present in factor I (Fig. 3). The N-terminal one is a factor I/membrane attack complex proteins C6/7 (FIMAC) module. In addition to factor I, this 70-residue module is only known to occur in complement proteins C6 and C7, which both exhibit two FIMAC copies at their C termini (Haefliger *et al.*, 1989). The FIMAC module is characterized by 10 conserved cysteine residues, all of which are assumed to be disulfide bridged. Its three-dimensional structure and function are unknown, although it has been suggested that it participates in protein–protein interactions in C6 and C7 (DiScipio, 1992).

The scavenger receptor Cys-rich (SRCR) module of factor I also occurs in one to four copies in diverse secreted and cell surface proteins, including the murine and bovine type I macrophage scavenger receptors, the CD5 and CD6 lymphocyte surface antigens, and the sea urchin speract receptor (Freeman *et al.*, 1990; Aruffo *et al.*, 1991). SRCR modules contain about

100 residues and are characterized by six highly conserved, disulfide-linked Cys and a number of other consensus residues with a distribution reminiscent of the consensus sequence of the immunoglobulin-like module. The factor I SRCR module is missing the fifth conserved Cys, but has an additional, nonconserved Cys residue between Cys 2 and 3. Thus, like other SRCR modules, that of factor I probably has three disulfide bridges.

Factor I also contains two contiguous low-density lipoprotein receptor class A (LDLRA) modules. These small modules (about 40 residues) were first identified in the LDL receptor (Südhof *et al.*, 1985) and in C9 (DiScipio *et al.*, 1984; Stanley *et al.*, 1985) and occur as arrays in LDL receptor and related surface receptors and in single copies in complement proteins C6, C7, C8α, C8β, and C9. LDLRA modules feature six conserved Cys and a cluster of acidic residues that is particularly conserved in the sequence Asp-Cys-Xxx-Asp-Gly-Ser-Asp-Glu that occurs at the C-terminal end. The conserved negatively charged residues of the module in the LDL receptor are thought to bind to closely spaced, positively charged residues of apoprotein E, a high-affinity ligand for the receptor. The structure of the first N-terminal LDLRA module from the human LDL receptor has already been determined (Daly *et al.*, 1995), indicating that many of the conserved acidic residues are located at one side of the molecule, providing support to the hypothesis that they are involved in a binding function.

IV. Structure and Function of Complement Proteases

A. Factor D

Factor D is the smallest and least abundant complement protein. It is similar to pancreatic serine proteases and has no additional domains or protein modules. The gene encoding human factor D is located on chromosome 19 (F. S. Rosen, personal communication), but its structure has not been reported. However, the structure of the gene for murine factor D, also termed adipsin, has been determined. It spans about 1.7 kb of DNA on murine chromosome 10, contains five exons, and has an organization similar to that of the gene encoding trypsin (Min and Spiegelman, 1986). Factor D catalyzes the cleavage of C3b-bound factor B, leading to the formation of the alternative pathway C3-convertase, C3bBb. It differs from other serine proteases in blood in that it apparently requires neither enzymatic cleavage for expression of proteolytic activity nor inactivation by a serpin-type inhibitor for its control. Instead, transition of factor D from the catalytically inactive to the active state seems to be mediated by fully reversible conformational changes, probably induced by its only natural substrate, C3bB. An initial understanding of the structural correlates

of these unusual features has emerged from structural and mutational studies (Volanakis and Narayana, 1996).

1. Primary and Tertiary Structure

The almost complete primary structure of factor D was initially determined at the protein level (Johnson *et al.*, 1984; Niemann *et al.*, 1984). More recently, the complete amino acid sequence was deduced from the nucleotide sequence of cDNA clones (White *et al.*, 1992). The single polypeptide chain of the mature protein consists of 228 amino acid residues with a calculated M_r of 24,376. No potential *N*-glycosylation sites are present in the amino acid sequence and no carbohydrate is detected in the purified protein (Tomana *et al.*, 1985). The primary structure shows 33–35% residue identity with pancreatic bovine trypsin, bovine chymotrypsin A, porcine elastase, and human neutrophil elastase (Volanakis and Narayana, 1996). Alignment of the factor D sequence with that of 35 other mammalian serine proteases (Greer, 1990) demonstrated that the majority of the invariable and conserved residues of the chymotrypsin-like family have been retained in factor D. In addition, four disulfide bridges that are highly conserved among serine proteases are also present in factor D (Narayana *et al.*, 1994). They form between half-cystine residues 42 and 58 ("histidine loop"), 168 and 182 ("methionine loop"), 191 and 220 ("bridge between primary and secondary substrate binding sites"), and 136 and 201 ("B–C chain bridge").

The factor D cDNA encodes the mature polypeptide chain preceded by a putative leader peptide and an additional seven residue sequence, AAPPRGR, which could represent an activation peptide. However, factor D purified from serum or urine of patients with Fanconi's syndrome (Volanakis and Macon, 1987) lacks an activation peptide, its N-terminal sequence, ILGG-, corresponding to the highly conserved one of active chymotrypsin-like serine proteases (Lesavre and Müller-Eberhard, 1978; Volanakis *et al.*, 1980). The discrepancy between the cDNA-encoded and the native factor D in serum was solved by the demonstration that expression of factor D cDNA in insect cells, using a baculovirus expression system, resulted in secretion of DFP-resistant, proteolytically inactive pro-factor D, consisting of two molecular forms with respective activation peptides AAPPRGR and APPRGR (Yamauchi *et al.*, 1994). Recombinant pro-factor D could be converted to its enzymatically active form by brief incubation with catalytic amounts of trypsin. Trypsin-activated pro-factor D exhibited the same N terminus and proteolytic activity as native serum factor D. No autoactivation of the zymogen was observed under experimental conditions favorable to its activation by trypsin. In addition, incubation of pro-factor D with an activator of the alternative pathway of complement

activation in the presence of human serum-depleted of factor D did not result in complement activation. Thus, no pro-factor D activating enzyme is present in human serum (Yamauchi *et al.*, 1994). Furthermore, expression of the same factor D cDNA in COS or CHO cells resulted in secretion of the serum form of the enzyme (Kim *et al.*, 1994). Taken together these data support the hypothesis that the activation peptide of pro-factor D is cleaved off within the secretory pathway of mammalian cells by a trypsin-like maturase or convertase (Vernet *et al.*, 1990). This is unlike other mammalian serine proteases and is similar to many prohormones and other proproteins that are also converted to their active forms before they are secreted (Steiner *et al.*, 1992). Insect cells are apparently deficient in the putative pro-factor D activating enzyme.

The crystal structure of factor D was solved by using a combination of multiple isomorphous replacement and molecular replacement methods (Narayana *et al.*, 1994). Two molecules, A and B, related by a noncrystallographic twofold axis are present in the triclinic unit cell. They are very similar to each other, but display distinct orientations of the side chains of a few key residues in their active centers. As expected from the conserved serine protease primary structure, both molecules of factor D have the characteristic chymotrypsin structural fold (Fig. 4, see color plate). The only major differences in backbone structure occur in surface loops connecting secondary structural elements. Despite its typical serine protease three-dimensional structure, factor D displays unique conformations of key catalytic and substrate-binding residues. These atypical structural features include residues of the catalytic triad, the substrate specificity pocket, and the nonspecific substrate-binding site of factor D.

Available high-resolution structures of several serine proteases have demonstrated that in the chymotrypsin family the spatial relationships among the side chains of the three residues of the catalytic triad, Asp^{102}, His^{57}, and Ser^{195} are invariable and crucial for catalysis (Perona and Craik, 1995). Nevertheless, in factor D the canonical orientation of the side chains of the catalytic triad residues has not been conserved (Fig. 5). In molecule A, the carboxyl of Asp^{102} is pointed away from His^{57} and is freely accessible to the solvent, whereas the geometry of the His^{57}–Ser^{195} dyad is identical to that of trypsin. In molecule B, the imidazolium of His^{57} is oriented away from Ser^{195}, having assumed the energetically favored *trans* conformation. The space filled in other serine proteases by the side chain of His^{57} is occupied in molecule B by Ser^{215}. A H bond formed between Ser^{215} and Asp^{102} contributes to the positioning of the side chain of Asp^{102} of molecule B in a typical serine protease orientation. In contrast, in molecule A, Ser^{215} is located inside the primary specificity pocket forming a H bond with Arg^{218}. Assuming that forms A and B are alternative conformations of factor

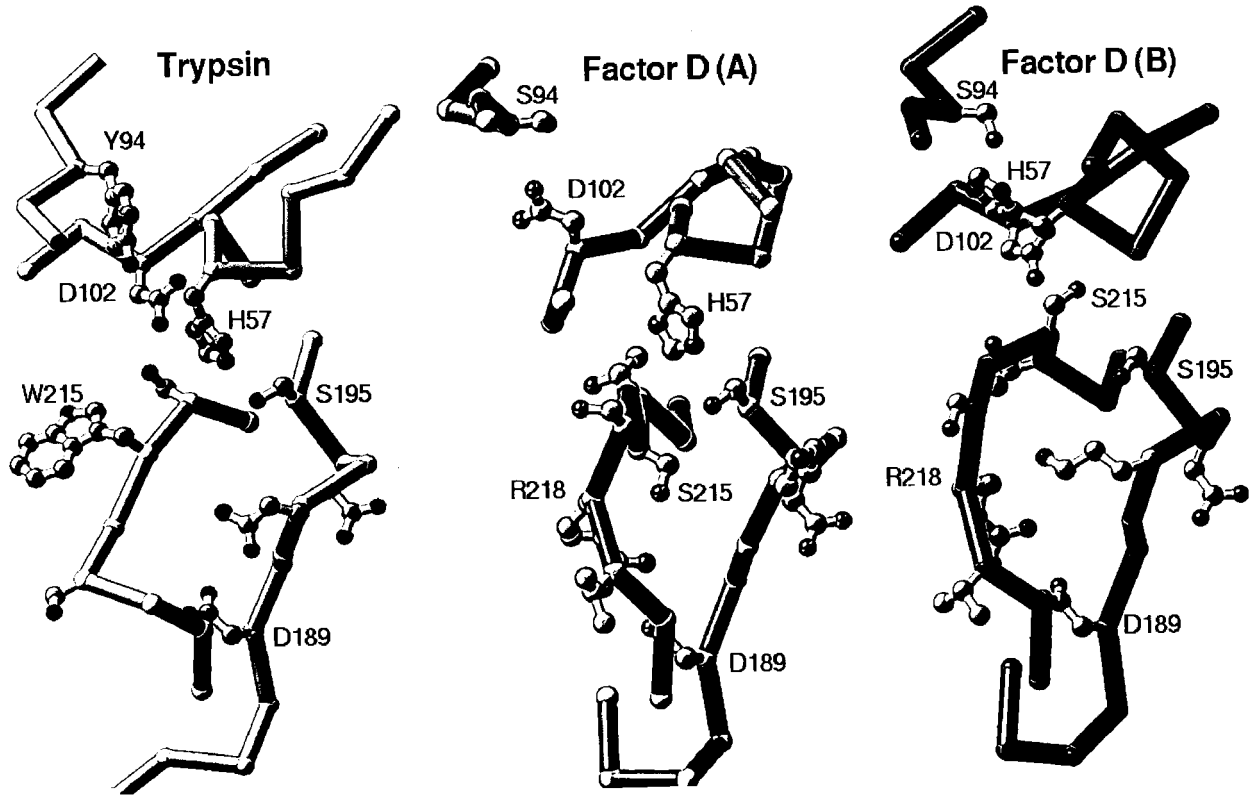


FIG. 5. Comparison of active center residues of trypsin, factor D molecule A, and factor D molecule B.

D, residues Thr^{214} and Gly^{216} appear to act as hinges, allowing Ser^{215} to swing in and out of the specificity pocket, thus forcing His^{57} to assume a *cis* or *trans* conformation, respectively. Neither of these two orientations of the catalytic triad residues is favorable for expression of catalytic activity, although molecule A, which has a correctly oriented His^{57}/Ser^{195} dyad, could be expected to express low-level activity. These considerations indicate the need for plasticity of the active center of factor D, allowing realignment of the catalytic triad residues via conformational changes.

Factor D cleaves only the Arg^{233}–Lys^{234} bond of its natural substrate factor B (Table I). Therefore, like all complement enzymes it belongs to the trypsin subfamily of chymotrypsin-like serine proteases, which as a rule have an aspartate at position 189 in the bottom of the specificity pocket. Extensive studies (Gráf *et al.*, 1988; Evnin *et al.*, 1990; Perona *et al.*, 1994) have established that the presence of a negative charge at the base of the primary specificity pocket is essential for substrate binding and catalysis by trypsin. Factor D also has an aspartate at position 189 (Figs. 2 and 5), but in both molecules A and B, Asp^{189} forms a salt bridge with Arg^{218}. This salt link probably restricts access of the side chain of the P_1 residue Arg^{233} of factor B to the negative charge of Asp^{189}. Thus, a conformational change leading to reorientation of Arg^{218} away from Asp^{189} seems necessary for optimal substrate binding and catalysis.

Superposition of the specificity pockets of factor D and trypsin (Kim *et al.*, 1995a) reveals that two of the walls formed by residues 189–195 and 225–228 have very similar backbone conformations in the two enzymes. However, the conformation of the third wall of the pocket of factor D, which is formed by residues 214–220, is considerably different from that of trypsin. Residues 214 and 215 of factor D are Thr and Ser, respectively, compared to the highly conserved Ser and Trp in trypsin. Residue 218 is Arg in factor D and Gly in trypsin and Val^{219} of factor D is missing from trypsin (Figs. 2 and 5). Compared to trypsin, the loop formed by residues 214–219 of factor D is substantially raised toward the solvent with the Arg^{218}–Asp^{189} salt bridge acting as a tether. This positioning results in considerable narrowing of the specificity pocket, which is illustrated by the crystal structure of complexes between factor D and its irreversible inhibitor 2,4-dichloroisocoumarin. Figure 6 (see color plate) shows a top view of the solvent-accessible surface of the complex, centered on the pocket. The left wall of the pocket is substantially raised toward the solvent and partially obstructs the opening of the pocket. The orientation of the 214–219 loop of factor D has additional implications for substrate binding because it results in a backbone conformation of Gly^{216} quite different from that of trypsin. As noted, Gly^{216} is an essential element of the nonspecific substrate-binding site, which forms a short antiparallel β sheet with residues P_1 to P_3 of the

substrate. Indeed, Gly^{216} is a crucial structural determinant of the correct positioning of the substrate scissile bond. The unusual disposition of these residues in factor D suggests that reorientation of loop 214–219 is necessary for efficient substrate binding and catalysis by factor D.

2. *Expression and Regulation of Catalytic Activity*

Results of active site mapping of factor D with peptide thioester substrates (Kam *et al.*, 1987) support the concept that the atypical conformation of key catalytic and substrate-binding residues of factor D are not compatible with the expression of high-level catalytic activity. A series of peptide thioesters, containing an Arg at the P_1 position and various groups and amino acids at P_2, P_3, P_4, and P_5, were used to investigate the specificity and reactivity of factor D. Of 12 dipeptides tested, only 3 containing Arg, Val, or Lys in the P_2 position were hydrolyzed at measurable rates. Lys is also present at the P_2 position of factor B (Table I). Z-Lys-SBzl was also shown to be cleaved by factor D (Kim *et al.*, 1994), indicating that, like trypsin, the P_1 site is reactive with both Arg and Lys residues. Extension of the peptide thioesters to include Gln at the P_3 and P_4 positions, which are also occupied by Gln in factor B, resulted in complete loss of reactivity. Thus, neither Bz-Gln-Lys-Arg-SBzl nor Bz-Gln-Gln-Lys-Arg-SBzl were hydrolyzed at measurable rates. Also, tripeptide thioesters containing four other amino acids at P_3 (Gly, Glu, Lys, Phe) did not react with factor D. In addition to this high degree of specificity, factor D exhibited an extremely low reactivity with peptide thioesters. Compared to C1s (McRae *et al.*, 1981), its functional homolog in the classical pathway, the reactivity (k_{cat}/K_m) of factor D was two to three orders of magnitude lower. Compared to trypsin, factor D was as much as 3×10^3 less efficient in hydrolyzing thioesters.

The reactivity of factor D with isocoumarins substituted with basic groups was similarly low (Kam *et al.*, 1992). These compounds are very effective inhibitors of trypsin-like serine proteases of the blood coagulation cascade (Kam *et al.*, 1988). Their mechanism of action involves the initial formation of an acyl-enzyme. By comparison to C1s, the inhibitory rates of factor D by its best inhibitors were three orders of magnitude lower. The inhibitory rates obtained with the best inhibitors of trypsin and coagulation serine proteases (Kam *et al.*, 1988) were five orders of magnitude higher than those measured for factor D.

The low reactivity of factor D with synthetic substrates and active site inhibitors is consistent with the atypical three-dimensional structure of its active center and supports the notion of an inactive conformation of resting-state factor D. Mutational analyses have identified structural determinants responsible for some of the unique features of the active center and the

resulting low esterolytic activity of factor D. Replacement of three residues, Ser^{94}, Thr^{214}, and Ser^{215}, with Tyr, Ser, and Trp, respectively, which are found in the corresponding positions of trypsin, resulted in a mutant enzyme with about 20-fold higher k_{cat}/K_m for hydrolysis of Z-Lys-SBzl (Kim *et al.*, 1995b). The increased reactivity could be accounted for by an increase in k_{cat} and could be directly attributed to a reorientation of the side chains of the catalytic triad residues. This was demonstrated by the high-resolution crystal structure of the S94Y/T214S/S215W mutant factor D, the catalytic triad of which had a typical serine protease three-dimensional structure (Kim *et al.*, 1995b).

Mutational replacement of all residues lining the primary specificity pocket of factor D with those present in trypsin did not result in increased esterolytic activity (Kim *et al.*, 1994, 1995a). This suggested that structural elements outside the pocket are important determinants of substrate specificity and reactivity. Previous studies on the substrate specificity of chymotrypsin by Gráf *et al.* (1988) arrived at a similar conclusion. It was subsequently shown that in addition to the binding pocket residues, surface loops connecting the walls of the pocket have a major effect on substrate specificity, although they do not directly come in contact with the substrate (Hedstrom *et al.*, 1992, 1994). Substituting one of these loops of factor D with the corresponding one of trypsin did not have a significant effect on esterolytic activity. However, combining this mutation with the specificity pocket mutations resulted in markedly increased reactivity with k_{cat}/K_m of about two orders of magnitude higher than wild-type factor D (Kim *et al.*, 1995a).

In conclusion, the mutational studies identified some of the structural elements responsible for the unusual active center conformation and the extremely low esterolytic activity of resting-state factor D. They include unique residues lining the specificity pocket (Lys^{192}, Thr^{214}, Ser^{215}, Arg^{218}, and Val^{219}), residues forming the surface loop 184–188 (Glu^{184}, Ser^{185}, Asn^{186}, Arg^{187}, Arg^{188}), and Ser^{94}. Results of esterolytic assays indicate that these residues act synergistically.

In sharp contrast to its low esterolytic activity, the proteolytic activity of factor D during activation of the alternative pathway is comparable to that of other complement enzymes. Factor D is the limiting enzyme in the activation sequence of the alternative pathway (Lesavre and Müller-Eberhard, 1978), but this is due to its low concentration in blood, which is maintained by a very fast (60% per hour) catabolic rate (Volanakis *et al.*, 1985; Pascual *et al.*, 1988). At 9–10 times physiological serum concentrations the enzyme becomes nonlimiting (Lesavre and Müller-Eberhard, 1978). The pronounced difference between the rates of hydrolysis of small synthetic amino acid and peptide thioesters and C3b-bound factor B can

be explained adequately by the proposal (Volanakis and Narayana, 1996) that the C3bB complex induces the conformational changes necessary for the realignment of the atypical active center residues of factor D. These changes probably are, at least in part, similar to those induced by the mutations discussed earlier. Native factor B and small peptide esters cannot induce these conformational changes apparently because they cannot form certain crucial contacts with the enzyme. A corollary to this hypothesis is that, following cleavage of C3b-bound factor B, the active center of factor D reverts to its resting-state inactive conformation. This mechanism and the inability of factor D to cleave uncomplexed factor B provide for the regulation of its proteolytic activity. Thus, the need for a circulating profactor D and an activating enzyme and also for a serpin-type inhibitor are obviated.

B. Factor I

Factor I cleaves three peptide bonds on the α' chain of C3b (Davis and Harrison, 1982; Medof *et al.*, 1982) and two bonds on the α' chain of C4b (Fujita *et al.*, 1978). Its action is limited to bimolecular complexes between C3b or C4b and the regulatory proteins factor H, C4b-binding protein, MCP, or CR1 (Seya *et al.*, 1995). By proteolytically cleaving C3b and C4b, factor I blocks the formation of the alternative pathway C3-convertase and of both the classical and the alternative pathway C5-convertases. It also generates C3b fragments, expressing important biologic activities. Factor I shares with factor D the property of having neither a circulating structural zymogen nor an inhibitor in the blood. Thus, like factor D, its proteolytic activity is probably regulated by reversible conformational changes.

1. Gene Organization

The gene encoding factor I (*IF*) is located on chromosome 4q25, telomeric of the gene encoding epidermal growth factor and centromeric of the interleukin 2 gene (Shiang *et al.*, 1989). It contains 13 exons and spans 63 kb of DNA (Vyse *et al.*, 1994). IF is unusual in that the first exon is small, (86 bp) and it is followed by a large 36-kb intron. There is a close association between *IF* exons and structural modules of the protein (Vyse *et al.*, 1994).

2. Protein Structure and Function

The primary structure of factor I has been determined from partial amino acid sequences (Davis, 1981; Yuan *et al.*, 1986) and from nucleotide sequences of cDNA clones (Catterall *et al.*, 1987; Goldberger *et al.*, 1987). The approximately 2.4-kb mRNA encodes a pre-pro-factor I polypeptide chain of 583 residues, which includes 18 residues apparently constituting

a leader peptide. The mature protein in blood is composed of two disulfide-linked polypeptide chains, heavy and light, of 50 and 38 kDa, respectively. The light chain consists of a serine protease domain. The two-chain structure is derived from the 565-residue pro-factor I single polypeptide chain by proteolytic removal of four basic amino acids, Arg-Arg-Lys-Arg, linking the two chains, which takes place intracellularly within the secretory pathway. Similar intracellular processing, involving the proteolytic excision of basic tetrapeptides by cathepsin-like enzymes, also converts pro-C3, pro-C4, and pro-C5 to the corresponding mature secreted proteins (Barnum *et al.*, 1989). In the case of factor I, the intracellular processing of the proprotein generates in the light chain a typical active serine protease N-terminal sequence, Ile-Val-Gly-Gly- (Fig. 2). It therefore corresponds to the cleavage of single peptide bonds that converts typical serine protease zymogens in blood to active two-polypeptide chain enzymes. Pro-factor I has never been identified in plasma, but it has been detected in biosynthetic studies using hepatoma cell lines and also under cell-free conditions (Goldberger *et al.*, 1984).

There are six potential *N*-glycosylation sites in the amino acid sequence of mature factor I, three on each chain. On the basis of a carbohydrate content of 27% (Goldberger *et al.*, 1984) and an approximate 23-kDa difference between the calculated size of the unglycosylated polypeptide and the one estimated for the mature protein, it seems likely that all potential sites are glycosylated.

Like most other serine proteases in blood, factor I has a modular structure. It is of interest that among complement proteins, only components of the membrane attack complex share modules with factor I. Starting from the N terminus, the heavy chain of factor I contains a factor I/membrane attack complex proteins C6/7 (FIMAC) module, a scavenger receptor Cys-rich (SRCR) module, and two low-density lipoprotein receptor class A (LDLRA) modules (Fig. 3).

The serine protease domain of factor I spans the entire length of the light chain between residues 322 and 565 of pro-factor I (Catterall *et al.*, 1987; Goldberger *et al.*, 1987). It is encoded by exons 9 to 13 of the *IF* gene, which have an organization similar to that of trypsin (Vyse *et al.*, 1994). Factor I has conserved most of the invariable amino acids of chymotrypsin-like serine proteases, including residues corresponding to Asp^{102}, His^{57}, and Ser^{195} of the catalytic triad, Asp^{189} of the specificity pocket, and Ser^{214}, Trp^{215}, and Gly^{216} of the nonspecific substrate-binding site. Overall, it exhibits the highest sequence similarity with tissue plasminogen activator (41% identical residues) and plasma kallikrein (37%). Among complement serine proteases, factor I is more similar with factor D (28% residue identity) (Goldberger *et al.*, 1987). Alignment of factor I with

serine proteases of known crystal structure (Perkins and Smith, 1993) has indicated that it has probably conserved the chymotrypsin fold. A small insertion and a small deletion occur at the protein surface and should not be expected to disturb the core β-sheet structure. The factor I serine protease domain has 11 Cys residues, 8 of which correspond to conserved serine protease disulfide bridges. In addition, Cys^{122} apparently forms a bridge with Cys^{309} of the heavy chain. This disulfide is homologous to bridges linking the A and B chains of C1r and C1s. Another disulfide bridge unique to factor I probably forms between Cys^{50} and Cys^{117}. Three potential *N*-linked glycosylation sites are present in the serine protease domain of factor I. They are all expected to be glycosylated (Goldberger *et al.*, 1987) and are located in loop regions probably exposed to the solvent (Perkins and Smith, 1993).

Factor I shares several properties with factor D. No structural zymogen and no inhibitor have been identified in blood for either enzyme. Both enzymes cleave their natural substrates only in the context of bimolecular complexes with cofactor proteins. Factor D has very low esterolytic activity (Kam *et al.*, 1987), and no reactive substrate was identified for factor I among 50 peptide thioesters examined (Kam *et al.*, 1992). This is probably unique among secreted serine proteases, all of which have been shown to hydrolyze thioester substrates, albeit with widely variable efficiencies. Finally, compared to trypsin and coagulation serine proteases (Kam *et al.*, 1988), the rates of inhibition of factor D and factor I by substituted isocoumarins were very low (Kam *et al.*, 1992). These structural and functional similarities have led to the suggestion that the proteolytic activities of both enzymes are regulated by similar mechanisms. The structural evidence supporting a reversible substrate-induced conformation of factor D, associated with expression of proteolytic activity, was reviewed earlier. A similar mechanism seems possible for factor I, although no structural evidence exists for an inactive, "resting-state" conformation of its active center. It is, however, relevant that diisopropylfluorophosphate, a mechanism-based inhibitor of all serine proteases, binds to and inactivates factor I only in the presence of C3b (Nilsson-Ekdahl *et al.*, 1990). This finding provides indirect evidence for a C3b-induced realignment of the catalytic center of factor I.

C. Factor B and C2

Factor B and C2 provide the catalytic subunits of the C3- and C5-convertases in the alternative and classical pathway, respectively. They are structurally and functionally similar single-chain glycoproteins that probably represent gene duplication products.

1. Gene Organization

Human factor B and C2 are encoded by single genes that are located in the class III region of the major histocompatibility complex on chromosome 6 (Carroll *et al.*, 1984). The 5′ end of the *C2* gene lies approximately 600 kb centromeric of the 5′ end of the HLA-B gene (Dunham *et al.*, 1987; Carroll *et al.*, 1987). The factor B gene (*Bf*) lies centromeric to the *C2* gene and has the same transcriptional orientation. The cap site of *Bf* is located only 421 bp downstream of the poly(A) signal of C2 (Wu *et al.*, 1987). This proximity is compensated for by the presence of a termination sequence or polymerase II "pause" site, which contains a GGGGGA direct repeat and is located 44 bp downstream of the C2 poly(A) site (Ashfield *et al.*, 1991). The nuclear factor MAZ binds to this site, contributing to termination from the *C2* gene and ensuring unimpeded initiation from the *Bf* promoter (Bossone *et al.*, 1992).

The *C2* and *Bf* genes contain 18 exons each and have very similar exon/intron organizations (Campbell and Bentley, 1985; Ishii *et al.*, 1993). The principal difference between the two genes is in their size: the *C2* gene occupies 18 kb of DNA as compared to 6 kb for *Bf*. The difference is due to the large size of some introns of the *C2* gene. The largest intron of *C2* follows exon 3 and contains a human-specific (Zhu *et al.*, 1994) SINE-type retroposon, derived from the human endogenous retrovirus HERV-K10 (Zhu *et al.*, 1992). This retroposon, termed SINE-R.C2, is associated with a variable number of tandem repeats (VNTR) locus, which gives rise to a multiallelic RFLP of the *C2* gene (Zhu and Volanakis, 1990). Several dimorphic RFLPs have also been described for the *C2* gene, whereas *Bf* is considerably less polymorphic (Cross *et al.*, 1985).

2. Protein Structure and Function

The complete primary structures of factor B (Mole *et al.*, 1984; Horiuchi *et al.*, 1993) and C2 (Bentley, 1986; Horiuchi *et al.*, 1989) have been determined from protein and/or cDNA sequencing. Factor B consists of 739 residues with a calculated M_r of 83,000 and C2 of 732 residues with a M_r of 81,000. The two polypeptides exhibit 39% amino acid residue identity. Factor B contains four sites for potential *N*-linked glycosylation, whereas C2 contains eight sites. The carbohydrate content of the two proteins, 8.6 and 15.9% for factor B and C2, respectively, indicates that all sites are occupied by oligosaccharides (Tomana *et al.*, 1985). The difference in glycosylation is probably responsible for the difference in molecular mass between the two mature proteins (factor B, 90 kDa; C2, 102 kDa).

a. The Modular Structure of Factor B and C2. Factor B and C2 have similar modular structures. Each polypeptide consists of, from the

N terminus, three CCP modules, a short connecting segment, a single VWFA module, a second short connecting segment, and a serine protease domain (Fig. 3). Transmission electron micrographs of factor B and C2 have shown similar three-lobed structures for both proteins (Smith *et al.*, 1984; Ueda *et al.*, 1987). In the assembled bimolecular C3-convertases, Bb and C2a were visualized as two-lobed structures bound to C3b and C4b, respectively, through a single lobe (Smith *et al.*, 1984). Apparently, the third lobe of each intact protein corresponds to the N-terminal fragment, Ba and C2b, which are cleaved off by the action of factor D and C1s, respectively. In addition to the three CCP modules and the connecting segment of their parent proteins, Ba and C2b also contain the seven N-terminal residues of the VWFA module.

The three CCP modules of factor B and C2 extend from the N terminus to residues 195 and 186, respectively. In both proteins each CCP module is encoded by a single exon and consists of 58–74 residues. The CCP/VWFA connecting segments contain 32 residues in factor B and 30 in C2. In both genes they are encoded by exon 5, which in addition encodes the 2–3 N-terminal residues of the VWFA modules. It is of interest that the S189F mutation within the connecting segment of C2 causes type II C2 deficiency, characterized by a block in C2 secretion (Wetsel *et al.*, 1996). It is of further interest that another mutation, G444R, which also causes type II C2 deficiency, is within the VWFA/serine protease domain connecting segment (Wetsel *et al.*, 1996). It thus appears that, at least in C2, the two connecting segments play a significant role in the overall folding and/or conformation of the polypeptide.

The VWFA modules of C2 and factor B extend from residues 217 to 432 and from 228 to 443, respectively. They are encoded by exons 6 to 10 of their respective genes. The metal ion-binding "MIDAS motif" that was described in the VWFA module of integrins (Lee *et al.*, 1995a) has been maintained in factor B and C2, both of which require Mg^{2+} for ligand binding. The putative Mg^{2+}-coordinating residues of factor B are Asp^{251}, Ser^{253}, Ser^{255}, Thr^{328}, and Asp^{364} and those of C2 are Asp^{240}, Ser^{242}, Ser^{244}, Thr^{315}, and Asp^{356}. Homology modeling of the factor B VWFA module (Fig. 7, see color plate), on the basis of the CR3 crystal coordinates, indicated that the overall structural folds of the two modules are quite similar (Tuckwell *et al.*, 1997). As expected, differences were mainly noted in the length of surface loops connecting secondary structural elements. As shown in Fig. 7, the loop connecting βD and α5 was substantially longer in factor B due to extension of helix α5 at its N terminus. Two other differences were the increased lengths of loops α3–α4 and α5–βE. The model of the factor B VWFA module and particularly its unique structural features were used as guidelines in a study on the ligand-binding site of factor B.

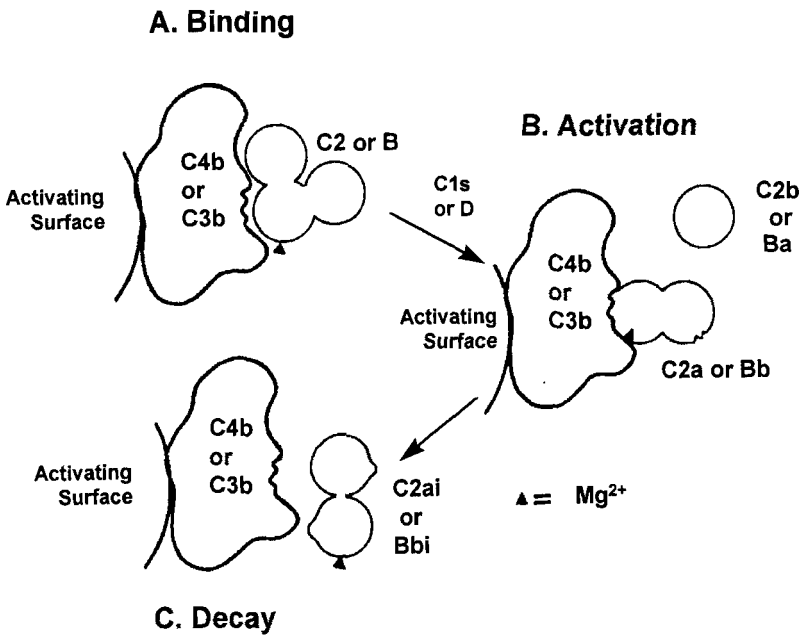


FIG. 8. Proposed model for the assembly and dissociation/decay of the C3-convertases in the classical and alternative pathways of complement activation. In the lectin pathway the C3-convertase is identical to the one formed in the classical pathway.

A hypothetical model (Fig. 8) for the assembly of C3-convertases has been proposed. It is based on previously presented experimental evidence and has been further supported by mutagenesis studies on factor B and C2. The model proposes that initial binding of factor B or C2 to activator-attached C3b or C4b, respectively, is mediated by two low-affinity sites, one on Ba or C2b and the other on the VWFA modules (Ueda *et al.*, 1987; Pryzdial and Isenman, 1987; Oglesby *et al.*, 1988; Volanakis, 1990). Mg^{2+} apparently acts as an allosteric effector of the site on the VWFA module (Fishelson *et al.*, 1983). The model also proposes that cleavage of factor B or C2 by factor D or C1s, respectively, induces a transient conformational change in the latter site, resulting in increased binding avidity for C3b or C4b, sequestration of Mg^{2+}, and expression of proteolytic activity for C3.

b. Ligand-Binding Sites. Initial evidence for the presence of a C4b-binding site on C2b was provided by the observation of a noncovalently linked C4b2b complex after C1s cleavage of mixtures of C4 and C2 (Nagasawa and Stroud, 1977; Kerr, 1980). In addition, it was shown (Nagasawa *et al.*, 1985) that purified C2b accelerated the dissociation of the C4b2a

complex. Further evidence for a C4b-binding site was subsequently provided by the demonstration that anti-C2b monoclonal antibodies (mAbs) inhibited binding of C2 to C4b (Oglesby *et al.*, 1988). Similar to C2b, evidence that Ba contains a C3b-binding site was also provided by the demonstration (Ueda *et al.*, 1987) that anti-Ba mAbs inhibited binding of factor B to C3b. Additionally it was shown that Ba could inhibit the formation of both the fluid-phase and the solid-phase C3bB complex. Indeed, specific binding of Ba to C3b, in a metal ion-independent fashion, was demonstrated using a cross-linking reagent (Pryzdial and Isenman, 1987).

More recently, two groups of investigators working independently provided more insights into the topology of the ligand-binding sites on the CCP modules of C2b and Ba through similar experimental approaches. Using a panel of C2/factor B chimeras, in which intact or partial CCP modules of factor B were substituted for the corresponding ones of C2 (Fig. 9), the epitopes of two inhibitory anti-C2b and anti-Ba mAbs were mapped on the second CCPs of C2 and factor B, respectively (Xu and Volanakis, 1997). Substitution of each intact CCP module of C2 resulted in substantial loss of hemolytic activity. It is of interest that chimeras containing the third CCP of factor B were resistant to C1s cleavage, probably due to subtle conformational changes of the region around the

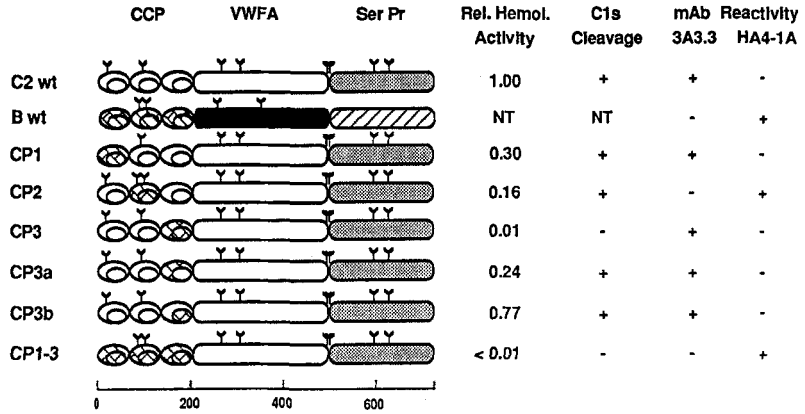


FIG. 9. Schematic representation of the modular structure and functional analysis of C2, factor B, and C2/B chimeras. CCP, complement control protein module; VWFA, von Willebrand factor type A module; Ser Pr, serine protease domain. Potential *N*-linked glycosylation sites are indicated by Y. C2/B chimeras are referred to by the number of the substituted CCP module(s); a and b indicate partial CCP3 chimeras. The scale at the bottom is for the amino acid residue number. NT, not tested; mAb 3A3.3 reacts with C2b and mAb HA4-1A with B. Modified from Xu and Volanakis (1997).

scissile peptide bond. Two partial CCP3 chimeras could be cleaved by C1s and one of them, involving substitution of residues 137 to 143 of C2, had substantially decreased hemolytic activity. The results indicate that all three CCP modules of C2 contribute structural elements to the C4b-binding site of C2b. A similar experimental approach was utilized to identify the topology of the C3b-binding site on Ba. A panel of factor B CCP mutants, in which small amino acid segments (4 to 10 residues) spanning all three CCPs of C2 were substituted for the corresponding ones of factor B, was constructed (Hourcade *et al.*, 1995). Functional analysis of the mutant panel revealed that two factor B regions were essential for C3b binding and hemolytic activity, one on the carboxyl end of the second CCP and the other in the first intercysteine region of the third CCP. Moreover, the residues Pro^{146} and Val^{152} of the second site seemed to be critical for the C3b–B interaction. Interestingly, as mentioned earlier, the homologous region of C2, i.e., the ^{137}SLGAVRT^{143} segment of the third CCP, was also involved in the C4b–C2 interaction. Taken together, ligand-binding data are in agreement with previous ones on complement receptors and regulatory proteins containing CCP modules, which have indicated that binding sites for fragments of C3 and C4 extend over two to four contiguous CCP modules (Ahearn and Fearon, 1989; Liszewski *et al.*, 1996).

Indirect evidence for the presence of a C4b-binding site on the VWFA module of C2 was initially provided by studies on the oxidation of C2 by I_2, which results in a longer half-life of the C4b2a convertase apparently due to increased binding avidity of C2a for C4b (Polley and Müller-Eberhard, 1967). The effect of I_2 was directly attributed to oxidation of the free thiol of Cys^{241} at the N-terminal region of VWFA (Parkes *et al.*, 1983). Oxidation does not result in incorporation of I_2 into C2 and no other free thiol is available for the formation of a disulfide bond. Thus, the most likely mechanism involves the formation of a sulfenyl iodide group. The latter becomes susceptible to nucleophilic attack by another amino acid side chain, replacing the iodide and forming an intramolecular covalent bond. Such mechanism would presumably result in the removal of the thiol from the surface of the molecule, a change that apparently allows the C4b-binding site to assume a conformation more favorable for interaction with C4b. Site-directed mutagenesis studies of that region of C2 demonstrated that substitution of Leu or Ala for Asp^{240} or Ser^{244}, respectively, resulted in more than a 100-fold decrease of C2 activity (Horiuchi *et al.*, 1991). Asp^{240} and Ser^{244} are two of the five invariable residues of the MIDAS Mg^{2+}-binding motif of C2 (Lee *et al.*, 1995a). Thus, these data directly implicated the putative Mg^{2+}-binding site of C2 in C4b binding. With respect to the VWFA module of factor B, indirect evidence for the presence of a C3b-binding site was originally provided

by the demonstration that the module contains the Mg^{2+}-binding site of factor B (Sánchez-Corral *et al.*, 1990). It was subsequently shown that alanine mutations of residues Asp^{251} and Ser^{255} of the MIDAS motif led to almost complete abrogation of factor B activity (Hourcade *et al.*, 1995). More recently, based on homology modeling of the factor B VWFA module, a series of chimeras were constructed by substituting surface loops surrounding the putative Mg^{2+}-binding site of the C2 VWFA module for the corresponding ones of factor B (Tuckwell *et al.*, 1997). Functional analysis of the chimeras indicated that the loops connecting the βA strand to the α1 helix (^{251}DGSDSIGASNFT^{262}, where underlining indicates Mg^{2+}-coordinating residues) and the βD strand to the α5 helix (^{364}DGLHNMGGDP^{373}) define regions participating in C3b binding. Enhancement of cobra venom factor binding after factor D cleavage was absent in the chimera involving replacement of the βA-α1 loop. The results suggested that the βA–α1 loop mediates the conformational regulation of the affinity of Bb for C3b. This finding is supported by the observation that oxidation of Cys^{241} in the homologous loop of C2 resulted in increased stability of the C4b2a complex. The presence of ligand-binding sites in the βA–α1 and βD–α5 loops was not only observed in the factor B VWFA module, but also in the corresponding loops of integrin αM and αL subunits and of von Willebrand factor (Michishita *et al.*, 1993; Edwards *et al.*, 1995; Kamata *et al.*, 1995; Lee *et al.*, 1995b).

c. Catalytic Activities. The serine protease domains of factor B and C2 extend from residues 457 and 447, respectively, to the C terminus. They are encoded by exons 11 to 18 of their respective genes. Exon 11 also encodes short connecting segments of 13 and 14 residues in factor B and C2, respectively, and exon 18 also encodes 3′-untranslated segments. The serine protease domains have conserved the main structural elements of the chymotrypsin-like family, including the catalytic triad and substrate-binding sites residues, except for the already mentioned absence of a negatively charged aspartate at position 189. This residue determines the substrate specificity for Arg and/or Lys peptide bonds of serine proteases with trypsin-like specificity. Alignment of factor B and C2 sequences with those of nine serine proteases of known high-resolution crystal structure (Fig. 2) suggested that they have probably also maintained the chymotrypsin structural fold (Perkins and Smith, 1993). However, factor B and C2 exhibit several differences from typical serine proteases, which probably subserve their highly specialized functions. One of their major unique features is the absence of the highly conserved N-terminal sequence that is generated during zymogen activation (Fig. 2). In other serine proteases

the positively charged α-N terminus folds into the interior of the protein and forms a salt bridge with the negatively charged carboxyl group of Asp^{194}. This molecular movement is accompanied by a rearrangement of the specificity pocket and expression of catalytic activity (Stroud *et al.*, 1975). In factor B and C2, cleavage leading to zymogen activation occurs at a distant site from the serine protease domain, which remains attached to the VWFA module. Thus, assumption of the active conformation must be achieved through a different mechanism. Additional major differences from canonical serine proteases include 12–13 residue insertions following residues 146 and 170 (Fig. 2). Comparison to serine proteases of known crystal structure indicates that both insertions are probably located at the protein surface and therefore they should not disturb the core antiparallel β-sheet structure (Perkins and Smith, 1993). Of the three walls of the substrate specificity pocket, the third ones of factor B and C2, which are presumably formed by residues 225–228, show striking amino acid sequence differences from other serine proteases. Most impressive are substitutions of Lys and Asn in factor B and C2, respectively, for the highly conserved Gly^{226} of typical serine proteases. It would be of interest to investigate the effect of the substitutions at this region on the specificity of C2 and factor B for C3 and C5.

Comparison to typical serine proteases indicates that factor B and C2 have maintained three of the highly conserved disulfide bridges. They form between Cys residues 42 and 58, 168 and 182, and 191 and 220 (Fig. 2). Two additional disulfide bridges are present. The first between Cys^{122} and the Cys corresponding to position 3 of chymotrypsinogen apparently forms between the serine protease domain and the VWFA/serine protease domain-linking segment. It is homologous to the disulfide bridge linking the A and B chains of C1r, C1s, and MASPs and the heavy and light chains of factor I. The final disulfide bridge between Cys^{125} and Cys^{139} appears to be unique to factor B and C2 and probably requires a structural rearrangement of the molecule to be formed (Perkins and Smith, 1993). The factor B serine protease domain has no *N*-linked glycosylation sites, whereas that of C2 has two. Two of them occur at residues 16 and 20, i.e., in the region forming the N terminus salt bridge with Asp^{194} in other serine proteases, again indicating a different conformation of this region of C2.

Full expression of the proteolytic activities of factor B and C2 only occurs in the context of the C3/C5-convertase complexes. A k_{cat}/K_m of 3.1 x 10^5 sec^{-1} M^{-1} was measured for cleavage of C3 by fluid-phase C3bBb, whereas the initiation convertase $C3_{H2O}Bb$ was slightly less efficient with k_{cat}/K_m of 1.6×10^5 sec^{-1} M^{-1} (Pangburn and Müller-Eberhard, 1986). For the classical pathway C3-convertase, C4b2a, a K_m of 1.8×10^{-6} M has been measured for C3 cleavage (Cooper, 1975). This is in the same range as

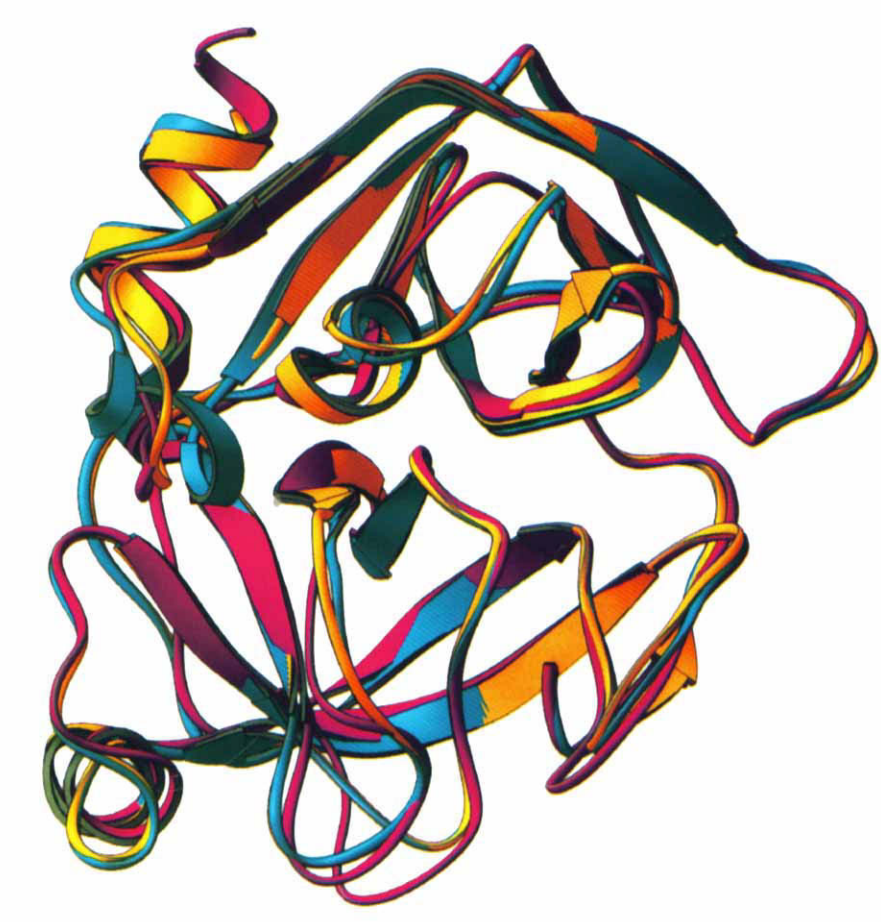
the K_m of 5.7×10^{-6} *M* measured for C3bBb (Pangburn and Müller-Eberhard, 1986). Uncomplexed Bb retains about 1% of the proteolytic activity of its C3b-complexed counterpart (Fishelson and Müller-Eberhard, 1984). In addition, low-level proteolytic activity has also been demonstrated for a 33-kDa fragment containing the serine protease domain of factor B (Lambris and Müller-Eberhard, 1984). Cleavage of C3 by this fragment is Mg^{2+} independent but is enhanced by the presence of C3b (Sánchez-Corral *et al.*, 1990).

Factor B and C2, as well as their fragments Bb and C2a, express esterolytic activity (Cooper, 1975; Ikari *et al.*, 1983) and their active sites have been mapped with peptide thioester substrates (Kam *et al.*, 1987). All synthetic substrates reactive with C2 contained the tripeptide sequences Leu-Ala-Arg or Leu-Gly-Arg, which correspond to the cleavage sites of C3 and C5, respectively (Table I). Tetrapeptide thioesters were better substrates for C2 than either tri- or pentapeptides whereas dipeptides were not hydrolyzed. C2a was more reactive than C2 and it hydrolyzed the pentapeptide thioester Z-Leu-Gly-Leu-Ala-Arg-SBzl four times better than C2. The best substrate for factor B was the dipeptide Z-Lys-Arg-SBzl. Bb was about one order of magnitude more reactive than factor B and its best substrate was the tetrapeptide Z-Gly-Leu-Ala-Arg-SBzl. Overall, C3-like substrates were considerably more reactive than C5-like substrates. Using their best substrates, the k_{cat}/K_m values of C2, C2a, factor B, and Bb were 8.6×10^3, 1.2×10^4, 1.4×10^3, and 9.2×10^3 sec^{-1} M^{-1}, respectively, as compared to 7.8×10^6 sec^{-1} M^{-1} measured for the hydrolysis of the most reactive thioester by trypsin.

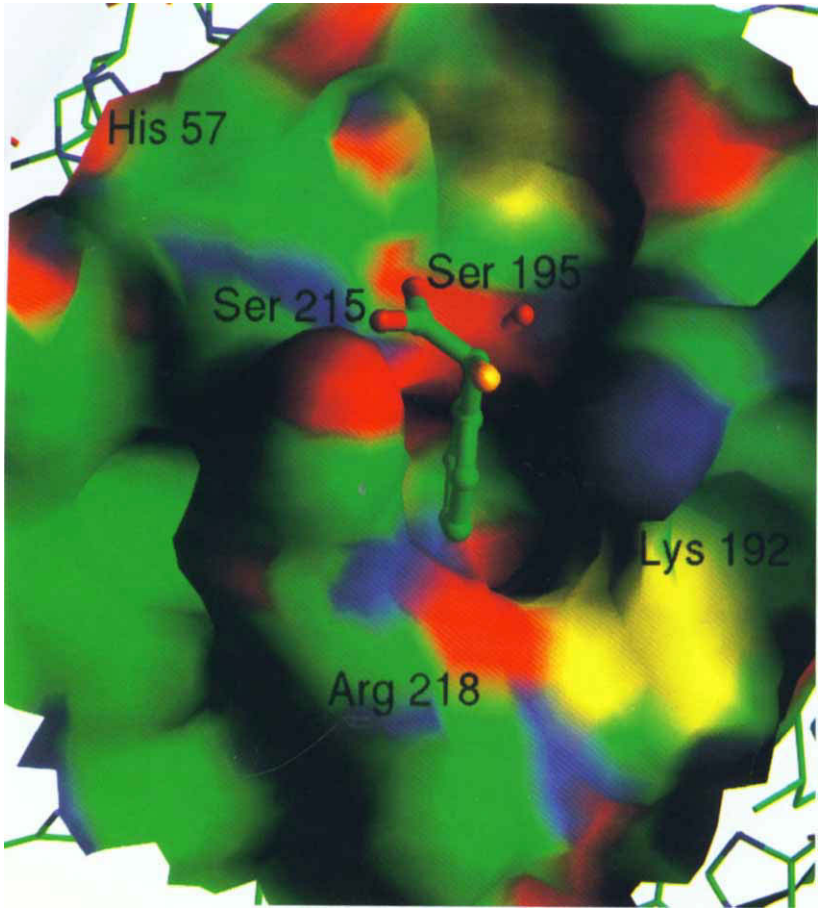
D. C1r AND C1s

1. Gene Organization

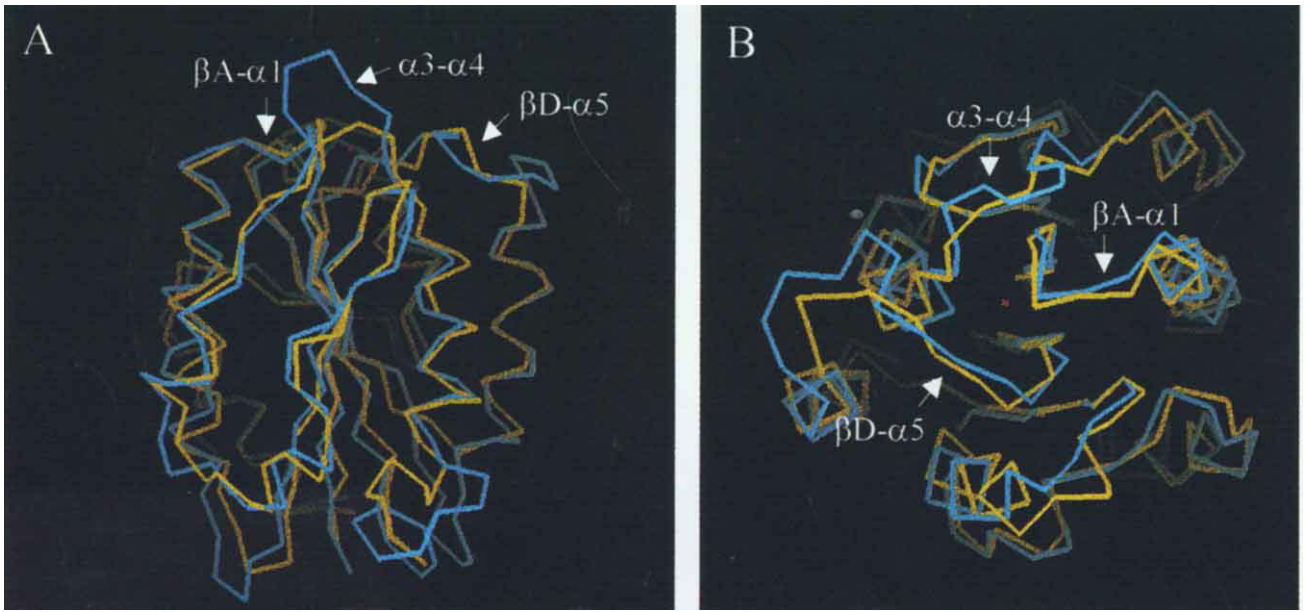
The genes encoding human C1r and C1s have been assigned to chromosome 12 and further located by *in situ* hybridization analysis in region p13 on the short arm of the chromosome (Van Cong *et al.*, 1988). The C1r and C1s genes are arranged in a tail-to-tail orientation and lie in close proximity, with a distance of about 9.5 kb between their 3′ ends (Kusumoto *et al.*, 1988). This unusual disposition supports the hypothesis of the occurrence within this short intergenic region of regulatory elements common to both genes, providing a plausible explanation for the observation that almost all of the hereditary deficiencies of C1r and C1s described so far are of the combined type (Loos and Heinz, 1986). The exon–intron structure of the human C1s gene has been mapped by electron microscopy of genomic DNA–cDNA hybrids, indicating that the protein is encoded by 12 exons (Tosi *et al.*, 1989). At variance with other known vertebrate serine proteases



CH. 4, FIG. 4. Superposition of the main chain structures of factor D molecule A (blue), factor D molecule B (yellow), and trypsin (magenta).



CH. 4, FIG. 6. Detail of the crystal structure of the complex between factor D and the irreversible inhibitor 2,4-dichloroisocoumarin. Top view showing the shallow and partially obstructed primary specificity pocket of factor D containing a covalently bound molecule of the inhibitor. Accessible surface is colored according to contributing atoms (C, green; O, red; N, blue; S,yellow).



CH. 4, FIG. 7. Superposition of Cα traces of the factor B VWFA module homology model (blue) and the integrin αM VWFA crystal structure (yellow), viewed either down the length of the β sheet (A) or from above (B). The Mg^{2+} ion of the αM module is shown as a red dot. The three loops substituted in the factor B/C2 chimeras are indicated by arrows. Reprinted with permission from Tuckwell *et al.* (1997). Copyright 1997, American Chemical Society.

in which residues of the catalytic triad are always encoded by different exons (Rogers, 1985), the C-terminal part of C1s, including a short segment encompassing the proteolytic activation site by C1r and the whole serine protease domain, is encoded by a single exon. This unusual feature is also shared by C1r (Endo *et al.*, 1996) and by haptoglobin, a serine protease homolog that lacks proteolytic activity (Tosi *et al.*, 1989). C1r, C1s, and haptoglobin exhibit additional striking similarities, both at the gene and protein level, supporting the hypothesis that these proteins may define a specific branch in the evolution of the serine protease family (Tosi *et al.*, 1989).

2. *Protein Structure and Function*

a. The Modular Structure of C1r and C1s. The complete primary structures of human C1r (Arlaud and Gagnon, 1983; Leytus *et al.*, 1986; Journet and Tosi, 1986; Arlaud *et al.*, 1987a) and C1s (Carter *et al.*, 1983; Spycher *et al.*, 1986; Mackinnon *et al.*, 1987; Tosi *et al.*, 1987) have been determined from protein and/or DNA sequencing. Both proteins are very similar with respect to overall structural organization. In their proenzyme form, C1r and C1s are single-chain glycoproteins containing 688 and 673 amino acids, respectively, and both are split upon activation, through cleavage of a single Arg-Ile bond (Table I), into the polypeptide chains A and B (the serine protease domain) that remain linked through a single disulfide bridge (Arlaud and Gagnon, 1985; Spycher *et al.*, 1986). C1r and C1s contain 12 additional disulfide bonds (10 in each A chain and 2 in each B chain). The complete disulfide bridge pattern of C1s has been determined (Hess *et al.*, 1991), but only some of these bonds have been formally identified in C1r (Arlaud *et al.*, 1987b).

The N-terminal A chains of C1r and C1s (446 and 422 amino acids, respectively) share 38% homology and exhibit the same type of modular structure, each comprising, from the C terminus, two CUB modules surrounding a single EGF-like module and a pair of contiguous CCP modules (Fig. 3). The latter are followed by a 15-residue segment homologous to the activation peptide in chymotrypsinogen, which connects the second CCP module to the serine protease domain in the proenzymes and is C-terminal to the A chain in the active proteases. This segment contains the cysteine residue involved in the disulfide bridge from the A chain to the serine protease domain. The CUB modules of C1r and C1s vary in length from 110 to 120 amino acids. The N-terminal CUB modules of each protein differ strikingly from each other with regard to their contents of charged residues, with a predominance of basic and acidic amino acids in C1r and C1s, respectively (Tosi *et al.*, 1987; Illy *et al.*, 1991). The CUB modules of C1r and C1s also exhibit different glycosylation patterns: whereas the

N-terminal C1s module is not glycosylated, one N-linked oligosaccharide is present in both C1r modules (at Asn residues 108 and 204) and in the second C1s module (at Asn residue 159). Based on mass spectrometry analyses, Asn^{159} of C1s has been shown to bear a complex-type biantennary, bisialylated oligosaccharide NeuAc2 Gal2 GlcNAc4 Man3 (Pétillot *et al.*, 1995). Although the precise structure of the carbohydrates linked to the CUB modules of C1r has not been determined, their monosaccharide contents suggest that they also belong to the complex type (Sim *et al.*, 1977).

The EGF modules of C1r and C1s contain 53 and 44 amino acids, respectively, and exhibit the consensus pattern characteristic of the particular subset of EGF-like modules involved in calcium binding (see Section III). This pattern includes an *erythro*-β-hydroxyasparagine in both C1r (at position 150) and C1s (at position 134), but hydroxylation is only partial (about 50%) in C1s (Arlaud *et al.*, 1987c; Thielens *et al.*, 1990a). In agreement with studies on other calcium-binding EGF modules (Selander-Sunnerhagen *et al.*, 1993), this posttranslational modification is probably not directly involved in calcium binding, given that recombinant C1s expressed in the baculovirus/insect cells system shows no detectable β-hydroxylation, but nevertheless retains its calcium-dependent interaction properties (Luo *et al.*, 1992). A very unusual feature of the EGF module of C1r is the large size of the loop comprised between the first two conserved cysteine residues (Cys^{129} and Cys^{144}), which contains 14 amino acids, whereas a maximum of seven residues is found in the corresponding segment of other EGF-like modules, the only other known exception being the 29th EGF module of the notch gene product from *Drosophila melanogaster* (Campbell and Bork, 1993; Wharton *et al.*, 1985). This loop also contains, at position 135, the single polymorphic site (Ser/Leu) identified in C1r from both protein and cDNA sequence analyses (Leytus *et al.*, 1986; Journet and Tosi, 1986; Arlaud *et al.*, 1987a), providing a molecular basis for the occurrence of two common alleles at the C1r locus (Kamboh and Ferrell, 1986). The EGF module of C1r (residues 123–175) has been synthesized chemically (Hernandez *et al.*, 1997) and its solution structure has been determined by NMR spectroscopy (Bersch *et al.*, 1998). The C-terminal part of the module possesses the characteristic EGF fold with a major and a minor antiparallel double-stranded β sheets, whereas the N-terminal part, including the extended loop between the first two cysteines, is disordered. The apparent flexibility of this loop and its high contents in charged residues suggest that it may participate via electrostatic contacts in module–module interactions within C1r or in protein–protein interactions within the C1 complex.

The CCP modules of C1r and C1s vary in length from 60 to 70 amino acids. There is more sequence homology between corresponding CCP

modules of C1r and C1s than between the first and second CCP modules of either protein. The second CCP module of C1s displays a large excess of negative charges and bears an *N*-linked oligosaccharide linked to Asn^{391}. Mass spectrometry analyses have revealed that this oligosaccharide belongs to the complex type and is heterogeneous, with the occurrence of a biantennary, bisialylated species (NeuAc2 Gal2 GlcNAc4 Man3), a triantennary, trisialylated species (NeuAc3 Gal3 GlcNAc5 Man3), and a fucosylated triantennary, trisialylated species (NeuAc3 Gal3 GlcNAc5 Man3 Fuc1), in relative proportions of approximately 1/1/1. This heterogeneity gives rise to three major types of C1s molecules with molecular masses of 79,318, 79,971, and 80,131 Da (Pétillot *et al.*, 1995). Three-dimensional homology models of the CCP modules of C1r and C1s have been built on the basis of the structural coordinates for the 16th and 5th CCP modules of human factor H (Rossi *et al.*, 1995; Lacroix *et al.*, 1997). In both proteins, the first and second CCP modules exhibit closer resemblance with the 16th and 5th modules of factor H, respectively. Each module shows a typical β-sandwich globular structure comprising five β strands, with significant modifications located in the C-terminal region of the modules and mostly occurring at the surface. Thus, compared to their templates, all CCP modules of C1r and C1s exhibit a major, six-residue insertion either forming a bulge or extending a preexisting bulge within one of the β strands.

The C-terminal B chains of C1r and C1s (242 and 251 amino acids, respectively) are serine protease domains that belong to the chymotrypsin-like family. They exhibit 45% sequence homology with each other and 25–35% homology with other serine protease domains. The three residues of the catalytic triad (His^{57}, Asp^{102}, and Ser^{195} in chymotrypsinogen) are surrounded by conserved sequences, although there are some exceptions (e.g., Pro^{198} in chymotrypsinogen is substituted by Val and Ala in C1r and C1s; see Fig. 2). Both C1r and C1s have an Asp residue at the S_1 subsite, indicative of trypsin-like specificity, consistent with the fact that both proteases cleave arginyl bonds in their natural protein substrates (Arlaud and Thielens, 1993). Thus, autolytic activation of proenzyme C1r and subsequent activation of C1s by active C1r both involve cleavage of Arg-Ile bonds. In the same way, active C1s cleaves a single Arg–Ala bond in C4, and a single Arg–Lys bond in C2 (Table 1). Active C1s has also been found to cleave other protein substrates, including type I and II collagens (Yamaguchi *et al.*, 1990), the major histocompatibility complex class I antigens (Eriksson and Nissen, 1990), and β_2-microglobulin (Nissen *et al.*, 1990). In this latter case, however, cleavage was shown to occur at a lysine residue. With respect to esterolytic activity, active C1r cleaves with low catalytic efficiency a restricted number of synthetic substrates containing a P_1 Arg or Lys residue. In contrast, C1s is an efficient esterase and cleaves

a wider range of substrates containing an Arg, Lys or, unexpectedly, a Tyr residue at the P_1 position, suggesting that, in addition to the anionic S_1 subsite, its specificity pocket may involve a hydrophobic component (McRae *et al.*, 1981; Arlaud and Thielens, 1993). C1r and C1s contain two of the disulfide bonds conserved in other chymotrypsin-like serine protease domains, namely the "methionine loop" and the disulfide bridge connecting the primary and secondary substrate-binding sites. In contrast, both proteases lack the "histidine loop" disulfide bridge present in all other known mammalian serine proteases (Arlaud and Gagnon, 1981). The serine protease domain of C1s bears no posttranslational modification, whereas that of C1r has two *N*-linked oligosaccharides attached to Asn residues 497 and 564, located in the regions following the His residue of the catalytic triad, and the Cys residue involved in the disulfide bridge connecting the serine protease domain to the A chain. Mass spectrometry analyses indicate that each position is occupied by heterogeneous complex-type biantennary species bearing either a single or two terminal sialic acid residues and one or no fucose residue, with a major species NeuAc2 Gal2 GlcNAc4 Man3 in both cases (Lacroix *et al.*, 1997).

Several three-dimensional homology models of the serine protease domains of C1r and C1s have been constructed, mainly using chymotrypsin as a template (Carter *et al.*, 1984; Fothergill *et al.*, 1989; Perkins and Smith, 1993; Rossi *et al.*, 1995; Lacroix *et al.*, 1997). Although C1r and C1s only exhibit 33–35% sequence identity with chymotrypsin, as much as 74–76% of their amino acids appear to be structurally conserved, as they are superposable within a limit of 1.5 Å (Rossi *et al.*, 1995; Lacroix *et al.*, 1997). Compared to chymotrypsin, the core of the C1r and C1s protease domains, including the catalytic triad, is highly conserved, whereas major modifications occur at the surface. In C1s these include two insertions of seven and eight residues forming extended loops at the entrance of the active site cleft, three deletions also occurring within the same area, and a five-residue extension at the tip of the C-terminal α helix of the protease (Rossi *et al.*, 1995). In the same way, the major insertions and all deletions in C1r occur in the vicinity of the active site cleft (Lacroix *et al.*, 1997), suggesting that in both proteases these modifications may be involved in substrate recognition. From a functional point of view, all three-dimensional models of the serine protease domains C1r and C1s are consistent with an activation mechanism involving formation of a salt bridge between the α-amino group of the N-terminal Ile of the serine protease domain and the carboxyl group of Asp^{194}, as this occurs in chymotrypsin.

b. Protein–Protein Interactions. A fundamental characteristic of C1r and C1s is that, during activation of the classical pathway, they do not

function as isolated proteases, but exert their catalytic activities within a calcium-dependent tetramer C1s-C1r-C1r-C1s, which itself binds to the nonenzymic protein C1q to form the C1 complex (Colomb *et al.*, 1984; Cooper, 1985; Arlaud *et al.*, 1987b; Schumaker *et al.*, 1987). Both proteases are therefore involved in various protein–protein interactions, both within the C1s-C1r-C1r-C1s tetramer and between the tetramer and C1q.

It is well established that the calcium-dependent C1r–C1s interactions involved in the assembly of the C1s-C1r-C1r-C1s tetramer are mediated by the N-terminal regions of the A chain of each protein (Villiers *et al.*, 1985). Both regions exhibit characteristic low-temperature transitions, with midpoints of 32°C for C1r and 37°C for C1s at physiological ionic strength. These transitions are abolished or shifted to higher temperatures in the presence of calcium ions (Busby and Ingham, 1987, 1988). Fragments C1rα (residues 1–208) and C1sα (residues 1–192) corresponding to these regions have been isolated by limited proteolysis with trypsin under controlled conditions (Thielens *et al.*, 1990b). These fragments each comprise the N-terminal CUB module, the EGF-like module, and the N-terminal disulfide loop of the second CUB module (see Fig. 3). Both fragments contain one high-affinity calcium-binding site, with K_d values of 32–38 μM, comparable to those determined for intact proteins. They also retain the ability to mediate calcium-dependent protein–protein interaction, as shown by the formation of C1rα–C1sα heterodimers, which bind two calcium atoms/mol, consistent with the fact that the C1s-C1r-C1r-C1s tetramer binds four calcium atoms/mol. Various studies performed on C1s suggest that the structural determinants required for calcium binding and calcium-dependent protein–protein interactions are contributed by both the N-terminal CUB module and the EGF module (Thielens *et al.*, 1990a; Illy *et al.*, 1991). Further experimental support for this hypothesis is provided by the observation that the isolated EGF module of C1r produced by chemical synthesis binds calcium, but with an apparent K_d of 10 mM, about 300 times higher than that measured for the whole C1rα fragment (Hernandez *et al.*, 1997). In the same way, a mutant C1r molecule deleted from the N-terminal CUB module has been expressed in a baculovirus/insect cells system and found to lack the ability to interact with C1s in the presence of calcium (Zavodszky *et al.*, 1993; Cseh *et al.*, 1996). That the N-terminal CUB module and the EGF module of C1r are indeed sufficient to mediate both calcium binding and subsequent interaction with C1s has been shown by functional characterization of the recombinant CUB–EGF module pair expressed in a baculovirus/insect cells system (N. M. Thielens, K. Enrié, M. Lacroix, A. F. Esser, and G. J. Arlaud, unpublished data). These data, along with current knowledge on other calcium-binding proteins involving EGF modules (Rao *et al.*, 1995; Sunnerhagen *et al.*, 1996;

Downing *et al.*, 1996), are consistent with the view that efficient calcium binding by C1r and C1s requires accessory structural elements located outside their EGF module. These are likely provided by the N-terminal CUB modules, which either contribute one additional ligand to complete the coordination sphere of calcium or stabilize the correct conformation of the calcium-binding site. This would result in the formation of a calcium-dependent CUB-EGF association that very likely represents the domain responsible for C1r–C1s interactions within the C1s-C1r-C1r-C1s tetramer.

C1s has the additional property to form calcium-dependent dimers in the absence of C1r. Again, this homologous C1s–C1s interaction is mediated by the N-terminal α region of the protein, but provides one extra calcium-binding site, as shown by the fact that both dimers C1s–C1s and C1sα–C1sα incorporate three calcium atoms/mol (Thielens *et al.*, 1990b). It has been suggested that this additional calcium atom may form an ion bridge between acidic residues located in between positions 20 and 64 of the N-terminal CUB module of each monomer (Illy *et al.*, 1991). In contrast, C1r does not have the ability to self-associate through its N-terminal region, but occurs naturally as a noncovalent dimer that forms the core of the C1s-C1r-C1r-C1s tetramer (Villiers *et al.*, 1985). Current knowledge on the structural determinants involved in the assembly of the C1r–C1r dimer is discussed in Section IV,D,2,c.

Less precise information is available about the sites of C1r and C1s that mediate interaction between the C1s-C1r-C1r-C1s tetramer and C1q. Indeed, assembly of the C1 complex appears to be a complex process involving multiple sites contributed by both C1r and C1s. Thus, the observations that proenzyme C1r alone binds weakly to C1q in the presence of calcium (Lakatos, 1987; Thielens *et al.*, 1994) and that the interaction between C1s-C1r-C1r-C1s and C1q is abolished by chemical modification of acidic amino acids of C1r (Illy *et al.*, 1993) provide support for a major involvement of this protein. However, the C1r–C1q interaction appears to be strengthened by fragment C1sα, as shown by the ability of the calcium-dependent C1sα-C1r-C1r-C1sα tetramer to form a stable and functional pseudo-C1 complex in the presence of C1q (Busby and Ingham, 1990; Thielens *et al.*, 1994). Based on these and other available data, a likely hypothesis is that assembly of the C1 complex involves primarily a binding site in the calcium-binding α region of C1r and that interaction with the corresponding C1sα region either induces a conformational change that enhances its affinity for C1q or provides an accessory binding site (Thielens *et al.*, 1994). Although the sites responsible for these interactions remain to be identified, it is very likely that the α regions of C1r and C1s, particularly their N-terminal CUB-EGF modules, play a major role in the interaction

with C1q and therefore represent key elements of the architecture of macromolecular C1. However, the hypothesis that the catalytic region of C1r may also participate in the interaction cannot be excluded, as the affinity of C1s-C1r-C1r-C1s for C1q is not modified on activation of C1s alone, but decreases significantly when both C1r and C1s, or C1r alone, are activated (Villiers *et al.*, 1982; Siegel and Schumaker, 1983; Lakatos, 1987). In addition to its structural role, various studies suggest that the C1rα region may participate, through a calcium-dependent intramolecular mechanism, in the regulation of autolytic activation of C1r (Thielens *et al.*, 1994).

Both solution scattering and electron microscopy studies strongly support the hypothesis that the isolated C1s-C1r-C1r-C1s tetramer has an elongated shape and folds into a more compact conformation on interaction with C1q (Tschopp *et al.*, 1980; Strang *et al.*, 1982; Boyd *et al.*, 1983; Perkins *et al.*, 1984). In agreement with other data (Siegel and Schumaker, 1983), electron micrographs of the chemically cross-linked C1 complex (Strang *et al.*, 1982) also suggest that the sites of C1q responsible for the interaction with C1s-C1r-C1r-C1s are located in the collagenous portion of the molecule, probably in the collagen-like arms. Such an interaction is featured in most of the low-resolution C1 models proposed so far (Cooper, 1985; Weiss *et al.*, 1986; Schumaker *et al.*, 1987; Arlaud *et al.*, 1987b; Perkins, 1989), in which further information may be found about current views of C1 assembly.

c. Catalytic Activities. Early studies based on limited proteolytic cleavage of C1r and C1s have allowed identification in each protease of the regions responsible for catalytic activity (Villiers *et al.*, 1985). The corresponding fragments (γ-B), derived from the C-terminal part of each protein, originate from cleavage of peptide bonds located within a short sequence stretch at the C-terminal end of the second CUB module and comprise both CCP modules, the 15-residue intermediary activation peptide, and the serine protease domain (see Fig. 3).

The γ-B fragment obtained by limited proteolysis of activated C1s with plasmin (residues 270–673) is monomeric and forms the outer portion of the C1s-C1r-C1r-C1s tetramer (Villiers *et al.*, 1985; Weiss *et al.*, 1986). Studies by differential scanning calorimetry are consistent with the occurrence in C1s γ-B of three independently folded domains, corresponding to the first and second CCP modules, and the serine protease domain (Medved *et al.*, 1989). The former unfold reversibly at about 60°C, whereas the serine protease domain melts at a lower temperature (49°C). From a functional point of view, the C1s γ-B fragment retains the catalytic properties of native activated C1s, as shown by its ability to cleave both synthetic

esters and the protein substrates C4 and C2. Studies based on baculovirus-mediated expression of truncated fragments from the C1s γ-B region have allowed insights into the functional role of its constituent modules (Rossi *et al.*, 1998). Two recombinant fragments, deleted either from the first CCP module (CCP_2-ap-SP) or from both CCP modules (ap-SP), were produced in a proenzyme form and both were cleaved by active C1r to generate enzymes expressing esterolytic activity and reactivity toward the C1 inhibitor comparable to those of native activated C1s. Likewise, both activated fragments retained C1s ability to cleave protein C2 in the fluid phase. In contrast, the C4-cleaving activity of CCP_2-ap-SP was reduced about 70-fold, whereas that of ap-SP was abolished. Thus, activation by C1r, reactivity toward the C1 inhibitor, and proteolytic activity toward C2 only involve residues located in the C-terminal part of C1s comprising the short activation peptide and the serine protease domain. In contrast, C4 cleavage requires substrate recognition sites located in both CCP modules. The latter finding is in agreement with previous observations that monoclonal antibodies directed to the γ segment of C1s inhibit its ability to cleave C4 (Matsumoto and Nagaki, 1986; Matsumoto *et al.*, 1989). Considering that C4b binding by complement receptor CR1 also involves two contiguous CCP modules (Krych *et al.*, 1991), it may be anticipated that the CCP modules of C1s bind to the C4b moiety of C4.

In the case of C1r, autolytic cleavage of the active protease, as well as limited proteolysis by various extrinsic proteases of different specificities, yields noncovalent $(\gamma\text{-B})_2$ homodimers that form the core of the C1r–C1r dimer and of the C1s-C1r-C1r-C1s tetramer (Arlaud *et al.*, 1986). Again, as observed for C1s, the autolytic C1r $(\gamma\text{-B})_2$ fragment (residues 280–688) retains the enzymic properties of intact active C1r, as shown by its ability to cleave and activate proenzyme C1s. The proenzyme form of these $(\gamma\text{-B})_2$ regions has also been obtained by limited proteolysis and exhibits the same autoactivation properties as native C1r, including similar activation kinetics and identical inhibition patterns, i.e., inhibition by 4-nitrophenyl-4′-guanidinobenzoate and insensitivity to diisopropylfluorophosphate (Lacroix *et al.*, 1989). However, unlike C1r, activation of the (γ-B)2 regions is totally insensitive to calcium ions, indicating that although these contain the structural elements required for intramolecular activation, they probably lack a regulatory element associated with the N-terminal calcium-binding region of C1r. The latter hypothesis is consistent with the observation that deletion of either the N-terminal CUB module or the EGF module from C1r significantly decreases the stability of the zymogen (Cseh *et al.*, 1996).

Studies based on the use of chemical cross-linking and homology modeling have provided insights into the structure of the catalytic regions of C1r and C1s (Rossi *et al.*, 1995; Lacroix *et al.*, 1997). Three-dimensional models of these regions have been constructed, indicating that, in both proteases, the second CCP module closely interacts with the serine protease domain on the side opposite to both the active site and the Arg–Ile bond cleaved on activation (Fig. 10). Such a configuration allows unimpeded access to both sites and is therefore consistent with activation and proteolytic activity of both proteases. In both C1r and C1s, chemical cross-linking yielded no information about the relative positioning of the first and second CCP modules, in keeping with the NMR solution structure of a pair of CCP modules from human factor H (Barlow *et al.*, 1993), indicating that there exists a significant range of angles of twist (131 ± 46°) of one module with respect to the other. If this feature applies to C1r and C1s, then the CCP_2 module/serine protease domain association may be expected to rotate about the long axis of CCP_2 with respect to CCP_1. Such a rotation may be a key element of C1 function, as it may, for example, allow the active serine protease domain of C1s to move toward the outside of the C1 complex and thereby gain access to its substrates C4 and C2 (Rossi *et al.*, 1995). With respect to the catalytic regions of C1r, chemical cross-linking of the $(\gamma\text{-B})_2$ fragment produced by autolytic cleavage of the active protease allowed identification of an intermonomer cross-link between Lys^{282} in the N-terminal region of the γ segment and Glu^{493} of the serine protease domain (Lacroix *et al.*, 1997). A three-dimensional model of the assembly of the activated $(\gamma\text{-B})_2$ dimer was derived from this information, indicating a loose "head-to-tail" association of the monomers, with the active sites facing opposite directions toward the outside of the dimer (Fig. 11). This configuration is fully consistent with enzymic activity of the active form of the dimer, as it allows access of both C1r active sites to the Arg–Ile activation sites in the C1s γ-B regions which, according to current C1 models (Weiss *et al.*, 1986; Arlaud *et al.*, 1987b), are expected to interact with opposite sides of the C1r $(\gamma\text{-B})_2$ dimer. In contrast, this configuration places the active site of each monomer about 77 Å away from the Arg–Ile activation site of the other monomer and is therefore not consistent with C1r autoactivation, as this requires a direct contact between these sites. These observations imply that dramatic conformational changes must take place in this region of C1r between proenzyme and active states. Again, such changes may occur, at least in part, through the previously mentioned rotation of one CCP module with respect to the other.

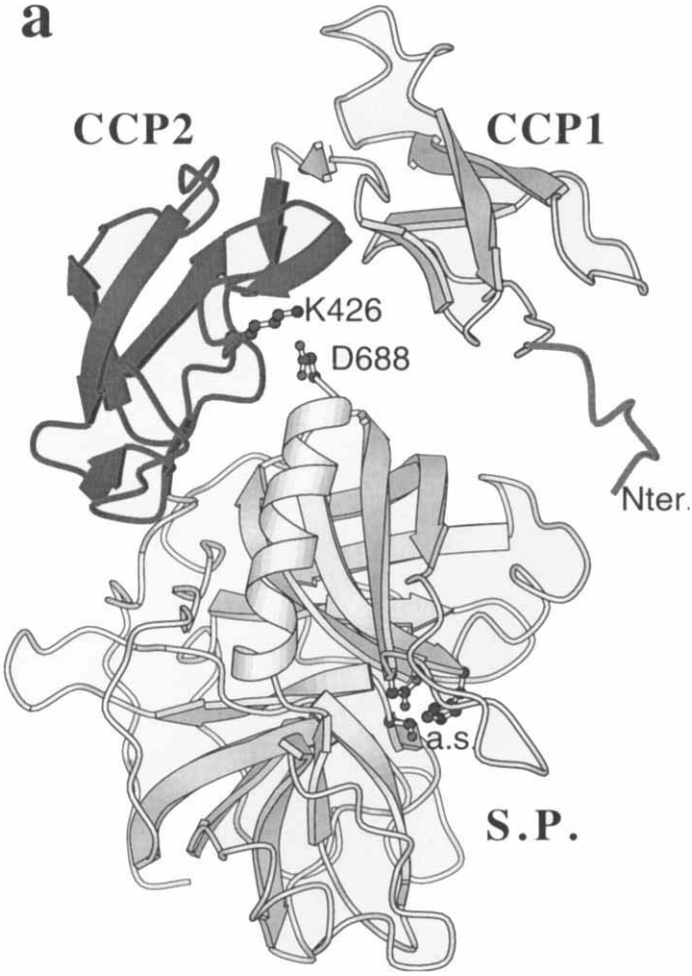


FIG. 10. Three-dimensional homology models of the catalytic regions of human C1r (a) and C1s (b). The models were generated using the Molscript program (Kraulis, 1991). β strands are represented by arrows and α helices by spirals. The respective locations of the first and second CCP modules (CCP1, CCP2) and of the serine protease domain (S.P.) are indicated. The side chains of the amino acids involved in domain–domain ionic bonds (K^{426}–D^{688} in C1r, K^{405}–E^{672} in C1s) and of the amino acids of the active site (a.s.) are shown. Nter and Cter indicate extensions at the N-terminal end of the C1r catalytic region and at the C-terminal end of the C1s catalytic region, respectively. Modified from Rossi *et al.* (1995) and Lacroix *et al.* (1997).

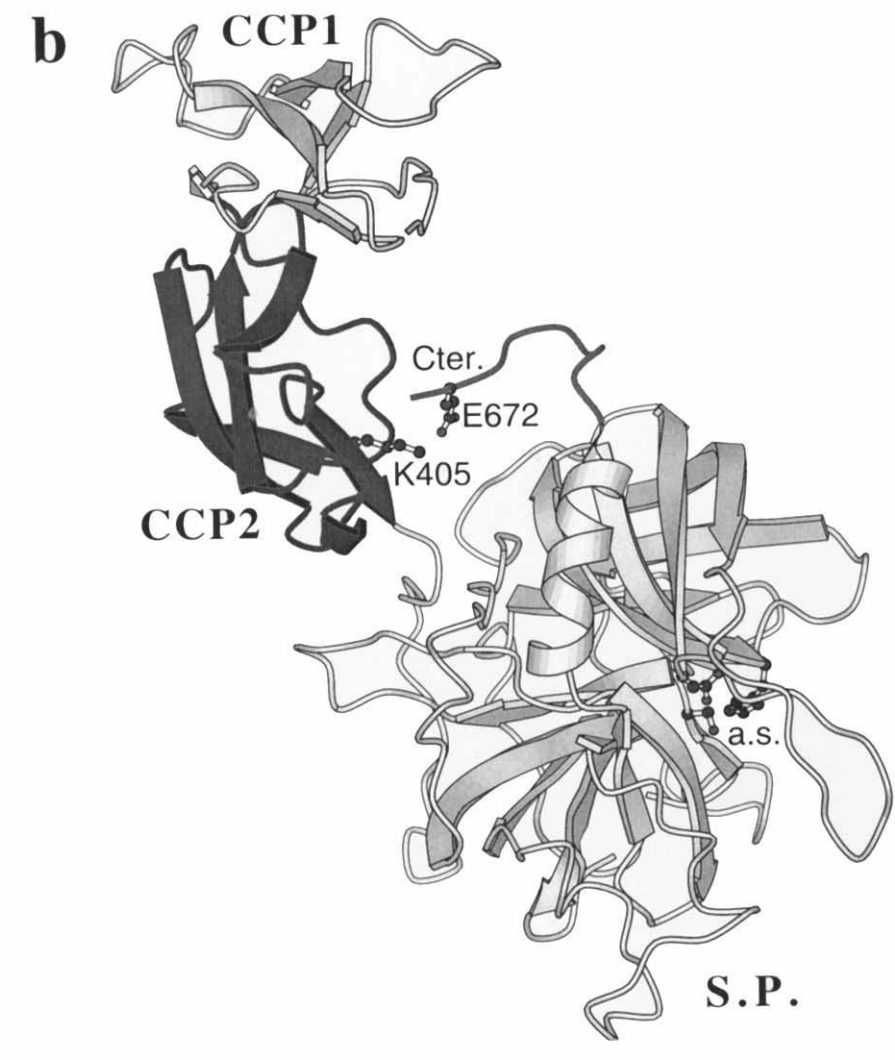


FIG. 10.—*Continued*

The proteolytic activity of C1 is controlled by C1 inhibitor, a member of the serine protease inhibitor (serpin) family that reacts stoichiometrically with the active sites of both C1r and C1s within activated C1 to form covalent protease–inhibitor complexes, resulting in inhibition and disassembly of the C1 complex (Ziccardi and Cooper, 1979; Sim *et al.*, 1979). Detailed information on the structure and function of C1 inhibitor and

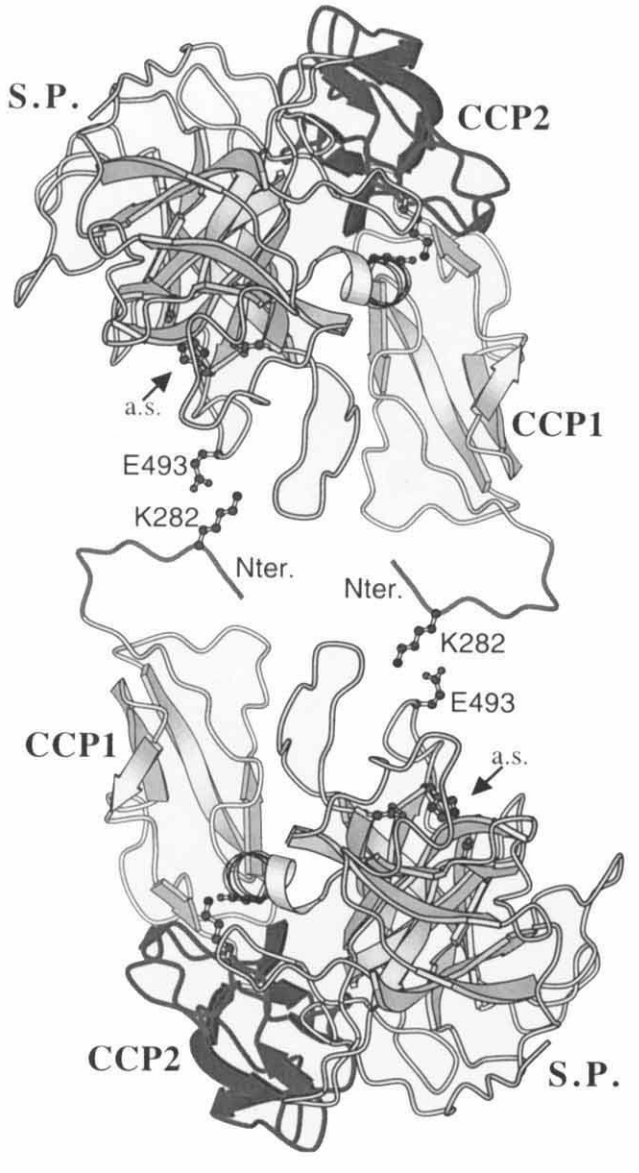


FIG. 11. A three-dimensional model of the assembly of the activated form of C1r catalytic regions. The model was generated using the Molscript program (Kraulis, 1991). Assembly of the dimer in a head-to-tail configuration is based on the occurrence of converse intermonomer ionic bonds between K^{282} of one monomer and E^{493} of the other monomer. a.s., active site. Modified from Lacroix *et al.* (1997).

its reaction with C1r and C1s may be found in several review articles (Cooper, 1985; Davis *et al.*, 1993).

E. Mannose-Binding Lectin Associated Serine Proteases

1. Gene Organization

The gene encoding the first MASP species discovered in human (thereafter called MASP-1) has been mapped by fluorescence *in situ* hybridization to region q27-q28 on the long arm of chromosome 3 (Sato *et al.*, 1994; Takada *et al.*, 1995). This location is clearly different from that of the C1r and C1s genes. Partial mapping of the exon–intron structure of the MASP-1 gene has also been performed (Endo *et al.*, 1996), indicating that, unlike C1r and C1s, the serine protease domain is encoded by at least six exons. Based on this information and other differences observed at the gene and protein level, it has been proposed that MASP-1 belongs to a branch of the phylogenetic tree of human serine proteases that is distinct, and possibly more ancient, than that defined by C1r, C1s, and haptoglobin (Endo *et al.*, 1996).

2. Protein Structure and Function

a. MASP-1. Although it was initially reported that mannose-binding lectin can bind and activate the proenzyme C1s-C1r-C1r-C1s tetramer (Lu *et al.*, 1990), it seems now established that MBL is preferentially associated with a distinct serine protease component (MASP). The MBL–MASP complex (also known as Ra-reactive factor) triggers the classical pathway of complement on binding to mannose and *N*-acetylglucosamine residues on certain microorganisms, thereby providing an activation mechanism (the "lectin pathway") distinct from that mediated by C1. MBL was originally found associated in human serum with a single MASP species (MASP-1), which could be dissociated from MBL in the presence of EDTA (Matsushita and Fujita, 1992). The primary structure of MASP-1 (originally called P100) has been deduced from DNA sequencing in both human (Takada *et al.*, 1993; Sato *et al.*, 1994) and mouse (Takahashi *et al.*, 1993; Takayama *et al.*, 1994). The proteins from both species are highly homologous, as they both contain 680 amino acids, share more than 87% sequence identity, and exhibit no insertions or deletions with respect to each other. Like C1r and C1s, they are activated through cleavage of a single Arg–Ile bond that splits the single-chain proenzymes into two disulfide-linked polypeptides of 429 and 251 amino acids. Also, human and mouse MASP-1 exhibit the same type of modular organization as C1r and C1s (Fig. 3). Thus, the N-terminal chain of MASP-1 contains the five modules and the intermediary activation peptide already described for C1r and C1s and, in view of the location of the cysteine residues at homologous positions, very

likely exhibits the same disulfide bridge pattern. The EGF-like module of MASP-1 also contains the particular consensus sequence characteristic of calcium-binding EGF modules, including the Asn residue (position 140) that undergoes β-hydroxylation in C1r and C1s. The N-terminal chain contains four potential *N*-glycosylation sites, located at identical positions in both human and mouse MASP-1, in the N-terminal CUB module (Asn^{30}), the EGF module (Asn^{159}), and the second CCP module (Asn^{366} and Asn^{388}). It should be mentioned that none of these sites is homologous to those occurring in C1r and C1s and that the presence of an *N*-linked oligosaccharide in the EGF module is unusual, as only *O*-linked oligosaccharides have been described so far in this type of module (Campbell and Bork, 1993).

The C-terminal serine protease domain of MASP-1 belongs to the chymotrypsin-like family and, given the presence of an Asp residue at position 189, is expected to display trypsin-like specificity. Unlike C1r and C1s, MASP-1 has conserved residue equivalent to Pro^{198} in chymotrypsinogen, as well as the two cysteine residues forming the "histidine loop" disulfide bridge. Comparative analysis of the whole proteins indicates that human MASP-1 exhibits 36 and 37% sequence identity with human C1r and C1s, respectively, indicating no preferential relationship with either protein. Both human and mouse MASP-1 were found to exhibit C1s-like proteolytic activity, as shown by their ability to cleave C4 and C2 (Matsushita and Fujita, 1992; Ji *et al.*, 1993). Further studies have provided evidence that, in contrast with C1s, human MASP-1 also cleaves complement protein C3 (Matsushita and Fujita, 1995), a property shared by murine MASP-1 (Ogata *et al.*, 1995). Both α_2-macroglobulin and C1 inhibitor have been reported to react with MASP-1 and to inhibit its proteolytic activity, suggesting that both inhibitors may be involved in the regulation of MASP-1 activity under physiological conditions (Terai *et al.*, 1995; Matsushita and Fujita, 1996).

b. MASP-2. A second MBL-associated serine protease (MASP-2) has been identified in human serum (Thiel *et al.*, 1997). Its primary structure has been mainly deduced from cDNA sequence analysis, indicating that the protein is secreted as a single-chain polypeptide of 671 amino acids, with no potential sites for N-linked glycosylation, and a possible polymorphism (Asp/Tyr) at position 356. MASP-2 has a modular organization homologous to that of MASP-1, C1r, and C1s (Fig. 3) and is probably split on activation at the Arg^{429}–Ile^{430} bond located in between the intermediary activation peptide and the serine protease domain. As in MASP-1, C1r, and C1s, the EGF-like module of MASP-2 possesses the consensus pattern associated with calcium binding, and the S_1 subsite residue in the serine

protease domain is an aspartic acid, also indicative of trypsin-like specificity. However, MASP-2, C1r, and C1s exhibit several common features that are not shared by MASP-1: (i) MASP-2 lacks the "histidine loop" disulfide bridge; (ii) as in C1r and C1s, its active-site serine residue is encoded by an AGY codon (where Y is T or C), whereas a TCN codon (where N is A, T, G, or C) is used in MASP-1; and (iii) Pro^{198} in chymotrypsinogen, which is conserved in MASP-1, is substituted by alanine in MASP-2. Based on these observations, it has been suggested that MASP-2, C1r, and C1s may have evolved from a MASP-1 ancestor (Thiel *et al.*, 1997). Whereas sequence identities among the four proteins are not significantly different (39–45%), similarity scores suggest a closer relatedness between MASP-2 and C1s (47%), and between MASP-1 and C1r (52%).

The MBL/MASP complex isolated from human serum by Thiel *et al.* (1997) was found to contain both MASP-1 and MASP-2, and all three components were found to coelute when submitted to gel filtration chromatography. In addition, MASP-1 and MASP-2 were separated by gel electrophoresis and, in contrast with previous findings (Matsushita and Fujita, 1992), only the latter was found to exhibit C4-cleaving activity. Thus, although it was originally assumed that MBL is associated with a single MASP species combining the functional properties of both C1r and C1s in C1, these latter experiments suggest the occurrence of two distinct proteases that would be functionally equivalent to C1r (MASP-1) and C1s (MASP-2). However, further analyses of the stoichiometry of the MBL/MASP-1/MASP-2 complex isolated by Thiel *et al.* (1997) and of the enzymic properties of the two proteases are required to get a clear picture of the assembly and activation of this newly discovered protease that may play a major role in innate immunity. The identification in the urochordate *Halocynthia roretzi* of the genes encoding two MASP-1-related proteases suggests that the lectin pathway has a more ancient origin than the classical and alternative pathways of complement activation (Ji *et al.*, 1997).

V. Summary and Perspectives

Proteases play several key roles in the complement system. In the classical and lectin pathways, C1r, C1s, and the MASPs are used to convert an initial recognition signal transmitted by C1q or the mannose-binding lectin, respectively, into proteolytic activity. In the same way, triggering of the alternative pathway results from acquisition of proteolytic activity by factor D. The active enzymes generated at the initial step of the three pathways are then used to produce additional proteases, the C3- and C5-convertases, that allow further amplification of the system and generation of biologically active peptides. Regulation is another facet of the role of complement

proteases. In addition to factor I, which mediates proteolytic control of the formation of the C3- and C5-convertases, the regulation of factor D catalytic activity by reversible substrate/cofactor-induced conformational changes provides a self-controlled mechanism of the activation of the alternative pathway.

To carry out their highly specialized functions, complement proteases have evolved unusual structural features in their serine protease domains and have acquired additional protein modules that apparently provide them with accessory binding sites for their substrates and cofactors and contribute to their extremely restricted specificity. The significant progress made in the elucidation of the structure and function of complement proteases has allowed insights into the molecular mechanisms involved in their activation and activity. This is best illustrated by the resolution of the three-dimensional structure of factor D, which provides a precise structural basis for the regulation of its catalytic activity. Although no experimental three-dimensional structure is available yet for the proteases of the C1 complex, models of their catalytic regions have been derived, which allow insights into the conformational changes occurring on C1r autoactivation and the mechanisms of substrate recognition by C1s. Also, studies have provided precise information on the modules of C2 and factor B involved in the assembly of the C3-convertases. In contrast, much less information is presently available on the structure of factor I and of the MASPs. The latter enzymes also need to be fully characterized functionally. From a general point of view, however, a major difficulty in solving the structure of complement proteases arises from their relatively large size, high carbohydrate content, and modular structure, which make them refractory to crystallization and/or high-resolution X-ray diffraction analysis. Therefore, novel strategies should be designed to tackle the problem of the resolution of the three-dimensional structure of complement proteases. One possible approach commonly used for other modular proteins is to dissect them into fragments corresponding to individual modules or arrays of modules in order to solve their structure either by X-ray crystallography or by NMR and precisely identify the structural correlates of their function. This can be achieved by the production of recombinant protein fragments using the available eukaryotic or bacterial expression systems. This methodology will also allow further mapping of functionally important residues through the use of site-directed mutagenesis. Then appropriate approaches will have to be devised to reconstitute each protease from its elementary pieces, which will likely require the combined use of different techniques, including lower resolution structural techniques such as electron microscopy and low-angle solution scattering. A further and difficult task will be to reconstruct the multimolecular proteases of complement (C1, MBL-

MASP, C3- and C5-convertases) in order to get high-resolution pictures of their assembly and function.

In addition to its well-established role as a major system in innate immunity, a number of studies have provided evidence for the participation of complement in the pathogenesis of disease. For example, several viruses, parasites, and bacteria have been shown to make use of membrane complement receptors to initiate cell infection (Cooper, 1998). Additional studies also provide evidence that the neurodegenerative process observed in Alzheimer's disease may result, at least in part, from direct activation of the classical pathway by the β-amyloid protein (Rogers *et al.*, 1992). In addition, various types of inherited or acquired deficiencies of complement proteins are associated with an increased susceptibility to pyogenic infections and immune complex disease (Morgan and Walport, 1991). These considerations underline the need to develop strategies to control complement activation. Because of their high specificity, complement proteases appear to be suitable targets for this purpose. Furthermore, their strategic position at different stages of the system offers the possibility to selectively block one of the activation pathways while keeping the others working. Undoubtedly, this will require the design of highly specific inhibitors based on a thorough understanding of the structural correlates of function of complement enzymes at the atomic level.

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Accessibility Control of V(D)J Recombination: Lessons from Gene Targeting

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I. Introduction

Because of the exquisite variability of the antigen receptors expressed at the surface of B and T lymphocytes, immunoglobulin (Ig) and the T-cell receptor (TCR), respectively, the immune system of vertebrates can recognize a virtually unlimited number of invading pathogens. In human and mouse, this extensive repertoire largely results from somatic DNA rearrangement events, referred to as V(D)J recombination, that assemble the various Ig and TCR variable region genes during the early stages of B and T lymphoid cell development. Major progress has been made toward the definition of the components of the V(D)J recombination machinery, as well as toward our understanding of the molecular mechanisms by which these factors mediate the rearrangement events. All the current evidence indicates that the same factors, and hence the same mechanisms, are responsible for V(D)J recombination at the various Ig and TCR genes. Nevertheless, it has long been recognized that the accurate targeting of V(D)J recombinase activity to restricted regions within the arrays of Ig and TCR gene sequences is dependent on the lineage and stage of differentiation of the lymphoid cell and, at specific loci, the allelic utilization. The molecular bases for this property, which is called recombinational accessibility, are essentially unknown. However, a large body of evidence, based mainly on cell transfection and transgenic mouse experiments utilizing recombination substrates, has suggested the existence of *cis*-acting regulatory sequences that control recombinational accessibility at the various Ig and TCR gene loci. Most of these sequences are closely associated with transcriptional regulatory elements, such as enhancers, promoters, and silencers. Until recently, the real impact of such sequences on V(D)J recombination at the endogenous Ig and TCR genes has been open to question due partly to the inherent drawbacks—uncontrolled copy number and sites of integration—of the classical transgenic approach. The situation has changed with the development of homologous recombination techniques, which have made it possible to directly test the influence of several types of regulatory sequences on V(D)J recombination *in situ*. This review focuses on these latter experiments and discusses the impact they have on

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our current understanding of the mechanisms of recombinational accessibility.

II. Basic Features

A. The V(D)J Recombination Reaction: Mechanistic Aspects, Target Sequences, and Recombination Machinery

Recombinational accessibility has to be appreciated in light of the spectacular progress made in our understanding of the molecular basis of the V(D)J recombination reaction. This has been made possible by several breakthroughs, including the development of sophisticated polymerase chain reaction (PCR) assays to identify and analyze broken DNA molecules representing V(D)J recombination intermediates in cells actively undergoing V(D)J recombination, application of these analyses to assay for rearrangement within artificial recombination substrates introduced into cells expressing the V(D)J recombinase activity, the definition of several essential components of the V(D)J recombination apparatus, and, finally, newly established *in vitro* cell-free recombination systems. Several detailed reviews on V(D)J recombination have been published, including some emphasizing the most recent progress, and the reader is urged to refer to these reviews for a more extensive and in-depth description of these various aspects (Lewis, 1994; Weaver, 1995; Bogue and Roth, 1996; Gellert, 1997).

1. The V(D)J Recombination Reaction and Its Products

Schematically, the V(D)J recombination process can be separated into two distinct phases. The first comprises the site-specific DNA recognition, synapsis, and (predominantly) coupled cleavage (see later) of two separate gene segments. Each cleavage results in the formation of a double-stranded break (DSB) of the DNA, of which one product consists of a blunt 5′-phosphorylated end, called the signal end (SE), whereas the other consists of a closed hairpin structure called the coding end (CE). The second phase consists of the differential processing and assembly of the CE and SE products, resulting in the irreversible alteration of the genomic structure at given Ig or TCR gene loci. Typically, CE are rapidly assembled into coding joints, creating the recombined structural gene, a process that involves nicking of the hairpins followed by extensive modifications (nucleotide deletion and/or addition) at the joined extremities. In contrast, signal joint formation is somewhat delayed (i.e., SE accumulate to some extent) and consists of the back-to-back fusion of the blunt-ended, unmodified SE. Most frequently, the rearranging gene segments lie in the same orientation, leading to the retention of the coding joint on the chromosome whereas the signal joint and intervening sequences are deleted as a circle. However,

when the two rearranging gene segments are oppositely oriented, the intervening sequences are inverted, linking physically the two final products within the chromosome. Because of the complex processing of the CE prior to coding joint formation, only a fraction of the resulting coding joints lead to productive rearrangement of the structural gene, whereas the remaining recombinants contain frameshifts. In addition to the canonical products just described, two types of noncoding junctions called open-and-shut and hybrid joints have also been observed at a significant frequency and correspond to the fusion of one SE to its flanking or to a *different* CE, respectively. Their existence has suggested that the intermediate cleaved products may be transiently held in a four-end complex. *In vitro* experiments have indeed provided evidence for the existence of different types of nucleo-protein complexes, composed of various components of the V(D)J recombinase, at distinct stages of the V(D)J recombination reaction (Hiom and Gellert, 1997; Agrawal and Schatz, 1997; Grawunder and Lieber, 1997).

2. *Target Sequences and Mechanistic Aspects of V(D)J Recombination*

In the mouse, seven endogenous genes are potential targets for V(D)J recombination events in developing lymphocytes, including Ig heavy (IgH) and light (IgLκ and IgLλ) chain genes and TCRα, β, γ, and δ chain genes (reviewed by Hood *et al.*, 1995). Depending on the locus, the rearrangement events involve either three gene segments [V (variable), D (diversity) and J (joining)], as is the case for IgH, TCRβ, and TCRδ genes, or two gene segments (V and J) in the case of IgLκ, IgLλ, TCRα, and TCRγ genes. Although IgH, IgLκ, IgLλ, TCRβ, and TCRγ genes are located at different loci of the mouse genome, remarkably enough, the genetic elements of TCRα and TCRδ genes are intermingled within a single gene locus (TCRα/δ locus). In their germline organization, Ig and TCR genes are spread out over relatively long distances, and rearrangements at endogenous antigen receptor loci can involve either proximate (a few hundred base pairs) or extremely distant (in the megabase range) fragments. In the vast majority of cases, rearrangements occur between gene segments of the same locus. However, interlocus rearrangements do occur at a low but significant frequency and are viewed as consequences of the genetic instability inherent to the V(D)J recombination process (Kirsch and Lista, 1997). For example, in the mouse, there is 1 Vγ-to-Jβ *trans* rearrangement for every 500 Vβ-to-Jβ rearrangements, resulting in a translocation between chromosomes 6 and 13. Also, in humans suffering from ataxia telangiectasia, an increase in the level of Vγ-to-Jβ *trans* rearrangements, resulting in an inversion on chromosome 14, has been correlated with an increased risk for the development of malignancies, particularly those of lymphoid origin.

This example points to the fact that while intralocus V(D)J recombination is relatively dependable, mistakes are made with, perhaps, pathological consequences (Tycko and Sklar, 1990; Lieber, 1993; Rabbitts, 1994).

Each of the various V, D, and J coding segments are bordered on one or both (in the case of D segments) sides by homologous recombination signal sequences (RSSs) that consist of a highly conserved palindromic heptamer, a less well-conserved AT-rich nonamer, and a spacer of 12 ± 1 or 23 ± 1 bp nonconserved sequences. *In vivo* experiments utilizing simple, artificial recombination substrates transiently transfected into cells expressing the recombinase have demonstrated that RSSs are the only sequences required for V(D)J recombination (Hesse *et al.*, 1989). Furthermore, the heptamer, nonamer, and spacer of the RSS are all required for efficient V(D)J recombination. The reaction is strongly biased toward rearrangements between fragments containing RSSs with spacers of different length, a restriction known as the 12/23-bp rule. Thus, the V(D)J recombination process is about 50-fold more efficient in the presence of a 12/23 RSS pair in comparison to 12/12 or 23/23 RSS pairs in plasmid-based recombination substrates. Similarly, using a ligation-mediated, PCR (LM-PCR)-based approach to detect SE resulting from cleavage of the recombination substrates, it is now possible to demonstrate that a 12/23 RSS pair is required for efficient SE formation whereas 12/12 and 23/23 RSS pairs (or a 12/23 RSS pair with one RSS mutated) as well as single RSS are all about 30-fold less efficient (Steen *et al.*, 1996). This is in good agreement with the 50 : 1 ratio mentioned earlier strongly suggesting that the 12/23-bp rule is exercised at the very first step of the reaction. Indeed, a Southern blotting approach to directly measure dual and single RSS cleavage *in vivo* has demonstrated that cleavage in the presence of a 12/23 RSS pair is coupled, although there was the presence of a single cut in about 10% of the products (Steen *et al.*, 1997). This property is intrinsically associated with recombinational accessibility, such that inaccessibility of one recombination partner to the recombinase leads to a significant decrease in both DSB formation at the other site and recombination, even if the other partner is accessible.

The results from *in vitro* experiments utilizing recombination-competent cell extracts recapitulate the *in vivo* results with respect to RSS utilization (Eastman *et al.*, 1996). This is also true when using recombinant RAG1 and RAG2 proteins (see later) in the presence of Mg^{2+} (Van Gent *et al.*, 1996). Conversely, the 12/23 rule is not reproduced in an *in vitro* reaction for the initiation of V(D)J recombination comprised solely of short oligonucleotides, recombinant RAG1 and RAG2, and Mn^{2+}. However, this reaction in which a single RSS is sufficient to initiate DNA cleavage has further simplified the analysis of the role of the RSS in DSB formation and has

generally confirmed the *in vivo* findings with respect to sequence requirements (Ramsden *et al.*, 1996; Cuomo *et al.*, 1996). Moreover, it has made it possible to dissect the different functions of the nonamer and heptamer sequences. Indeed, an isolated heptamer sequence is capable of accurately targeting the precise site of the DNA cleavage, albeit at a low level, whereas the nonamer appears to increase the efficiency of the reaction. It has been argued that the functionality of the heptamer may also involve some DNA unwinding at the junction between the RSS and the coding sequences. Thus, factors that may favor such unwinding and/or which help to localize unwinding factors near the rearranging RSS may play a role in recombinational accessibility. Finally, in addition to the RSS, it is also clear that the first few nucleotides at the coding flanks can strongly influence V(D)J recombination, both in terms of efficiency of the reaction and of the structure of the recombination products (Gerstein and Lieber, 1993; Ezekiel *et al.*, 1995, 1997; Nadel and Feeney, 1997).

3. *Recombination Machinery*

Considerable advances made by a number of laboratories have established the products of the *RAG1* and *RAG2* genes as the central components of the V(D)J recombination machinery (reviewed by Alt *et al.*, 1992; Schatz *et al.*, 1992; Schatz, 1997). These two proteins are simultaneously expressed exclusively in lymphoid cells at precisely the same time as antigen receptor gene rearrangement, and both are necessary for V(D)J recombination. Mice in which either one or the other of the *RAG* genes has been inactivated by gene targeting have no mature B or T cells and lack any products of V(D)J recombination (Mombaerts *et al.*, 1992b; Shinkai *et al.*, 1992). In these mice, V(D)J recombination is not even initiated. In addition, *RAG1* and *RAG2* cDNAs are all that are required for the rearrangement of a recombination substrate when cotransfected in expression vectors into fibroblasts, indicating that they are the only lymphoid-specific factors required for V(D)J recombination, while the rest of the recombination machinery is ubiquitous. Interestingly, large portions of the sequences of both RAG molecules (notably at the N terminus of RAG1 and the C terminus of RAG2) are dispensable for recombination in the cotransfection system just described (Silver *et al.*, 1993; Sadofsky *et al.*, 1993, 1994; Cuomo and Oettinger, 1994; Kirch *et al.*, 1996). However, *in vivo* experiments using extrachromosomal recombination substrates or the endogenous IgH locus has demonstrated that some basic motifs in the N-terminal region of RAG1 are necessary for full recombinase activity, suggesting that these sequences may participate in the formation of a recombination complex that facilitates the rearrangement process (McMahan *et al.*, 1997; Roman *et al.*, 1997).

As already discussed, an *in vitro* recombination assay has been developed that requires only a single RSS, purified RAG1 and RAG2 proteins, and divalent cations. This system utilizes the RAG core proteins (i.e., the portion of both RAG proteins essential for recombination activity), as the full-length molecules are highly insoluble. Experiments using this system demonstrated that RAG1 and RAG2 are sufficient for the initial phase of the reaction, including DNA recognition, nicking of the DNA, and strand transfer (hairpin formation) (McBlane *et al.*, 1995). Analyses using either surface plasmon resonance or an *in vivo* one-hybrid DNA binding assay demonstrated that the initial DNA recognition event is mediated principally by RAG1 and the nonamer (Spanopoulou *et al.*, 1996; Difilippantonio *et al.*, 1996). RAG2 shows no DNA-binding activity, but its localization to the RSS depends on the presence of RAG1. Finally, RAG1-mediated recruitment of RAG2 is more efficient with a 12-bp as compared to a 23-bp spacer signal. As briefly mentioned already, in the presence of Mg^{2+}, the *in vitro* reaction reproduces the 12/23-bp rule and coupled cleavage. Interestingly, inclusion of high mobility group (HMG) proteins in the reaction stimulates cleavage and RAG binding to the 23-bp RSS and also increases the efficiency of coupled cleavage when both signal sequences are present (Sawchuk *et al.*, 1997; Van Gent *et al.*, 1997). These results suggest that architectural components assist the catalytic components of the V(D)J recombinase at the cleavage site. Thus, it has been argued that by helping to bend the longer (23-bp) spacer DNA, HMG proteins assist the RAG core proteins in formation of the synaptic complex and may contribute to the enforcement of the 12/23 rule *in vivo*. However, their presence within some forms of SE-containing complexes unlikely to contain bent DNA (see Agrawal and Schatz, 1997) also suggests that HMG proteins may serve to stimulate binding of the recombination apparatus to its target sequences, much in the same way as they have been described to increase sequence-specific binding for several transcription factors (Zwilling *et al.*, 1995; Zappavigna *et al.*, 1996).

Results from the Gellert laboratory, where they have achieved the complete V(D)J recombination reaction in a cell-free system, demonstrate that RAG1 and RAG2 are important for the latter stages of V(D)J recombination as well as for the cleavage step (Ramsden *et al.*, 1997). RAG1 and RAG2 stimulated coding joint formation by up to 50-fold compared to when they were removed before the joining step of the reaction. In contrast, signal joint formation was inhibited by RAG1 and RAG2 under the same conditions, suggesting that the SE were trapped in a synaptic complex and unavailable for processing. These results are consistent with previous studies demonstrating a strong correlation between the presence of RAG1 and

RAG2 and the persistence of SE *in vivo* (Ramsden and Gellert, 1995; Livak and Schatz, 1996).

The ubiquitous factors that mediate the second phase of V(D)J recombination are less well understood, although they appear to be largely derived from the DNA DSB repair machinery (reviewed by Weaver, 1995; Lieber *et al.*, 1997). They include the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) as well as the DNA-binding subunit of DNA-PK, Ku70, and Ku80/Ku86. Mice carrying a mutation in DNA-PKcs (scid mice) demonstrate a severe defect in V(D)J recombination, particularly in the formation of coding joints, as well as a defect in general DSB repair. The mutation has been identified as a single base pair alteration in the *scid* C-terminal kinase domain (Danska *et al.*, 1996). A frameshift mutation N-terminal of this domain, as described in scid Arabian horses, yields a more severe phenotype where both signal and coding joint formation are affected (Shin *et al.*, 1997). Moreover, mice in which Ku86 has been targeted show a similar defect in signal and coding joint formation in lymphocytes (Zhu *et al.*, 1996). Altogether, these findings support a role for DNA-PKcs activity in the formation of both signal and coding joints, although the precise mechanism is not yet understood.

Another factor shown to be important for signal and coding joint formation is that encoded by the *XRCC4* gene (Li *et al.*, 1995). Mutations in this gene also lead to a severe deficiency in the ability to make signal and coding joints during V(D)J recombination as well as sensitivity to ionizing radiation. The XRCC4 gene product shares homology with no known protein and has no enzymatic activity. Two studies, however, by the Lieber and Jackson laboratories provide a role for XRCC4 in V(D)J recombination (Grawunder *et al.*, 1997; Critchlow *et al.*, 1997). Both groups demonstrated that DNA ligase IV specifically interacts with the XRCC4 protein. Furthermore, the Lieber study demonstrated that DNA ligase IV-mediated ligation of DSB breaks in an *in vitro* assay was stimulated five- to eight-fold in the presence of the XRCC4 protein. These results provide strong evidence that the function of XRCC4 may be to recruit and/or stimulate DNA ligase IV during the final phase of V(D)J recombination.

Finally, terminal deoxynucleotidyl transferase (TdT) also plays a role in V(D)J recombination, as it is responsible for nucleotide addition (N nucleotide) to the CE (Gilfillan *et al.*, 1993; Komori *et al.*, 1993). Its role appears less fundamental to the V(D)J recombination process as mice with a null mutation in the *TdT* gene show no obvious immune defects (Gilfillan *et al.*, 1995). Unexpectedly, the rare coding joints found in Ku86-deficient mice lack N addition, suggesting that TdT activity is somehow dependent on Ku-86 (Bogue *et al.*, 1997). Although many of the factors that make up the V(D)J recombination machinery have been described, it is quite

probable that others remain unidentified. Furthermore, with the exception of the RAG proteins, the mechanism by which they act or how they associate is not known. This may rapidly change with the development of *in vitro* systems capable of achieving the joining processes of the second phase of the V(D)J recombination reaction (Cortes *et al.*, 1996; Ramsden *et al.*, 1997).

B. Recombinational Accessibility: Definition, Putative Regulatory Elements, and Molecular Aspects

The tissue specificity of *RAG1* and *RAG2* gene expression is probably the major factor restricting V(D)J recombination to the cells of the immune system, given that *RAG1* and *RAG2* gene cotransfection is sufficient to provide nonlymphoid mammalian cells (such as fibroblasts) with V(D)J recombination activity when assayed on introduced artificial recombination substrates. Importantly, however, in such transfected cells, endogenous antigen receptor genes remain unrearranged. This clearly indicates that, at the level of complexity of the endogenous recombination substrates, the RSSs alone are not sufficient to target V(D)J recombination, implying higher order regulation of this process. As detailed later, it appears that in developing B and T cells, specific molecular mechanisms do exist that target the common recombinase activity to appropriate Ig or TCR loci in a lineage-specific, developmentally ordered, and allelically excluded manner (reviewed by Ferrier *et al.*, 1989b; Okada and Alt, 1994; Sleckman *et al.*, 1996). It is believed that such tight regulation of V(D)J recombinase-mediated rearrangements helps ensure the specificity of the immune response. Although the molecular basis for this level of control is still a matter of debate, it is clear that recombinase targeting must occur at the level of the substrate locus, a concept known as "recombinational accessibility."

1. Several Levels of Regulation at Ig and TCR Gene Loci; Relationships to Chromatin

V(D)J recombination in prelymphocytes appears to be regulated at three distinct levels: (i) Cell lineage, i.e., antigen receptor gene rearrangement only occurs within the appropriate cell lineage. Thus, complete (V-to-DJ or V-to-J) Ig gene rearrangement is a property of B lineage cells whereas TCR gene rearrangement is characteristic of T lineage cells. (ii) Stage of cell differentiation, i.e., within a given cell lineage, gene rearrangement is an ordered process that depends on the developmental stage of the cell. For example, in the vast majority of developing B cells, IgH gene rearrangement and expression usually precede IgL gene recombination and expression, with IgLκ rearrangement generally occurring before IgLλ rearrange-

ment. Similarly, in developing T cells, TCRβ, γ, and δ gene rearrangement and expression are early events, usually occurring before possible TCRα gene recombination and expression. Moreover, rearrangement of gene segments within a given locus are often temporally regulated, such that at IgH and TCRβ loci, the D-to-J joining step occurs first followed by the appendage of a V segment onto the preformed DJ unit. (iii) Allelic exclusion, i.e., at particular loci, productive rearrangement on one allele results in the arrest of further rearrangement on the remaining allele, contributing to allelic exclusion of antigen receptor gene expression, a fundamental property of the lymphoid system.

Given the fact that Ig and TCR genes are assembled by an activity common to both B and T cells that selectively targets highly conserved RSSs within the various Ig and TCR genes, the different levels of control just mentioned are generally explained by specific changes in the access of the V(D)J recombinase to Ig and TCR gene loci and/or segments. This model implies the existence of differences in the chromatin structure at the various Ig and TCR gene loci, depending on the lineage and developmental stage of the cell that renders the antigen receptor loci differentially accessible to the recombination machinery. Evidence that V(D)J recombinase access to specific RSSs is indeed a regulated property of lymphocyte chromatin has been provided by the demonstration that when purified nuclei from *RAG*-deficient mice are treated *in vitro* with a nuclear extract made from a source with high levels of RAG activity, Ig and TCR genes are differentially accessible to DSB formation, depending on the type and stage of differentiation of the lymphoid cells used to prepare the nuclei (Stanhope-Baker *et al.*, 1996).

It is worth noting that while recombinational accessibility is highly regulated, it is not absolute. For example, D-to-J recombination at the IgH μ locus sometimes occurs in T cells (Born *et al.*, 1988). In addition, IgL chain rearrangement has been observed in the absence of IgH gene expression and TCRα rearrangement has been observed in the absence of TCRβ expression (Ehlich *et al.*, 1993; Mombaerts *et al.*, 1992a). Finally, rearrangement on both alleles of normally allelically excluded loci has also been observed (Balomenos *et al.*, 1995). These examples illustrate the multiplicity of situations observed with respect to recombinational accessibility depending on the locus and/or type of segment. Accessibility thus appears to be a complex phenomenon, not totally immune from the occasional regulatory misstep. This may reflect the fact that the control of recombinase targeting apparently involves multiple *cis*-regulatory elements that may dictate unique nucleo-protein interactions at individual Ig and TCR loci, as detailed next.

2. *cis-Acting Sequences Mediating Recombinational Accessibility*

The recombinational accessibility model has predicted the existence of *cis*-acting regulatory sequences able to modulate access of the recombinase to a given Ig or TCR gene locus (Alt *et al.*, 1987). Because there is a strong correlation generally observed between induction of germline transcription and V(D)J recombination at antigen receptor loci (e.g., see Yancopoulos and Alt, 1985; Schlissel and Baltimore, 1989; Alessandrini and Desiderio, 1991; Fondell and Marcu, 1992; Goldman *et al.*, 1993), *cis*-acting transcriptional regulatory elements were viewed as likely candidates. These would include notably enhancer, promoter, and silencer elements (Ernst and Smale, 1995a,b). It was therefore logical to look for assays in which the influence of such regulatory elements on the rate of recombination of adjacent variable gene segments could be evaluated. Initially, this has been done by constructing recombination substrates containing transcriptional regulatory elements or mutated versions of those sequences. These substrates were subsequently tested for their recombination potential following introduction into lymphoid cells expressing the V(D)J recombinase activity by either transient or stable transfection or by the generation of transgenic mice (Sleckman *et al.*, 1996). Among these various approaches, the latter offers the advantages that the introduced DNA is integrated into the host genome, it is exposed to physiological levels of V(D)J recombination activity, and it is subjected to the modifications induced by developmentally regulated *trans*-acting factors during the development of the various lymphoid cell lineages. Most of these experiments have addressed the role of transcriptional enhancers from the various Ig and TCR genes. The general outcome of these studies is that in the absence of known enhancer sequences (or in case of their mutation at specific protein-binding sites), the level of V(D)J recombination within the integrated substrates is negligible, whereas it is significantly increased by the *cis* addition of a lymphoid gene enhancer (Watrin *et al.*, 1994; Sleckman *et al.*, 1996, and references within). The one exception is found at the TCRδ locus, as Vδ-to-Dδ recombination occurred in the absence of the known TCRδ enhancer and was not affected by its presence (Lauzurica and Krangel, 1994a,b; Hernández-Munain *et al.*, 1996; Lauzurica *et al.*, 1997). Taken together, the case of Vδ-to-Dδ assembly notwithstanding, these studies suggest that transcriptional enhancers play a critical role in the initiation of recombinational accessibility. However, transgenic experiments have suggested that enhancers alone may not always be sufficient to impart lineage and/or developmentally ordered specificity to the rearrangement of associated gene segments and that their effect is sometimes dependent on the trangenic mouse line studied. For example, results obtained with either TCRβ or TCRδ en-

hancers demonstrated that there was a variable level of, respectively, D-to-J or V-to-D rearrangement of the corresponding recombination substrates in B cells, which was, nevertheless, lower than the level of the same rearrangement in T cells (Capone *et al.*, 1993; Okada *et al.*, 1994; Lauzurica and Krangel, 1994a,b). However, V-to-DJ or VD-to-J rearrangement was strictly confined to the T-cell lineage. These results point toward the existence, at endogenous antigen receptor gene loci, of other *cis*-regulatory elements, which together with the enhancers confer accurate cell lineage and temporal specificity to the modulation of recombinational accessibility. Indeed, transgenic experiments using an IgLκ recombination substrate, which includes the intronic κ enhancer (see later), argued for the existence of such elements within the more recently identified IgLκ 3′ enhancer (Hiramatsu *et al.*, 1995). In addition, other transgenic experiments have demonstrated the activating role of other types of *cis*-acting elements on V(D)J recombination, including the promoter-associated sequences of a V gene, as well as sequences capable of downmodulating rearrangement, which may contain transcriptional silencer elements (Lauster *et al.*, 1993). Finally, nonenhancer-associated sequences have been identified, which have been claimed to contribute to lineage-restricted rearrangement of the given transgenes (Shutter *et al.*, 1995; Janowski *et al.*, 1997; Cavelier *et al.*, 1997).

3. *Molecular Basis of Recombinational Accessibility*

So far, the molecular basis of V(D)J recombinase accessibility remains poorly understood. Given the evidence that access of the V(D)J recombinase to specific RSSs is a regulated property of lymphocyte chromatin, several hypotheses have been proposed to explain how enhancers, and hence the nuclear factors that bind to them, may impact on chromatin and V(D)J recombination, including lineage-and/or stage-specific activation of (i) germline transcription (which could act either directly or by means of the germline transcripts) and (ii) additional processes leading to the alteration of the chromatin structure (i.e., nucleosome positioning, CpG methylation). However, despite extensive studies, little progress has been made so far in confirming or invalidating any of these hypotheses. Lymphoid gene enhancers have actually been implicated in *cis* activities other than transcription, including the establishment of local factor access (Jenuwein *et al.*, 1993) or of regional demethylation (Lichtenstein *et al.*, 1994). Similar to the story for transcription, analyses of endogenous Ig and TCR loci, as well as recombination reporter transgenes, have indeed correlated demethylation and changes in chromatin structure (as defined by the increased DNA sensitivity to endonucleases) with ongoing V(D)J recombination (e.g., see Blackman and Koshland, 1985; Burger and Radbruch,

1990; Goodhardt *et al.*, 1993; Engler *et al.*, 1993; Demengeot *et al.*, 1995; Hashimoto, 1989; Ferrier *et al.*, 1989a). However, because all of the data linking these factors with V(D)J recombination are correlative, it is impossible to determine if they are the cause or the consequence of recombinational accessibility. Furthermore, it is difficult to precisely determine the causal factor as transcriptionally active loci are also generally hypomethylated and in a structurally accessible status whereas, conversely, changes in chromatin structure influence the methylation-dependent repression of gene activity (reviewed by Bird, 1992; Siegfried and Cedar, 1997). The correlative studies are even more difficult to interpret in the cases of recombination reporter transgenes because of the fact that transgenes usually integrate into the chromatin in large tandem arrays and not all of the integrated copies undergo rearrangement. Consequently, although recombination-competent transgenes are generally found to be transcribed, demethylated, and sensitive to endonucleases, in most cases it has been difficult to demonstrate with absolute confidence the actual status of the rearranging copies (e.g., see Lauster *et al.*, 1993). Finally, a few exceptions exist suggesting that the processes of recombinational accessibility and germline transcription may be independent (Hsieh *et al.*, 1992; Goodhardt *et al.*, 1989; Kallenbach *et al.*, 1993; Alvarez *et al.*, 1995). However, these experiments either were done with recombination substrates that were not integrated into the chromatin and for which the requirements for gene assembly may be quite different or, when performed using integrated transgenes, were difficult to rigorously interpret because it was not always possible to analyze transcription at the cell stage where V(D)J recombination actually takes place. Even more puzzling, a few cases were found where transcribed and hypomethylated transgenes were not rearranged; in some instances, transcription was also detected at the stage were active recombinase is present (Okada *et al.*, 1994; Fernex *et al.*, 1995). Therefore, transcription (and hypomethylation) may not be sufficient per se for V(D)J recombination. These findings raise the intriguing possibility that *cis*-acting regulatory sequences and *trans*-binding factors may play a more specific role in the process of rearrangement by, for example, interacting with components of the V(D)J recombination apparatus, thus helping to stabilize and/or activate some forms of the recombination complex, a hypothesis not necessarily exclusive to those mentioned earlier. In agreement with this, lymphoid transcriptional regulatory elements, notably enhancers, share common motifs that bind factors expressed in both B and T lineage cells (reviewed by Staudt and Lenardo, 1991; Leiden, 1993). The possibility that a subset of factors that bind to Ig and TCR enhancers may promote recombination by directly interacting with the recombination complex during some specific step(s) of the recombination reaction could also ex-

plain the surprising observation that reporter transgenes carrying certain IgH enhancer ($E\mu$) mutants display enhancer-dependent transcription and demethylation in immature B cells but no V(D)J rearrangement (Fernex *et al.*, 1995; C. Fernex and P. Ferrier, unpublished data).

III. Role of *cis*-Acting Elements: Trial of the Homologous Recombination Approach

Cell transfection and transgenic experiments have inherent drawbacks that are related to the randomness of the integration site and concatenation of multiple integrated copies. When analyzing the role of *cis*-regulatory elements in a process such as V(D)J recombination, both may be a source of potential artifacts leading to misinterpretation of the results. For example, transgene-associated regulatory sequences may behave differently than their endogenous counterparts due to the different chromosomal context and/or their multimerization within the transgenic integration array. Based on these assumptions, conclusions from transgenic experiments on the critical role that elements such as enhancers may play within endogenous Ig and TCR genes have been open to question. Fortuitously, the development of homologous recombination techniques that theoretically permit one to manipulate virtually any genomic sequence has offered the opportunity to address this issue directly. Indeed, in the past few years, gene targeting has been applied to the analysis of various types of *cis*-acting Ig and TCR gene regulatory elements with respect to their role in V(D)J recombination and gene expression. Although one should remain cautious given the still limited number of published experiments, a picture of the hierarchical organization of the regulatory sequences has begun to emerge. In addition, these experiments have provided some information on the molecular mechanisms of recombinational accessibility. Finally, these first experiments have helped to emphasize the fact that this powerful approach is not exempt from its own technical drawbacks.

A. Technical Aspects and Concerns

Gene targeting has become a widely used approach in genetics, based notably on the ability to produce mice carrying a precise gene mutation(s) (Capecchi, 1989; Koller and Smithies, 1992). Briefly, following transfection of embryonic stem (ES) cells with an appropriate DNA construct, the cells are placed under selection. Surviving clones are then screened for the presence of the desired homologous recombination event and the cells harboring the correct recombinant are injected into mouse blastocysts. Following implantation into host female mice, the injected blastocysts give rise to chimeric progeny that can be analyzed directly or used to achieve germline transmission of the mutation to generate heterozygous and even-

tually homozygous mutant mice. In the classical, most widely used approach, the sequences of interest within the targeted region are disrupted (or replaced) by a cassette consisting of a selectable marker gene (usually the *neo*^{r} gene) placed under the control of an ubiquitously expressed promoter. The selectable cassette is left in place in the selected ES cells and, consequently, in every ES-cell derived tissue of the resulting mutant mice where it can be strongly expressed. For those who wish to address issues on V(D)J recombinational accessibility, this approach should be a matter of concern as the expressed cassette is likely to influence chromatin structure at the targeted locus, notably in the developing lymphocytes.

In fact, efforts have been made to circumvent this potential problem, either by using a *neo*^{r} gene whose expression is driven by a promoter that is expected not to be active in lymphoid cells (Takeda *et al.*, 1993) or, more reliably, by removing the selectable cassette from the targeted locus once production of the mutant ES cells (or mice) has been achieved. Several complex strategies have been used to achieve removal of the selectable marker gene, including the so-called "hit and run" and "tag and exchange" approaches (Hasty *et al.*, 1991; Valancius and Smithies, 1991; Askew *et al.*, 1993). Removal of the selectable marker gene has become more convenient and easy to perform, thanks to the adaptation to gene targeting of bacterial- and yeast-derived recombination models, the so-called Cre/*loxP* and FLP/*FRT* systems, respectively (reviewed by Kilby *et al.*, 1993; Kühn and Schwenk, 1997). In these systems, the selectable cassette in the targeting construct is flanked by specific recombination sequences (the *loxP* or *FRT* sites, each of which is 34 bp) which, when in the appropriate orientation, can be recombined by the action of the specific (Cre or FLP) recombinase in such a way that the intervening sequences are deleted. Thus, expression of the corresponding recombinase in ES cells (by transfection of an expression vector) or in tissues of the mutant mice (by crossing with the appropriate transgenics) eliminates the intervening cassette from the targeted locus, leaving behind a single copy of the *loxP* or *FRT* site. Current evidence suggests that such sequences are neutral with respect to their effect on local chromatin structure (see Ferradini *et al.*, 1996).

Several examples of gene targeting experiments at various loci, including antigen receptor loci, in which the functions of *cis*-regulatory elements have been tested either following the removal of the selectable cassette or not, have clearly demonstrated the possible interference of the introduced cassette on gene expression, generally manifested as an inhibitory effect (Fiering *et al.*, 1995; McDevitt *et al.*, 1997; Xu *et al.*, 1996; also see later). It is assumed that this effect is the result of competition between the promoter sequences driving expression of the selectable marker genes and endogenous regulatory sequences at the targeted locus. This finding

mandates that, in future experiments, these potential drawbacks be taken into consideration. Because the following experiments were carried out using gene targeted animals where the selectable marker gene was either left in or removed, the results must be interpreted cautiously.

B. Analysis of Enhancer Elements of Lymphoid Genes by Gene Targeting

Studies published to date on the analysis of lymphoid gene enhancers by the gene targeting approach have concentrated on five distinct well-established elements, including two from TCR genes (the TCRβ and TCRα gene enhancers) and three from Ig genes (the IgH intronic enhancer and the IgLκ intronic and IgLκ 3′ enhancers). The position of each of these elements within their respective locus is schematized in Figs. 1 and 2, respectively. Each element was previously shown to increase dramatically the level of V(D)J recombination of associated variable gene segments within transgenic recombination substrates (Sleckman *et al.*, 1996, and references within). Gene targeting of the TCRβ enhancer is discussed first because it is the only locus with only one known transcriptional enhancer. Thus the interpretation of the resulting phenotype is more straightforward than those of gene targeting experiments at the other antigen receptor loci.

1. Analysis of the TCRβ Gene Enhancer

A single transcriptional enhancer element has been described at the murine TCRβ gene locus (Krimpenfort *et al.*, 1988; McDougall *et al.*,

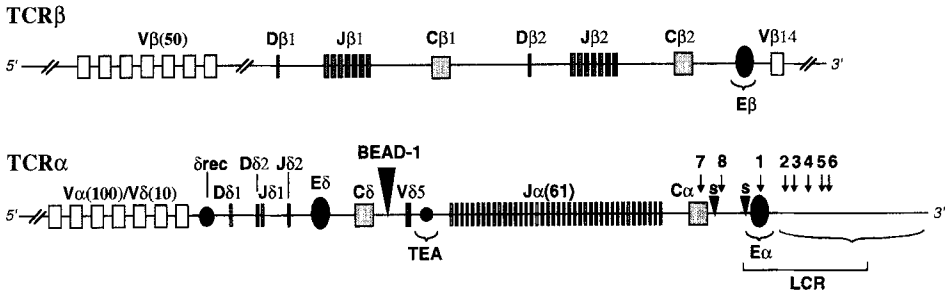

FIG. 1. Schematic diagram of *cis*-acting elements of TCRβ and TCRα loci targeted by homologous recombination. V region genes are designated by white rectangles, D and J segments by dark gray bars, and C region genes by light gray rectangles. All *cis*-acting elements are marked in black. Transcriptional enhancers appear as large ovals. At the TCRα locus, the TEA promoter appears as a circle and the transcriptional silencers (S) and BEAD-1 element as arrowheads. DNase I hypersensitive sites (1 to 8) of the LCR at the TCRα locus are indicated by arrows. Targeted regions discussed in this review are underscored with an open bracket and labeled below the diagram of the specific locus. This figure is not drawn to scale.

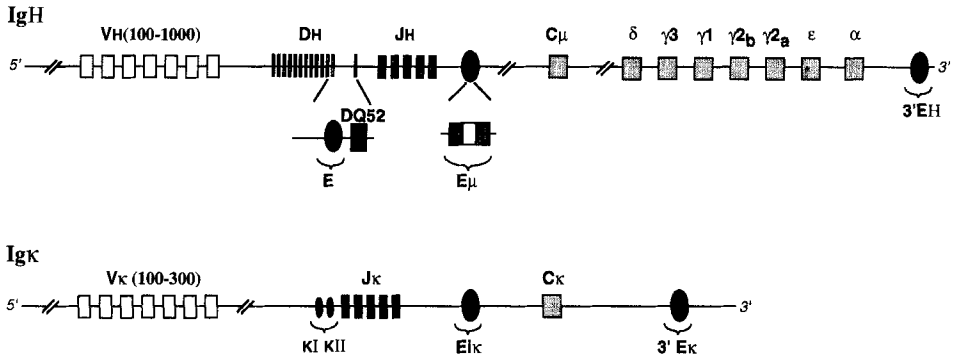

FIG. 2. Schematic diagram of *cis*-acting elements of IgH and IgLκ loci targeted by homologous recombination (not drawn to scale). Labeling is as in Fig. 1 with the exceptions that, at the IgH locus, MAR sequences associated with the Eμ enhancer are indicated by shaded rectangles flanking the core enhancer indicated by a white rectangle; at the IgLκ locus, KI and KII sites appear as small ovals.

1988). TCRβ enhancer sequences are included within a 560-bp fragment (referred to as Eβ) located at one extremity of the ~0.5-Mbase TCRβ gene locus, between the Cβ2 constant gene region and the inverted Vβ 14 gene segment (Fig. 1). Two groups have reported on the effects of the Eβ fragment deletion on rearrangement and expression of the TCRβ locus (Bories *et al.*, 1996; Bouvier *et al.*, 1996). In both studies, the 560-bp Eβ fragment on one TCRβ allele was first replaced with a selectable *neo*^{r} cassette flanked by *loxP* sites, using homologous recombination in ES cells. Afterward, the *neo*^{r} cassette was deleted from the targeted chromosome by transfection of a Cre expression vector into the recombinant ES cell clones. The heterozygously modified (Eβ$^{+/-}$) ES cells were then injected into either blastocysts from *RAG2* deficient (*RAG2*$^{-/-}$) mice (Bories *et al.*, 1996) or into blastocysts from wild-type mice (Bouvier *et al.*, 1996). Because the *RAG2*$^{-/-}$ mice lack T and B lymphocytes due to the fact that the TCR and Ig genes cannot be rearranged, those lymphocytes present in the resulting *RAG2*$^{-/-}$ chimeras must be derived from the injected ES cells. The use of this so-called "*RAG2*-deficient blastocyst complementation" (Chen *et al.*, 1993a) thus offered the opportunity to analyze directly the resulting chimeric mice for the consequences of the engineered Eβ$^{+/-}$ mutation (Bories *et al.*, 1996). In the other study, the analyses were performed following germline transmission of the Eβ$^{+/-}$ mutation and production of heterozygous (Eβ$^{+/-}$) and homozygous (Eβ$^{-/-}$) animals (Bouvier *et al.*, 1996). Irrespective of the strategy used to produce the mutant mice, analyses of lymphocytes in the two studies gave consistent results with

respect to DNA rearrangement events. Namely, V(D)J recombination at the targeted TCRβ allele(s), as analyzed by the presence of specific TCRβ coding and signal joints, was dramatically impaired at both the Dβ-to-Jβ and the Vβ-to-DJβ joining steps, but appeared not to be affected at the nontargeted TCRβ allele as well as at other TCR or Ig gene loci. Moreover, the Eβ targeted deletion also resulted in a profound decrease in TCRβ transcription as tested by sensitive reverse transcriptase (RT)-PCR assays, although a few Vβ germline transcripts could be detected in E$\beta^{-/-}$ thymocytes. In the same samples, no transcripts from TCR DJβ and VβDJβ rearranged genes were found. Finally, whereas the various lymphocyte populations from heterozygous mice (E$\beta^{+/-}$, *RAG2*$^{-/-}$ chimeras and E$\beta^{+/-}$ mice) were found in normal numbers and percentages as compared to wild-type mice (one TCRβ allele being functional), homozygous (E$\beta^{-/-}$) animals were deprived selectively of lymphocytes expressing a TCRβ chain at their surface, indicating that in the homozygous state the Eβ deletion selectively blocks $\alpha\beta$ T-cell development. Of note is the fact that the phenotype of these mice with respect to T-cell development is essentially identical to that of the TCR$\beta^{-/-}$ mouse in which the *neo*^{r} gene has been inserted in place of >15 kb of genomic DNA sequences extending from the Jβ1.3 segment down to the Cβ2 exons (Mombaerts *et al.*, 1991, 1992a). Taken together, these results formally establish the critical role that enhancer sequences within the Eβ fragment play in the *cis* activation (and/or the completion of the V(D)J recombination process; see later) at the mouse TCRβ gene locus during $\alpha\beta$ T-cell development. In addition, these results suggest that Eβ can act, with respect to V(D)J recombination, on sequences up to 20 kb away as there is a substantial decrease in Dβ1-to-Jβ1 recombination. It is unclear, however, whether Eβ can act on more distant sites (e.g., Vβ gene segments) as the Dβ-to-Jβ rearrangement is rate limiting at the mutated allele.

The study by Bories and colleagues (1996) also describes the analyses of chimeras produced following injection into *RAG2*$^{-/-}$ blastocyts of ES cells in which Eβ has been replaced by a DNA fragment containing the IgH intronic enhancer (Eμ). This element, located downstream of the JH cluster (see Fig. 2), is known to activate transcription from Ig and TCR gene promoters within transgenes in both B and T cells (Grosschedl *et al.*, 1984). In addition, within a transgenic TCRβ recombination substrate, Eμ can promote Dβ-to-Jβ joining in both lineages (Ferrier *et al.*, 1990). Two types of *RAG2*$^{-/-}$ chimeras were produced using selected ES cells in which the flanking *neo*^{r} gene has either been deleted (EμRΔ) or not (EμRNeo). By Southern blotting analysis, a significant level of Dβ-to-Jβ rearrangement was found in the T lymphocytes (albeit lower than when Eβ was left in place) but not in the B lymphocytes from the EμRΔ chimeric

mice. No Dβ-to-Jβ rearrangement was found in T or B lymphocytes from the EμRNeo mice. Thus, after removal of the *neo*^{r} gene, the inserted Eμ can stimulate endogenous TCRβ gene rearrangement in place of Eβ. Unexpectedly, in view of the fact that there was no Dβ-to-Jβ rearrangement in B cells, Northern blot analysis detected constant-region β chain (Cβ) hybridizing transcripts in total RNA from splenic B cells of the EμRΔ chimeras, indicating that replacement of Eβ with Eμ can activate germline transcription of the TCRβ locus in B cells. Although germline transcription in V(D)J recombinase-expressing B-cell progenitors from the same mice remains to be formally demonstrated, this finding is in line with previous reports that describe enhancer-dependent transcriptional activation in the absence of V(D)J recombination within a transgenic TCRβ substrate (Okada and Alt, 1994; Fernex *et al.*, 1995). However, the stronger level of Eμ-driven Dβ-to-Jβ rearrangement in T versus B cells from the EμRΔ chimeras contrasts with the previous transgenic studies in which such a rearrangement occurred equally well in the two lineages. The simplest explanation for these contrasting results is to postulate that additional negative elements are present at the TCRβ locus (i.e., absent from the TCRβ recombination transgene), which, in B lineage cells, downmodulate V(D)J recombination. Finally, it is noteworthy that the effect of the Eβ to Eμ replacement was only manifest in lymphocytes of the EμRΔ chimeras but not the EμRNeo chimeras, underscoring the inhibitory effect that insertion of a selectable marker gene and/or its expression can exert on *cis* recombination at the TCRβ locus.

2. *Deletion of the TCRα Enhancer*

As already mentioned, the elements that encode the TCRα and δ proteins are found in a single gene locus (Hood *et al.*, 1995; also see Fig. 1). The Vα and Vδ segments are found in the most 5′ portion of the locus, and the Jα segments and Cα gene lie in the most 3′ region. The Dδ and Jδ segments and the Cδ gene lie between these regions and, as a result, are excised on Vα-to-Jα rearrangement. Accordingly, recombinase targeting in this locus must be tightly regulated within the developmental context of the cell as uncommitted T cells differentiate along either an αβ or an γδ lineage pathway (reviewed by Kisielow and von Boehmer, 1995). The initial events that occur in this locus include germline transcription and rearrangement of the TCRδ gene in thymocytes that are not yet committed to either lineage. These cells then proceed along developmental pathways that lead to either γδ or αβ cells. Cells that differentiate along the γδ pathway fail to undergo Vα-to-Jα rearrangements (such events would delete the rearranged TCRδ gene), whereas cells that differentiate along the αβ pathway initiate germline transcription of the TCR-Jα region and

undergo Vα-to-Jα rearrangements. These rearrangements proceed to completion on both alleles failing to exhibit properties of allelic exclusion. This differential targeting of the TCRα/δ locus during T-cell development is likely regulated by *cis*-acting elements within the locus. As schematized in Fig. 1, these include the TCRα and δ enhancers (Eα and Eδ) (Winoto and Baltimore, 1989b; Gill *et al.*, 1991), a promoter of germline TCR-Jα transcription (TEA) (de Chasseval and De Villartay, 1993), two silencer elements proposed to silence the effect of Eα in γδ cells (Winoto and Baltimore, 1989a), a locus control region (Diaz *et al.*, 1994), and an insulator (BEAD-1) that lies between the Cδ gene and the Jα gene segments (Zhong and Krangel, 1997).

The Cre-*loxP* strategy has been used to delete a 1.1-kb fragment containing Eα, without disrupting the silencer elements 5′ or the LCR 3′ of the enhancer (Sleckman *et al.*, 1997). Eα was replaced on both alleles in ES cells with a *neo*^{r} selectable cassette, flanked by *loxP* sites, by culturing the selected heterozygous recombinants in increasing concentrations of the drug G418. Subsequently, the *neo*^{r} cassette was removed following transfection of the ES cells with a Cre-expressing plasmid. The ES cell-derived T cells were studied using *RAG*2-deficient blastocyst complementation. In addition, heterozygous recombinant ES cells were used to generate chimeras for germline transmission, and the resulting (+/−) animals were bred to generate mice homozygous for the targeted allele. These mice were subsequently crossed with mice carrying a Cre-expressing transgene to achieve deletion of the *neo*^{r} cassette. Both mice generated by *RAG*2-deficient blastocyst complementation and germline transmission were used for subsequent analysis. In either case, homozygous deletion of Eα resulted in a major reduction in the level of Vα-to-Jα recombination compared to that in wild-type or Eα$^{+/-}$ mice, whereas TCRδ rearrangement was unaffected. Accordingly, thymocyte development is blocked at the CD4$^+$CD8$^+$ double positive (DP) cell stage in Eα$^{-/-}$ mice, just prior to the time when TCRα would be expressed. Furthermore, analysis of mice heterozygous for the targeted allele, as well as hybridomas generated from the peripheral T cells of such mice, demonstrated that the affect of the Eα deletion on recombination acts in *cis* as only the targeted allele was affected. Interestingly, the targeted allele of one hybridoma had rearranged, suggesting that there is a low level of TCRα rearrangement in the absence of Eα. In agreement with this observation was the fact that Eα$^{-/-}$ mice did contain αβ T cells in the periphery. However, analysis of TCRβ$^+$ T-cell hybridomas demonstrated severely restricted Vα usage. In addition to the effects on recombination at the TCRα locus, homozygous deletion of Eα resulted in the loss of germline transcription from the TEA promoter, which normally occurs during the CD4$^-$CD8$^-$ double negative

(DN) to DP cell stage transition, immediately before the onset of TCR-Jα rearrangement (Wilson *et al.*, 1996). Furthermore, an unexpected reduction in transcription of the rearranged TCRδ alleles was observed in $\gamma\delta$ T cells (as well as $\gamma\delta$ T hybridomas) derived from the E$\alpha^{-/-}$ mice.

Taken together, these results demonstrate that Eα plays an important role in TCRα rearrangement, although a low level of rearrangements can occur in its absence. In addition, it is required for full Vα gene utilization, suggesting that it can operate over large distances. Finally, although Eα does not appear to be required for TCRδ rearrangement, demonstrating the temporal and spatial partitioning of the TCRα/δ locus, it is required for optimal expression of rearranged TCRδ genes in $\gamma\delta$ T cells, suggesting that partitioning of this locus is not absolute

3. *Analysis of the IgH Intronic Enhancer*

Serve and Sablitzky (1993) used the "hit and run" procedure to delete a 1-kb DNA fragment containing the Eμ core enhancer region as well as two flanking matrix attachment regions (MARs) from the IgH gene locus (also see Fig. 2). Using this procedure, which involves two successive steps of drug selection, it was possible to isolate clones of mutated ES cells in which the Eμ-containing fragment has been replaced by an irrelevant 20-bp oligonucleotide, while removing from the targeted locus the selectable marker genes (*neo*^{r} + thymidine kinase genes) introduced by the initial homologous recombination event. Clones of selected ES cells were injected into wild-type blastocysts to produce chimeric mice. Analyses of the effects of the targeted mutation on IgH gene rearrangement and expression were performed on cell-sorter purified splenic B cells from the resulting chimeras, taking advantage of differences in surface IgD allotypes between ES cell-derived and host (blastocyst)-derived B cells. Using semiquantitative PCR analyses, the authors were able to demonstrate that 15–30% of the mutated loci remained in a germline configuration in ES cell-derived B splenocytes, implying a significant inhibition of V(D)J recombination at the targeted allele (usually, all IgH loci are rearranged in mature B cells from normal mice). Obviously, the inhibition of V(D)J recombination at the targeted allele in this study is less striking than that observed for the TCR Eβ or Eα deletions. Yet, while a fairly high proportion (65–80%) of the mutated alleles in the ES-derived splenic B cells underwent D-to-J_H rearrangements, V_H-to-DJ_H rearrangements were much less frequent (3–6%). In contrast, all the nonmutated alleles within the sorted B cells were found to carry either V_HDJ_H (in their vast majority) or DJ_H rearrangements. A careful PCR analysis of the latter (DJ_H) rearrangements indicated that most of them were likely due to contaminating, host-derived wild-type B cells. However, a few DJ rearrangements were truly

carried by the wild-type allele in ES-derived B cells, implying that those cells were purified based on the expression of the V_HDJ_H rearranged, mutated allele. It was known from studies of cell lines of B-cell origin that spontaneous deletion of the IgH intronic enhancer does not obligatorily affect Ig gene expression (Wabl and Burrows, 1984; Zaller and Eckhardt, 1985). The study of Serve and Sablitzky (1993) thus shows that even in the absence of the Eμ-containing fragment, V_HDJ_H rearrangement can occur and the rearranged gene is transcriptionally active and can be expressed at the protein level. This finding suggests that homozygous deletion of Eμ would still permit the development of some B cells, although this would have to be confirmed. Nevertheless, this study shows that Eμ is an important *cis*-acting element required for efficient V(D)J recombination in B lymphocytes, particularly for V_H-to-DJ_H rearrangement.

The IgH intronic enhancer region has also been analyzed by gene targeting approaches in the laboratory of F. W. Alt (Chen *et al.*, 1993b). In this study, the 1-kb (Eμ + MARs) containing fragment was replaced by a *neo*^{r} gene under the control of a strong promoter (pGK). In addition, the same pGK-neo^r cassette was used to mutate each of the Eμ flanking sequences. Thus, an overlapping 0.7-kb fragment downstream of Eμ, including associated 3′ MAR sequences, was replaced, whereas the Eμ 5′ MAR sequences were simply moved away from the enhancer by insertion of the pGK–neo^r cassette. The various mutated ES cells (from mice of the 129 strain) were used to inject C57BL/6J blastocysts and produce chimeras. From these animals, pre-B-cell lines were established by A-MuLV infection of bone marrow; a high proportion (>90%) of the cell lines were from ES cell origin due to the more efficient A-MuLV transformation of cells from 129 rather than C57BL/6J origin. Comparison, in individual pre-B-cell lines, of the rearrangement status of the normal and mutated alleles by Southern blotting analysis and their expression by Northern blot and nuclear run-on assays gave the following results. (i) Replacement of the 0.7-kb region downstream of Eμ by the pGK–neo^r cassette has no obvious effects on *cis* rearrangement or expression of the altered allele. (ii) In striking contrast, replacement of the 1-kb Eμ-containing fragment resulted in a *cis*-acting block in J_H rearrangement in most of the cell lines that were analyzed. (iii) Surprisingly, the same effect was observed in transformants derived from the *neo*^{r} gene insertion in the MAR sequences 5′ of Eμ. Additional analyses of the transcriptional and methylation status of the mutated alleles also indicated that Eμ replacement led to *cis* inhibition of both germline transcription and demethylation, whereas the 5′ insertion led to *cis* inhibition of germline transcription but not demethylation. Therefore, the authors conclude that the integrity of the Eμ and upstream flanking sequences is required for efficient rearrangement at the J_H locus and that demethyl-

ation of this locus does not necessarily make it a good substrate for V(D)J recombination. However, they point out the tight correlation between germline transcription and rearrangement of the J_H locus. Finally, these results must be interpreted in light of the fact that the experiments were carried out in the presence of the *neo*^{r} gene, with the attendant problems already discussed.

In summary, the two studies led to the conclusion that the μ enhancer is important for efficient V(D)J rearrangement but is not absolutely required. The quantitative differences between the two studies may well be due to the existence of an expressed *neo*^{r} gene in the latter but not the former study, although differences due to the type of cells that were analyzed in the two studies (pre-B versus mature B cells, the latter possibly being able to accumulate DJ_H rearrangements) cannot be excluded. The lowered efficiency of V(D)J recombination in the absence of Eμ suggests that the main function of Eμ, with respect to this process, may be to act in concert with other regulatory *cis*-acting elements to increase recombinational accessibility at the IgH locus. One possibility is an enhancer immediately 5′ of DQ52 (Kottmann *et al.*, 1992, 1994). It has been suggested that this enhancer may play an important role in increasing recombinational accessibility prior to D-to-J_H rearrangement. Indeed, preliminary results have shown that mice in which the DQ52 region was replaced by a single *loxP* site demonstrated a general reduction in (i) the recombination frequency at the mutated allele and (ii) the number of B cells that expressed antigen receptors originating from this allele (Pelkonen *et al.*, 1997). An additional possibility would be the enhancer elements ($3'E_H$) that lie downstream of the Cα gene (Lieberson *et al.*, 1991; Dariavach *et al.*, 1991). In a study where some of these sequences were replaced by the *neo*^{r} gene, no deficiency of V(D)J recombination was reported; instead, there was an effect on IgH switch recombination and constant region germline transcription of certain IgH constant region genes (Cogné *et al.*, 1994). Although these findings imply that the $3'E_H$ sequences are not required for IgH gene assembly, it would be informative to study this process using mice in which both Eμ and $3'E_H$ have been mutated on the same allele. Disruption of $3'E_H$ sequences by a *neo*^{r} gene in an Ig-producing cell line that already lacks Eμ was shown to result in the abolition of IgH gene expression, demonstrating the ability of these sequences to function efficiently over a large distance in the absence of Eμ (Lieberson *et al.*, 1995). Finally, although it is generally accepted that the *trans*-acting, factor-binding motifs within Eμ are critical for V(D)J recombination, it is not yet known whether the promoter of the so-called Iμ transcripts (Su and Kadesh, 1990) or at least one of the MAR elements and associated regulatory sequences (Scheuermann and Chen, 1989; Ariizumi *et al.*, 1993; Herrscher *et al.*,

1995) play an additional role as the two Eμ gene targeting studies included these elements as well.

4. Deletion of the Igk Intronic and 3′ Enhancers

Two enhancer elements have been described at the IgLκ locus, which are located on either side of the unique Cκ region and are referred to as the IgLκ intronic enhancer and the IgLκ 3′ enhancer (Fig. 2). These two elements have been individually subjected to replacement by a *loxP* flanked *neo*^{r} cassette by the Baltimore and Alt groups, followed by *neo*^{r} gene deletion using the Cre/*loxP* approach (Xu *et al.*, 1996; Gorman *et al.*, 1996). In a previous study, the IgLκ intronic enhancer was replaced with the selectable *neo*^{r} gene in the Rajewsky laboratory (Takeda *et al.*, 1993).

In the Baltimore study, the intronic IgLκ enhancer (Eiκ), together with flanked MAR sequences (740-bp fragment: MiEκ), was replaced on both alleles in ES cells with a pGK-*neo*^{r} selectable cassette, flanked by *loxP* sites (EκNI) (for details, see Xu *et al.*, 1996). Subsequently, the *neo*^{r} cassettes were removed following transfection of the ES cells with a Cre-expressing plasmid, resulting in a single *loxP* site on both alleles (EκND). The ES cell-derived B cells were assayed for IgLκ gene rearrangement, IgLκ cell surface expression, and the ratio of IgLκ versus IgLλ expression, using *RAG2*$^{-/-}$ blastocyst complementation. These analyses showed that B cells derived from the EκND-mutated ES cells have reduced levels of κ rearrangement, normal levels of κ expression, and a κ:λ ratio of 1:1 (the usual ratio in the mouse is >20:1). In addition, λ-producing hybridomas from EκND B cells displayed little κ rearrangement. This is an unusual finding in light of the fact that in 97% of normal λ-expressing B cells, the κ locus of at least one allele has either gone through one or more rounds of nonproductive rearrangement or been deleted through a particular V(D)J recombination reaction called RS rearrangement (Shimizu *et al.*, 1991). In addition, while 45% of κ-producing hybridomas derived from normal mice demonstrated κ rearrangement on both alleles, most of the κ-producing hybridomas derived from the EκND mice had undergone κ rearrangement on only one allele. Thus, the ratio of κ:λ-producing cells of 1:1 underestimates the true effect on κ rearrangement of the MiEκ deletion. Finally, in agreement with previous transgenic studies (Betz *et al.*, 1994), MEiκ sequences do not appear necessary for efficient transcription of the rearranged κ gene as the surface level of IgLκ expression on EκND-derived B cells was equivalent to that on normal B cells. In contrast to the results observed for EκND-derived B cells, the EκNI-derived B cells lacked IgLκ rearrangement and virtually no κ-expressing B cells were found in these mice. These latter results are entirely consistent with the Rajewsky study in which the intronic enhancer was similarly replaced with

a *neo*^{r} selectable cassette (Takeda *et al.*, 1993). Altogether these analyses indicate that MAR and Eiκ sequences contribute to the process of κ rearrangement but are not strictly necessary.

Interestingly, targeting of the 808-bp fragment of the IgLκ 3′ enhancer (see Fig. 2) yielded similar results (Gorman *et al.*, 1996). In this study, heterozygous ES cell lines containing a *loxP*-flanked *neo*^{r} gene in place of the IgLκ 3′ enhancer were used to generate mouse lines carrying the mutation in their germline (3′EκN/N). The *neo*^{r} cassette was then removed by mating the mutant mice with a transgenic strain expressing Cre. The resulting mice (3′E$\kappa\Delta/\Delta$) were assayed for IgLκ gene rearrangement, IgLκ cell surface expression, and the ratio of IgLκ versus IgLλ expression of resting and activated splenic B cells. As seen in the previous study, there was a dramatic decrease in the κ:λ ratio (2:1) resulting from both a decrease in the level of κ^+ and an increase in the level of λ^+ B cells. Furthermore, analysis of heterozygous mice showed a reduction in rearrangement of the targeted allele, and in accord with the previous study, both κ and RS rearrangements were reduced in λ^+ 3′E$\kappa\Delta/\Delta$ B cells, albeit to a lesser degree. Interestingly, there was a differential effect of the 3′Eκ deletion on expression of rearranged IgLκ genes in resting versus LPS activated splenic B cells. Although there was a threefold decrease in the level of IgLκ RNA transcripts in resting 3′E$\kappa\Delta/\Delta$ B cells as compared to normal B cells, the 3′Eκ deletion had only a minimal effect on κ expression in LPS-activated mutant B cells. This finding is surprising in light of the fact that transgenic studies suggested that the IgLκ 3′ enhancer plays a significant role in high level expression of rearranged κ transgenes containing the MiEκ element (e.g., see Betz *et al.*, 1994) and points toward the possibility that other *cis* elements (i.e., besides enhancers) may be present in the endogenous IgLκ locus. Also, the 3′Eκ deletion does not appear to disturb lineage as well as temporal specificity of IgLκ endogenous rearrangement (F. W. Alt, personal communication), in disagreement with the conclusions from a previous transgenic study on the very same element (Hiramatsu *et al.*, 1995). Finally, mice derived from mutated ES cells still harboring the *neo*^{r} selectable cassette showed a similar, but more pronounced, phenotype. Altogether these results indicate that, as in the case of the Eiκ element, the 3′ IgLκ enhancer is critical for establishing the κ:λ ratio in the mouse, but is not absolutely required for κ gene rearrangement or for normal κ expression in activated B cells.

It is striking to note that gene targeting of the two major κ enhancers yielded such similar results, suggesting that both carry out largely overlapping functions. Furthermore, it is interesting that neither appears to play a major role in regulating transcription of rearranged IgLκ genes. However, it is also possible that, given the seemingly overlapping functions of the

two enhancers, the remaining enhancer is able to compensate for the transcriptional function of the deleted one. It is most probable that the two enhancers act in concert, possibly along with other *cis*-acting elements at the κ locus (see later), to regulate recombinational accessibility and transcription. To completely appreciate the relative contributions of each, it will first be necessary to carry out a double gene targeting experiment to delete both enhancers on the same allele. It is tempting to speculate that such an experiment would yield a far more striking effect than the studies just described.

C. Targeted Mutation of Other Types of *cis*-Regulatory Elements

The gene targeting experiments of antigen receptor transcriptional enhancers described earlier clearly demonstrate that these are critical *cis*-acting elements required for recombinational accessibility. In each case, however, these experiments have suggested that there may also be other *cis*-acting elements that are required for the precise control of V(D)J recombination. Consequently, more recent gene targeting experiments on such elements have been performed and are described for IgLκ and TCRα/δ loci as well as yet uncharacterized elements at the TCRγ locus.

1. Definition of a Rearrangement-Enhancing Element Upstream of Mouse Jκ Gene Segments

Transgenic mouse experiments using a portion of the chicken IgLλ gene as a recombination substrate have defined *cis*-regulatory sequences located within the Vλ–Jλ intervening gene region endowed with rearrangement-enhancing activity (Lauster *et al.*, 1993). Specifically, it was proposed that these sequences, when bound by nuclear factors during chicken B-cell differentiation, transiently derepress a silencer located in between the Vλ and Jλ segments. The silencer sequences have been shown to constitutively counteract V(D)J recombination in transgenic studies as well as transcription (as measured by CAT assays) in cell transfection studies. The model argues implicitly that the result from this activity would then be Vλ-to-Jλ rearrangement on one derepressed allele only, thus contributing to the process of allelic exclusion at the IgLλ locus.

Sequences homologous to those in the chicken locus exist just 5′ of the IgL-Jκ cluster in the mouse genome. These two palindromic sequences, called KI and KII, were previously found to interact with a factor(s) present in nuclear extracts from B-cell lines, but not those from other lymphoid as well as nonlymphoid cell lines (Weaver and Baltimore, 1987). Ferradini and colleagues (1996) have used homologous recombination techniques, including the Cre-*loxP* system, to introduce, within the KI and KII se-

quences, specific mutations predicted to abolish DNA–protein interactions based on the results of previous methylation interference assays.[1] Southern blotting and PCR analyses led the authors to estimate that, in heterozygous animals, the rearrangement frequency on the targeted allele is reduced (by around 80%) in comparison to the wild-type allele. However, levels of germline Jκ transcripts and CpG methylation were found to be similar between wild-type and mutant alleles. Therefore, the authors propose that mutation of the KI and KII sites acts in *cis* to reduce Vκ-to-Jκ rearrangement without preventing accessibility of the Jκ locus, as monitored by germline transcription, methylation status, and the baseline level of V(D)J recombination. However, the importance of these two sites on the differentiation process of Igκ-expressing cells still needs to be precisely defined since, surprisingly enough in view of the inhibition found in the heterozygous situation, homozygous mice for the KI-KII mutations have peripheral B cells in normal numbers with an unmodified $\kappa:\lambda$ ratio. The authors handle this conundrum by suggesting that while transcriptional enhancers act as accessibility factors by altering chromatin structure, KI-KII participate in the regulation of Vκ-to-Jκ rearrangement. The precise role hypothesized for KI-KII is as an antisilencer, which lifts repression on one allele during B-cell differentiation, thus allowing recombination and subsequent feedback inhibition of recombination of the other allele. Further experiments will need to be carried out in the future to confirm this model.

2. *Effect of the TEA Deletion*

T early α (TEA) transcripts have been defined as germline transcripts that initiate upstream of the TCR-Jα cluster within the TCRδ/α locus (De Villartay *et al.*, 1987; Shimizu *et al.*, 1993). In developing thymocytes, germline TEA transcription is activated during the transition from the $CD4^-CD8^-$ DN to the $CD4^+CD8^+$ DP cell stages, just prior to the onset of Vα-to-Jα rearrangement (Wilson *et al.*, 1996; Villey *et al.*, 1997). This property has led to the hypothesis that TEA transcription (and/or the transcripts themselves) is required to target the recombination machinery to the TCR-Jα cluster. The TEA element is composed of a promoter region and a single gene exon located within a 2-kb DNA region 5′ of the most 5′ Jα (Jα61) gene segment (de Chasseval and De Villartay, 1993; also see Fig. 1).

To verify this hypothesis, the TEA element has been replaced by a recombined *loxP* site in ES cells (Villey *et al.*, 1996). Mice heterozygous

[1] In this study, a control experiment was performed to evaluate the influence of the remaining *loxP* site following Cre-mediated excision on V(D)J recombination within the IgLκ locus, in the absence of any mutation at the KI and KII sites. It was found that a single *loxP* site does not affect by itself the rearrangement frequency, implying that this site is neutral with respect to recombinational accessibility.

and homozygous for the targeted mutation were produced and analyzed with respect to TEA transcription, Vα-to-Jα rearrangement, and αβ T-cell development. The $TEA^{-/-}$ animals demonstrated normal numbers of αβ T cells, immediately ruling out an absolute requirement for TEA in inducing Vα-to-Jα rearrangement. In addition, although no TEA transcripts were found in T cells from animals crossed with $RAG2^{-/-}$ mice and treated with anti-CD3 (to promote differentiation to the DP cell stage), transcripts that hybridized with a Cα-specific probe were detected, suggesting that other *cis*-acting elements capable of regulating recombinational accessibility may be scattered within the TCR-Jα cluster. Interestingly, however, Southern blot analyses using a probe specific for a 5′ region of the TCR-Jα cluster demonstrated that while purified T cells from wild-type mice had no remaining 5′ TCR-Jα germline sequences (both TCRα alleles undergoing deletional Vα-to-Jα rearrangement), purified T cells from $TEA^{+/-}$ mice retained a significant level of such sequences at the targeted allele, suggesting that much of the TCR-Jα cluster was unrearranged in $TEA^{+/-}$ T cells. However, when a similar analysis was carried out on T-cell hybridomas derived from the same mice, there was no detectable germline TCR-Jα DNA. This seeming contradiction is explained by the fact that excised DNA circles generated from Vα-to-Jα rearrangement persist in purified T cells whereas they do not in hybridomas, and hence, the detected germline sequences are actually found on the DNA circles (Livak *et al.*, 1995; Livak and Schatz, 1996). This conclusion was supported by performing (i) quantitative Southern blotting analyses and (ii) pulsed field gel electrophoresis on $TEA^{-/-}$ versus wild-type thymocyte DNA. Thus, in $TEA^{-/-}$ thymocytes, it was observed that there was (i) a large reduction in the utilization of the upstream Jα segments for V(D)J *cis* recombination (Jα61 to Jα53—spanning 7 kb on the 5′ side of the TCR-Jα cluster) and (ii) the unrearranged segments were not retained within the TCRα/δ gene locus. Altogether, these findings confirm that the TEA mutation compromises rearrangement on the most 5′ TCR-Jα segments, strongly suggesting that the TEA element contributes in targeting recombinase activity to a narrow window within the TCR-Jα cluster. This was further substantiated by comparative RT-PCR analysis of mature (VαJαCα) transcripts generated from either $TEA^{-/-}$ or wild-type T cells throughout the TCR-Jα cluster. This approach revealed that, in $TEA^{-/-}$ T cells, the most 5′ Jα segments were underutilized, with Jα utilization increasing as one progresses 3′ along the cluster. In fact, the most 3′ Jα segments were used more than those in wild-type mice, suggesting that there may also be a compensation mechanism for the decreased 5′ Jα segment usage or that another *cis*-regulatory element(s) on the 3′ side of the TCR-Jα cluster, such as the TCRα enhancer or LCR (see later), exerts a greater effect on

recombinational accessibility of the 3′ Jα segments in the absence of the TEA element.

These are the first experiments that identify a recombination *cis*-activating element, the activity of which is exerted within a local area. These results raise the question of whether similar elements are spread over the entire TCR-Jα gene cluster. It is interesting to note in this regard that despite the fact that the Jα cluster spans 70-kb, of which only 6% are coding sequences, there is a significant level of sequence homology between species, implying that there may be important functional elements within this region (Koop and Hood, 1994). If this were the case, it would offer a molecular explanation for the observed synchronous recombination of the two alleles along the entire TCR-Jα cluster (Malissen *et al.*, 1992). More generally, this study suggests a role in recombinational accessibility for similar elements in the other Ig and TCR gene loci and, particularly, for the promoters upstream of the various V gene segments.

3. *Other cis-Regulatory Elements That May Influence Ordered Rearrangements*

a. A Locus Control Region (LCR) Downstream of the TCRα/δ Locus? A set of DNase I hypersensitive sites, including one within the well-characterized TCRα gene enhancer (Eα), has been identified downstream of the TCRα constant region. On the basis of transgenic mouse experiments, it was reported that all of the HS sites were required in addition to Eα to confer integration site-independent, copy-number-dependent, and tissue-restricted expression of associated transgenes (Diaz *et al.*, 1994; Ortiz *et al.*, 1997). These properties are characteristic of a type of *cis*-regulatory element known as a locus control region (LCR), initially described within the β-like globin gene complex (Martin *et al.*, 1996, and references within). By analogy with the β-globin LCR, which has been shown to participate in organizing the chromatin configuration of the entire β-like globin gene locus, Winoto and colleagues have proposed a model in which the TCRα downstream sequences, acting as an LCR element, could be shared by the two TCRδ and TCRα genes and play a major role in the control of the differential recombination and expression of the two parts of this locus (Diaz *et al.*, 1994).

This model has been tested by targeted deletion of a 10-kb region immediately 3′ of the Eα element, including most of the HS sites responsible for LCR activity in transgenes (Hong *et al.*, 1997; also see Fig. 1). The *neo*^{r} gene was left in place at the mutated locus. Possibly because the targeted deletion also affected the third exon of a closely located, inversely oriented gene, DAD1, no animals homozygous for the mutation could be obtained. Instead, the heterozygous mutants were mated with TCRα$^{-/-}$

(Mombaerts *et al.*, 1992a) or TCR$\delta^{-/-}$ (Itohara *et al.*, 1993) mice, so that only the TCR chains from the mutated allele could be expressed. No difference was found in the development of $\alpha\beta$ and $\gamma\delta$ cells in the double mutated animals either in the thymus or in the periphery when compared to control wild-type mice. Furthermore, in the whole thymus, the expression of the targeted TCRα allele as measured by RNase protection and flow cytometry was unaffected. The only identified consequence of the mutation seems to be a modest reduction in the number of mature thymocytes expressing high levels of TCRα. One interpretation that the authors provide to explain the minor effect of the LCR deletion is that the transcription of the residual *neo*^{r} gene may render the chromatin accessible to the recombination machinery, in affect replacing the normal function of the LCR at the targeted TCRα/δ locus. Although this interpretation runs counter to the usual effect of an inserted *neo*^{r} cassette on gene expression (Olson *et al.*, 1996), β-globin LCR activity (Kim *et al.*, 1992), or V(D)J recombination (see earlier), it is not out of the realm of possibility. In any case, the credibility of the TCRα LCR is at stake unless this hypothesis can be verified by removing the selectable cassette after targeting.

b. Putative cis-Regulatory Elements Associated with Vγ Gene Segments? During thymic ontogeny in the mouse, Vγ-to-Jγ recombination in the TCRγ1 gene cluster is ordered in such a way that Vγ gene segments rearrange in the following order: Vγ3 → Vγ4 → Vγ2 (reviewed by Raulet *et al.*, 1991). Interestingly, the order of appearance of each Vγ gene reflects the position of the individual Vγ segments relative to the Jγ1 segment on the chromosome and is maintained in TCRδ knockout mice that do not express the TCR$\gamma\delta$ receptor. These findings suggest that ordered rearrangement at the TCRγ1 locus reflects a temporally programmed intracellular mechanism rather than a cellular selection process. To further investigate the mechanism responsible for such an ordered rearrangement, Sunaga *et al.* (1997) used targeted deletion of the intermediate Vγ4 gene, using the Cre-*loxP* system. Thus, in the mutant mice, the Vγ2 gene is located close to Vγ3. However, the mutant mice showed the same temporal profile of rearrangement of the remaining Vγ genes as wild-type mice, and no significant difference in the level of germline transcripts of the Vγ3 and Vγ2 genes was found in mutant versus control animals. Therefore, the temporal order of TCRγ1 gene rearrangement during thymic ontogeny seems to be controlled by neither the distance nor the relative order of the individual Vγ genes relative to Jγ1. This would favor control by *cis*-regulatory elements associated with the individual Vγ segments rather than the competition of these segments for a putative 3′ distal regulatory element.

IV. Targeted Mutation of *trans*-Regulatory Factors

Many of the *cis*-acting elements, including enhancers, that have been demonstrated to play a role in regulating V(D)J recombinational accessibility have been characterized with respect to the *trans*-acting DNA-binding proteins with which they interact (reviewed by Staudt and Lenardo, 1991; Leiden, 1993). Thus, another potential approach to examining the regulation of recombinational accessibility within the antigen receptor gene loci would be to use gene targeting to inactivate the *trans*-acting factors that bind to the *cis*-regulatory elements. To date, this approach has not borne much fruit because disruption of the corresponding genes has either had no effect on V(D)J recombination, possibly due to compensation by a related family member, or had such a major effect on general and/or lymphoid development that it is difficult to make any conclusion on the role of the factor on V(D)J recombination (Shivdasani and Orkin, 1996; Clevers and Grosschedl, 1996; Georgopoulos, 1997). In exception to this situation, analysis of gene targeted IL-7 receptor α chain (*IL7-Ra*$^{-/-}$) mice suggests that IL-7 or another IL-7R-binding ligand may play a critical role in activating V(D)J recombination (reviewed by Candéias *et al.*, 1997a).

IL-7 had already been shown to play a critical role in B- and T-cell development as mice treated with anti-IL-7 antibodies, as well as mice in which the *IL-7* gene has been inactivated by homologous recombination, demonstrate severely repressed B- and T-cell development (Grabstein *et al.*, 1993; Sudo *et al.*, 1993; Peschon *et al.*, 1994; von Freeden-Jeffry *et al.*, 1995). It has, however, been difficult to separate the possible trophic and mechanistic effects of IL-7 on B- and T-cell development, thus confounding the analysis of V(D)J recombination in *IL-7R*$\alpha^{-/-}$ mice. Nevertheless, the combined results of the *IL-7R*α chain gene targeting experiments suggest that the presence of IL-7Rα seems to be most critical for TCRγ gene rearrangement and expression, whereas it appears to aid rearrangement of IgH and TCRβ genes, but is not strictly necessary; it does not appear to be required for TCRα and TCRδ gene rearrangement (Candéias *et al.*, 1997a, and references within).

Initial reports indicated that TCRγ rearrangement in IL-7R$\alpha^{-/-}$ mice was absent or severely reduced whereas TCRδ gene rearrangement was unaffected, leading to a complete lack of mature $\gamma\delta$ T cells (Maki *et al.*, 1996; Candéias *et al.*, 1997b). However, a more recent report suggests that the situation is more complicated; whereas TCRγ rearrangements involving some Vγ genes were absent, rearrangements utilizing other Vγ genes were present, albeit at lower levels as compared to those in wild-type controls (Perumal *et al.*, 1997). Thus, the lack of $\gamma\delta$ T cells in these mice appeared to also result from the lack of transcription of the rearranged

TCRγ products. Although the precise role of IL-7Rα in V(D)J rearrangement at the TCRγ locus has yet to be clarified, these reports support a role for IL-7Rα in activating TCRγ gene rearrangement and expression.

A possible mechanism has been invoked for the effect of IL-7R signaling on the regulation of TCRγ rearrangement and/or expression. Normally, IL-7R-mediated signal transduction leads to the activation of the JAK3/STAT5 signaling pathway. Interestingly, there is a lack of STAT5-binding activity in IL-7R$^{-/-}$ mice (Perumal *et al.*, 1997). In light of the fact that the TCRγ locus contains several transcriptional enhancer elements (Spencer *et al.*, 1991; Vernooij *et al.*, 1993), of which some include STAT5 binding motifs, it is possible that disruption of the *IL-7Rα* gene and the subsequent lack of STAT5 activation can explain at least the lack of transcription of the rearranged TCRγ genes if not the reduction in V(D)J recombination at this locus as well. This set of studies is intriguing because they represent a possible connection between signaling at the plasma membrane and activation of V(D)J recombination, and may provide us with additional clues as to the identity of some of the nuclear DNA-binding factors involved in recombinational accessibility.

V. Biological Consequences

A. Different Types of *cis*-Regulatory Elements Contribute to the Control of V(D)J Recombinational Accessibility

This overview of the analysis of V(D)J recombination regulatory elements by the gene targeting approach clearly illustrates the importance of *cis*-acting transcriptional regulatory elements, particularly enhancers, during the onset of V(D)J recombinational accessibility at endogenous antigen receptor gene loci. Thus far, all gene targeting experiments of enhancers from either Ig or TCR gene loci have resulted in, at least, a strong inhibition of *cis* recombination throughout the targeted allele. Consequently, in cases where homozygous targeted mice were produced and analyzed, the enhancer mutation was shown to result in a significant block in lymphocyte development within the relevant lymphoid lineage and at the expected stage of the given cell differentiation pathway. Conversely, when similarly analyzed, other putative *cis*-regulatory elements such as a promoter (TEA), antisilencer (KI, KII sites), or LCR (TCRα LCR) seem to have a more localized or limited effect. Strikingly, in the case of the TCRβ enhancer, which may well be the only element of this type at the TCRβ gene locus, the effect of the targeted deletion was maximal as no traces of VβDJβ coding products could be found at the targeted allele (and hence no TCRβ expressing cells in homozygously Eβ-deleted mice), even after removal of the *neo*^{r} gene. The existence of at least two well-defined enhancer elements

at other loci (i.e., TCRα/δ, IgH, and IgLκ) might thus be responsible for the fact that, in those cases, single enhancer deletion resulted in a less substantial effect. Indeed, individual deletion of the two IgLκ enhancers gave very similar phenotypes in terms of the level of inhibition of V(D)J recombination. Therefore, it is quite likely that enhancers are generally critical elements for modulating recombinational accessibility at a given locus.

However, other targeting experiments such as, for example, the TEA deletion and the subsequent lack of utilization of the most 5′ Jα segments in recombination events strongly suggest that enhancers must cooperate with additional *cis*-regulatory elements to exert their recombinational activity. The strong inhibition of TCR–Jα recombination within Eα-deleted TCRα/δ loci would nevertheless indicate that, even at the local level, the function of these additional *cis*-regulatory elements and the corresponding enhancer are not redundant or equivalent. One essential feature of recombinational accessibility at some genomic regions may therefore depend on the coordinated utilization of two hierarchically distinct *cis* elements where a long range-acting element (i.e., the Eα enhancer) is required for the action of a local accessibility factor (i.e., the TEA promoter). The selective recombinational effect observed in T versus B lineage cells following replacement of Eβ by Eμ coupled to the complete lack of TCRβ rearrangement in the absence of the TCRβ enhancer may indicate that a similar requirement is in effect at the TCRβ gene locus. Likewise, the inhibitory effect on *cis* recombination of *neo*^{r} gene insertion upstream, but not downstream, of the Eμ element may result from the disruption of such a cooperative interaction at the IgH locus. It is expected that coordinated utilization of distinct elements within the same locus would contribute to enforce tight control of, for example, *cis* versus *trans* locus rearrangement and would account, at least in part, for the multiplicity of situations described with respect to recombinational accessibility at the various antigen receptor gene loci.

B. Molecular Basis of V(D)J Recombinational Accessibility

Our understanding of how regulatory elements, particularly enhancers, influence V(D)J recombinational accessibility is still limited. Notably, the long-standing question of the role, if any, of germline transcription (and/or transcripts) in the onset of rearrangement at given loci has not been resolved. In all gene targeting studies on transcriptional regulatory elements that have been reported so far, the resulting inhibitory effect on V(D)J recombination occurs exclusively in *cis* as only the targeted allele is affected and as the presence of a wild-type element on the nontargeted allele in the heterozygous mice is not sufficient to rescue the rearrangement

process. These findings suggest that the germline transcripts do not play a role per se in targeting the V(D)J recombinase activity, unless one invokes a model involving allele-specific transcripts analogous to those implicated, for example, in the process of X inactivation (Lyon, 1996; Willard, 1996). Moreover, it is worth emphasizing that, when analyzed, activation of germline transcription at some specific regions (i.e., upstream of J segments) also appears to be strongly inhibited following gene targeting of enhancer elements. Likewise, the localized inhibitory effect resulting from the TEA promoter mutation is best correlated to a parallel downregulation of TEA germline transcription (Villey *et al.*, 1997). For all these reasons, transcription cannot at this point be ruled out as playing a role in V(D)J recombinase targeting. The transcription process may contribute among other things to the establishment and/or maintenance of an accessible chromatin structure.

The observation in several cases of ongoing transcription in the absence of the obligatory formation of V(D)J coding joint products (e.g., in mutant mice in which Eβ has been replaced by Eμ—with the possible problem that only mature B cells were analyzed—see Section III,B,1) seems to eliminate a function that would solely be the consequence of the transcriptional enhancing activity conferred by these sequences. Another more consensual model predicts that nuclear factors bound at enhancer sequences may, in conjunction with similar or different factors bound to other *cis* elements, modify the chromatin structure of adjacent regions to render them more accessible to complex machineries such as the transcriptional and V(D)J recombinational complexes (Jenuwein *et al.*, 1993). McMurry *et al.* (1997) provide evidence to support this model. Recombinational accessibility, as assayed by DSB formation, was analyzed in transgenic mice with a human TCRδ locus minigene that also included the TCRδ enhancer, mutated forms of the enhancer, or no enhancer. It has already been established that Eδ appears to be important for VDδ-to-Jδ but not Vδ-to-Dδ recombination (Lauzurica and Krangel, 1994a,b; Hernández-Munain *et al.*, 1996; Lauzurica *et al.*, 1997). In this novel study, the minigenes that harbored a functional Eδ enhancer demonstrated abundant DSB 3′ of the Dδ and 5′ of the Jδ segments, whereas in the minigene without the enhancer or with a mutated enhancer there was a strong reduction of DSB 5′ of Jδ while the DSB 3′ of Dδ were unaffected (McMurry *et al.*, 1997). Thus, the presence of Eδ appeared to render the Jδ segments accessible to the recombinational machinery in a way that may have been dependent on transcription factor binding, whereas, interestingly, the Dδ segment was already accessible, perhaps due the presence of another unidentified *cis*-acting factor. In addition to this transgenic study, preliminary analyses of thymocytes from Eβ-mutated mice suggest that access of the V(D)J recombinase to the TCRβ D-J regions, as assayed

by the formation of DSB products, is not strongly impaired at the Eβ targeted alleles (W. Hempel, P. Stanhope-Baker, M. Schlissel, and P. Ferrier, unpublished data). Interestingly, the frequency of coding and signal joint formation is further reduced in the same cells, suggesting that Eβ may also play a role during a specific step(s) of the V(D)J recombination reaction. Activities that may be considered include possibly a role in synapse formation or in the recruitment of DNA repair enzymes to the synaptic complex.

Although very little is known about the mechanism by which transcriptional enhancers and other *cis*-acting elements regulate recombinational accessibility, there is no disputing their importance in the process of V(D)J recombination. Furthermore, we are starting to obtain our first mechanistic glimpses by analyzing the recombination intermediates in transgenic and gene targeted mice. Further experiments featuring more restricted gene targeting or gene replacement (knock-in) studies using various appropriately mutated sequences, coupled with the careful analysis of the resulting recombinational intermediates, should shed further light on the precise role of transcriptional enhancers and other *cis*-acting elements in regulating recombinational accessibility and V(D)J recombination.

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Note added in proof. Since the submission of this review for publication, several important studies have been published which concern the material presented. These include two studies on the effect of targeting the *Ku70* gene on V(D)J recombination and lymphoid cell development (Ouyang *et al.*, 1997, *J. Exp. Med.* **186,** 921–929; Gu *et al.*, 1997, *Proc. Natl. Acad. Sci. U.S.A.* **94,** 8076–8081), as well as the effect of purified Ku70 on DNA ligation *in vitro* (Ramsden and Gellert, 1998, *EMBO J.* **17,** 609–614). In addition, two new studies have been published demonstrating complete V(D)J recombination *in vitro* (Leu *et al.*, 1997, *Immunity* **7,** 303–314; Weis-Garcia *et al.*, 1997, *Mol. Cell. Biol.* **17,** 6379–6385). Finally, newly published work focuses on the effect of IL-7R deficiency on V-to-DJ recombination at the IgH locus (Corcoran *et al.*, 1998, *Nature* **391,** 904–907).

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Interactions between the Immune System and Gene Therapy Vectors: Bidirectional Regulation of Response and Expression

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I. Introduction

Gene therapy has opened many exciting possibilities for treating or modifying diseases or for altering normal physiology to elucidate molecular and cellular interactions (Crystal, 1995). Since some of the earliest attempts to transfer genes, it has been recognized that specific B- and T-cell responses against components of viral vectors limit gene expression and may induce detrimental or undesirable immunopathological consequences (Yei *et al.*, 1994b; Trapnell and Gorziglia, 1994). In retrospect, this should not have been surprising, considering that the immune system has evolved to be poised to respond to invading microbial insults. More recently, it has become clear that these immune responses consist not only of conventional antigen-specific lymphocyte responses, but also nonspecific and innate mechanisms inherent to lymphocytes, neutrophils, macrophages, natural killer (NK) cells, and even organ parenchymal cells. Although the antivector immune response is clearly a significant determinant of vector persistance and efficacy, the antitransgene product response is likewise emerging as an important consideration in evaluating and designing transfer vectors. More detailed analysis of the immune response demonstrates that beyond simple destruction of virus-infected cells or elimination of antigen, there are specific interactions between cytokines and the promoter elements of gene therapy vectors that regulate gene expression. Specific interactions also exist between vector DNA motifs and innate immune responses and between vector protein products and the molecules that mediate immune responses. Both of these latter types of interactions demonstrate that while host immunity regulates vector expressions, the vectors themselves can regulate immunity in numerous ways. Data are now emerging that the fundamental cellular processes important for transcriptional expression of exogenous nucleic acids intersect class I and class II major histocompatibility complex (MHC) antigen-processing pathways at several steps. These separate areas are examined in the present review and are analyzed with respect to how some aspects of gene therapy may benefit from these immune responses (e.g., tumor immunization), and how greater under-

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standing of immune responses will be required to enhance the utility of other aspects of gene transfer (e.g., gene replacement).

II. Immunity to Vectors

If gene therapy is to be successful, one of the most significant impediments to be overcome is the host immune response to the vector. This is particularly true in the case of adenoviral vectors that induce an exuberant immune response, and is especially problematic for human gene therapy applications in that most individuals have preexisting immunity to adenovirus (Wilson, 1996; Trapnell and Gorziglia, 1994; Wilson and Kay, 1995; Hogg and Hegele, 1995; Thiele *et al.*, 1989). Adenoviral vectors are appealing for *in vivo* gene therapy because of their capacity to transduce a wide variety of cell types, including nonreplicating cells. Further, unlike retroviral vectors, the potential risk of insertional mutagenesis is avoided as adenoviral vectors are not known to integrate into the host genome. However, this may also be a disadvantage in definitively correcting genetic defects, as repeated administration of an adenoviral vector would be required.

Many of the initial adenoviral gene transfer studies utilized adenoviral vectors encoding cystic fibrosis transmembrane conductance regulator (CFTR), administered to the lung as a model for the treatment of cystic fibrosis (Trapnell and Gorziglia, 1994; Yei *et al.*, 1994b). Early efficacy studies using the cotton rat demonstrated that acute lung inflammation occurs within 24 hr of adenoviral administration (Yei *et al.*, 1994b). The inflammatory response peaks at 3 to 4 days, with the initial inflammation consisting of a neutrophil-dominated infiltrate followed by a lymphocytic infiltrate at 7 to 10 days. Further, administration of adenoviral vector can cause vascular inflammation, activation, and proliferation (Newman *et al.*, 1995). Newman *et al.* (1995) demonstrated that exposure of arteries to replication-deficient adenoviral vectors results in pronounced T-cell infiltration, upregulation of the intercellular adhesion molecule (ICAM) and the vascular cell adhesion molecule (VCAM), and neointimal hyperplasia of the vasculature. Studies have shown that the host immune response to adenoviral vector is transient, dose dependent, and less pronounced than that seen with wild-type adenovirus (Trapnell and Gorziglia, 1994). Host immune responses to adenoviral vectors have been seen in many species and with various transgenes, suggesting that the adenoviral vector itself, rather than the transgene product, is primarily responsible for eliciting the immune response (Trapnell and Gorziglia, 1994). To further prove this point, Yang *et al.* (1996a) used a β-galactosidase encoding adenoviral vector delivered to the liver of mice to demonstrate that although the induction of cytotoxic T lymphocytes (CTLs) to both viral antigens and the transgene

product is observed, CTLs to viral antigens alone are sufficient to destroy virus-infected hepatocytes. Inflammation and loss of transgene expression can be explained by the development of class I-restricted CTLs to viral antigens, which target the genetically modified cells, to extinguish transgene expression by destroying the cell. Similarly, instillation of E1-deleted adenovirus into mouse lung also elicits MHC class I-restricted, $CD8^+$, CTL responses, leading to loss of transgene expression by mechanisms that included destruction of virus-infected cells (Yang *et al.*, 1995b; and 1996c). In these lung studies, transgenic mice tolerant to the transgene were used to prove that CTLs specific for viral antigens only are the primary contributor to loss of transgene expression (Yang *et al.*, 1996c). Similar principles have been confirmed in adenovirus-mediated gene transfer to muscle. Vilquin *et al.* (1995) demonstrated that mouse muscles infected with adenovirus-encoding β-galactosidase showed massive infiltration of macrophages, NK cells, and $CD8^+$ T cells. Further, the mRNA levels of granzyme B and interferon (IFN)-γ were increased 6 days after vector injection, indicating that the lymphocytes were activated. Thus, adenoviral reactive, CD8+ T cells are important effector cells limiting transgene expression in many different systems.

The precise antigens for adenoviral vector reactive, CD8+ T cells have not yet been identified. However, investigators have defined the immune response to wild-type adenovirus in mice (Mullbacher *et al.*, 1989). In wild-type adenovirus, the viral CTL response is directed against early rather than late viral proteins (mainly E1A and E1B). This same preference for early viral gene products was also demonstrated in a rat model (Routes *et al.*, 1991). Epitope targets of CTLs appear to reside on viral structural proteins that are expressed from E1-deleted vectors (Yang *et al.*, 1994a,c, 1995b). Immunological detection of low levels of adenoviral hexon and fiber proteins suggests that low level basal expression of adenoviral late genes can provide targets for CTL recognition of E1-deleted adenoviral vector-infected cells (Yang *et al.*, 1994c). Despite the importance of adenoviral specific CTL, it is noteworthy to consider that antigen-nonspecific immune responses, such as NK cells or cytokine-mediated inhibition of transcription (*vide infra*), also contribute to loss of transgene expression.

Even though it is clear that CD8+ CTL are major effectors in eliminating adenovirus-infected cells, CD4+ T cells also contribute to the antiadenoviral immune response. Studies indicate that the activation of CD4+ T cells by viral capsid proteins is necessary for full realization of effector function of CD8+ T cells (Yang *et al.*, 1995a,c). CD4+ T cells contribute to the maturation of CD8+ CTL and secrete IFNγ, which sensitizes the virus-infected cells to CTLs through upregulation of MHC class I (Doherty *et al.*, 1992). Further, CD4+ T cells aid in generating a humoral antiadenoviral immune

response by providing help in the form of cytokines to B cells (Fig. 1). Adoptive transfer experiments in immunodeficient mice evaluated the role of CD4+ cells in the antiadenoviral immune response (Yang and Wilson, 1995). Yang and Wilson (1995a) found that CD4+ cells alone are capable of destroying virus-infected hepatocytes, and depletion of CD4+ cells leads to prolonged transgene expression. Similarly, DeMatteo *et al.* (1996) confirmed these findings by demonstrating prolonged expression of β-galactosidase transgene expression in the livers of CD4-depleted mice. In addition, *in vitro* assays showed a decreased proliferative response to adenoviral antigens in $CD4^+$-depleted animals. However, the eventual loss of transgene expression coincided with the development of adenovirus-specific CTL activity *in vitro* (DeMatteo *et al.*, 1996). Finally, studies utilizing immune-deficient mice by Dai *et al.* (1995), Acsadi *et al.* (1996), and Zsdengeller *et al.* (1995) further proved that T-cell-dependent, antigen-specific immunity is the principal factor limiting the duration of primary transgene expression.

Although cellular immune responses are responsible for limiting primary transgene expression, humoral immune responses are responsible for limiting the effectiveness of secondary administration of vector. MHC class II-dependent activation of T helper cells and B cells to capsid proteins of adenovirus leads to the production of antiviral neutralizing antibody following primary exposure to virus (Yang *et al.*, 1995b). These capsid specific antibodies bind to virus and prevent entry during a second infusion. Humoral antibody responses to adenovirus vectors have been demonstrated in a variety of animal models, including mouse (Yang *et al.*, 1995b), rat (Yei *et al.*, 1994a), rhesus monkey (Zabner *et al.*, 1994), and humans (Zabner *et al.*, 1996). Little is known about the role of antibody responses in the destruction of virus-infected cells *in vivo*. However, it has been demonstrated that B-cell-mediated events do not participate in the destruction of hepatocytes *in vivo*, despite the production of antiviral and anti-transgene antibodies (Yang *et al.*, 1996a). In addition, it has also been shown that administration of ultraviolet (UV)-inactivated adenovirus leads to a full humoral response without CTL development (Yang *et al.*, 1995b). Because adenovirus is a nonenveloped virion that does not express viral antigens on the host cell surface, this finding sugests that viral particles can directly enter the class II MHC antigen-processing pathway and induce $CD4^+$ T helper responses for B-cell antibody production. A summary model of immune responses to viral vectors is presented in Fig. 1.

Mastrangeli *et al.* (1996) have proposed a novel strategy to circumvent adenoviral humoral immunity by alternating the use of adenoviral vectors belonging to different subgroups. Neutralizing antibodies against one adenovirus serotype do not cross-react with an adenovirus belonging to a second serotype in a manner that blocks infection and gene expression. Thus, in a

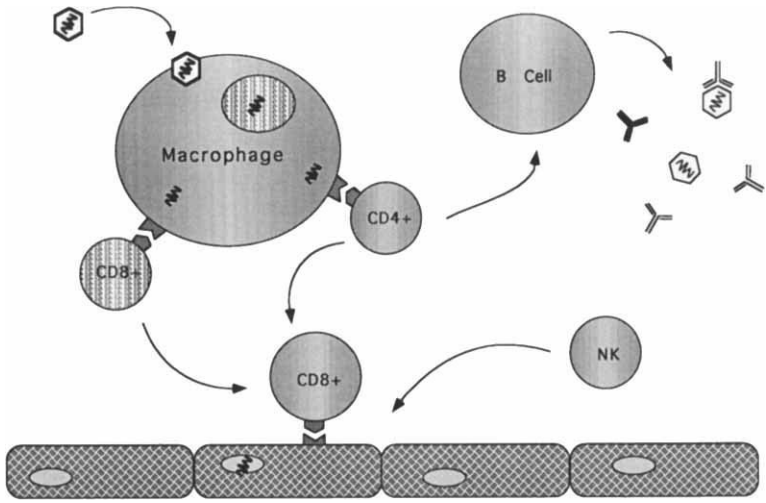

FIG. 1. Viral vectors infect cells and deposit their genomes into the nucleus. The genome encodes viral and transgene proteins that are expressed and presented by MHC class I molecules to $CD8^+$ T cells. At the same time, $CD4^+$ T cells are activated by viral capsid proteins, processed, and presented in the context of MHC class II molecules. Activated $CD4^+$ T cells produce cytokines that help in the development of $CD8^+$ cytolytic T cells, resulting in the elimination of genetically modified cells. In addition, cytokines produced by $CD4^+$ T cells assist capsid-specific B cells, resulting in the secretion of neutralizing antibodies that prevent the secondary administration of vector.

rat model these investigators demonstrated that effective expression from secondary adenoviral administration can be achieved with an adenoviral vector belonging to a different subgroup than the first administration.

Despite all the evidence for antigen-specific adenoviral immunity, Worgall *et al.* (1997) suggest that there are actually two phases of adenoviral vector elimination. A late, previously recognized, immune-specific elimination and an early innate immune elimination. Experiments demonstrated that following intravenous administration, 90% of adenoviral vector is eliminated from the mouse within the first 24 hr (Worgall *et al.*, 1997). This loss is independent of dose, transgene expression, and is also seen in immunodeficient animals. Thus, it appears that innate immune mechanisms, particularly related to Kupfer cells, may also contribute to the elimination of vector and loss of transgene expression. This is commensurate with previous studies documenting antigen-nonspecific components to the antiadenoviral immune response (Yei *et al.*, 1994b; Newman *et al.*, 1995; Vilquin *et al.*, 1995).

Initial adenoviral studies used first-generation vectors rendered replication defective by deletion of E1 region genes, which control viral replication and the expression of other early and late genes (Trapnell and Gorziglia, 1994). However, it is now known that E1-deleted adenoviral vectors can still express low levels of both early and late viral genes, which induce a significant immune response (Yang *et al.*, 1994a). Thus, second-generation adenoviral vectors have been constructed, which in addition to being E1 deleted, have a temperature-sensitive mutation within the E2A-encoded, DNA-binding protein (Engelhardt *et al.*, 1994). This E2A mutation reduces late gene expression and further cripples replication. Studies have shown that *in vivo* administration of this second generation vector results in a decreased CTL response and an increased duration of transgene expression compared to first-generation adenoviral vectors (Engelhardt *et al.*, 1994), yet leakiness of the temperature-sensitive mutation and virus-independent activation of the major late promoter limit the performance of these second generation vectors. Thus, these modifications in adenoviral vectors do not fully eliminate immunogenecity. An alternative approach has been to express adenoviral immunosuppressive E3 proteins to prevent the immune response (Lee *et al.*, 1995; Ilan *et al.*, 1997) More recently, adenoviral vectors fully deleted of all viral genes have been described (Fisher *et al.*, 1996; Kochanek *et al.*, 1996). These new generation adenoviral vectors are deleted of all viral open reading frames, contain only the essential *cis* DNA regulatory elements, and are propagated in 293 cells in the presence of E1-deleted helper virus. Clemens *et al.* (1996) demonstrated prolonged expression of a full-length dystrophin transgene in neonatal mice utilizing such an adenoviral vector. It will be interesting to see how these new adenoviral vectors perform in fully immunocompetant adult animals and in other systems, but improved production and purification strategies need to be developed before this vector system will be fully effective *in vivo*.

Studies have shown that various immunosuppressive protocols, such as depletion of CD4+ T cells by monoclonal antibody or administration of tacrolimus at the time of adenoviral-mediated gene transfer, can result in prolonged transgene expression and a marked decrease in the appearance of neutralizing antibody (Yang *et al.*, 1995c; DeMatteo *et al.*, 1996; Lochmuller *et al.*, 1995), yet global immunosuppressive regimens such as these are not clinically practical solutions to inhibiting the antiviral vector immune response. For this approach to be successful, a regimen needs to be developed that results in only transient immunosuppression with minimal effects on preexisting immunity and does not result in immunological tolerance to wild-type adenovirus. Costimulatory signals among T cells, B cells, and other antigen-presenting cells may provide such targets for intervention. Kay *et al.* (1995) demonstrated that blockade of the CD28/

B7 interaction with CTLA4Ig resulted in markedly prolonged expression (greater than 5 months) of adenoviral-transduced genes and markedly attenuated antibody production. Similarly, Yang *et al.* (1996b) showed that a transient blockade of T-cell activation by administration of an antibody to CD40 ligand at the time of adenoviral vector-mediated gene transfer led to a stabilization of transgene expression and a diminished production of neutralizing antibody, even allowing for readministration of vector. Thus, approaches that interfere with costimulatory pathways such as CD28/B7 and between CD40/CD40L can attenuate antibody production and cellular immunity to adenoviral vectors and may allow for sufficient transgene expression to produce a significant biologic effect.

Despite the present interest in adenoviral vectors, retroviral vectors have been the vehicle of choice in many gene therapy trials, primarily due to their ability to stably integrate into the host genome, ensuring sustained gene expression (Gordon and Anderson, 1994). The majority of the retroviral vectors being used are derived from murine leukemia retroviruses (Miller and Rosman, 1989; Cornetta *et al.*, 1991; Jolly, 1994). The biology of these viruses is well understood, yields moderate titers, and variants can infect human cells (Gordon and Anderson, 1994; Miller and Rosman, 1989; Cornetta *et al.*, 1991). At present, most current gene therapy protocols with retroviral vectors use an *ex vivo* approach in which the cells to be transduced are isolated from a patient, genetically modified *in vitro*, and then reintroduced into the patient (Gunzburg and Salmons, 1996). Because no viral proteins are expressed after transduction, there is little concern about generating an immune response to the retroviral vector, although this does not exclude the possibility of generating a response to the transgene. This *ex vivo* approach has its limitations. It can only be applied to cells that are easily isolated and cultured, a time-consuming and costly procedure that requires specialized equipment (Gunzburg and Salmons, 1996). Thus, it would be advantageous to develop *in vivo* approaches to introduce the retroviral vector into host cells.

Initial studies *in vivo* demonstrated that intravenous gene transfer using recombinant retrovirus suffers from very low infectious viral titer, caused in part by complement-mediated inactivation of the retrovirus in serum (Gunzburg and Salmons, 1996). Inactivation of nonprimate-derived, type C retrovirus in human serum is an antibody-independent mechanism involving activation of the classical complement pathway, through direct binding of C1q to the retroviral envelope protein p15E, which leads to complement-mediated virolysis in human sera (Cooper *et al.*, 1976; Bartholomew *et al.*, 1978; Bartholomew and Esser, 1980). The inactivation depends both on the type of retrovirus and on the origin of the producer cells (Takeuchi *et al.*, 1994). For example, murine leukemia virus (MLV) produced from

mouse cells is more readily inactivated by human serum than MLV produced from human or mink cells. Thus, careful consideration must be given to the choice of packaging cell lines with respect to both the viral envelope that is expressed by the line and the species origin of these cells. It may be advantageous to construct vector systems based on retroviruses that are not inactivated by human complement such as HIV-1 and HTLV-I (Banapour *et al.*, 1986; Hoshino *et al.*, 1984).

Rother *et al.*, (1995b) reported on another mechanism of complement-mediated type C retrovirus inactivation that is initiated by the binding of a natural occurring antibody against the α-galactosyl moiety expressed on the amphotropic MLV retroviral envelope. The authors also showed that MLV-derived ecotropic retroviral particles expressing the α-galactosyl epitope are inactivated in human serum by anti-α-galactosyl antibody. The α-galactosyl epitope has also been detected on the surface of the eastern equine encephalitis virus and the Friend murine leukemia virus (Snyder and Fleissner, 1980). Thus, involvement of the α-galactosyl epitope in the serum sensitivity of other type C retroviruses or of enveloped viruses in general seems likely.

The inactivation of both amphotropic and ecotropic retroviral particles is dependent on anti-α-galactosyl antibody (Rother *et al.*, 1995b). This is also the primary ligand responsible for hyperacute rejection of xenogeneic transplants (Dalmasso *et al.*, 1992). Downregulation of the α-galactosyl epitope on the surface of murine retroviral particle producer cells renders them resistant to inactivation by human serum (Rother *et al.*, 1995b). The inactivation of murine-derived retroviral vectors in human serum is a formidable barrier to many potential applications of *in vivo* retroviral-mediated gene therapy. Thus, Rother *et al.*'s (1995b) description of anti-α-galactosyl antibodies suggests a technique to interfere with complement activation, when using retroviral vectors for *in vivo* gene therapy applications where exposure to human complement is unavoidable. These investigators demonstrated that retroviral particles could be protected from complement-mediated inactivation by the addition of soluble Galα1-3Gal to serum samples. In addition, they showed that pretreatment of human serum with functionally blocking monoclonal antibodies that target terminal complement components effectively protects retroviral particles from inactivation (Rother *et al.*, 1995a). These reagents could be valuable for certain applications of *in vivo* gene therapy in humans where exposure to complement is unavoidable. Finally, the generation of retroviral vector packaging cell lines that do not express the α-galactosyl epitope would allow for the production of retroviral particles that survive complement-mediated inactivation in human serum.

Although the majority of gene therapy studies have focused on retroviral and adenoviral vectors, several other viral vectors have been developed based on herpes simplex virus (HSV) (Ali *et al.*, 1994; Glorioso *et al.*, 1995), vaccinia virus (Lee *et al.*, 1994; Lattime *et al.*, 1996), SV-40 (Strayer, 1996), adenoassociated virus (AAV) (Smith, 1995; Flotte and Carter, 1995), Sindbis virus (Dubensky *et al.*, 1996; Johanning *et al.*, 1995), and HIV (Parolin and Sodroski, 1995; Naldini *et al.*, 1996). All of these vectors have their own inherent advantages and disadvantages. For example, HSV shows great promise as a gene delivery vector in the treatment of neurological disorders due to its tropism for neural tissue and its ability to reside in neurons in a latent state that does not appear to affect normal physiology (Glorioso *et al.*, 1995). However, despite successful prolonged neural transgene expression (Andersen *et al.*, 1992), a high level of toxicity associated with HSV vectors has been demonstrated (Ho *et al.*, 1995; Sabel *et al.*, 1995). AAV vectors incorporate into genomic DNA and may have reduced immunogenicity, permitting long-term gene expression (Flotte *et al.*, 1993; Conrad *et al.*, 1996; Kaplitt *et al.*, 1994b, 1996; Arnold *et al.*, 1997), although it is uncertain if immune responses will limit AAV expression. HIV-derived lentivirus vectors are appealing in that, unlike current retroviral vectors, HIV-derived vehicles have the ability to deliver genes into nondividing cells *in vivo*, which greatly increases the applicability of retroviral vectors for human gene therapy (Parolin and Sodnoski, 1995; Naldini *et al.*, 1996). Because most of these viral constructs have been developed relatively recently, little is known about the immune response to the vector. However, information about immune responses to unmodified viruses suggests that these vectors will generate vigorous humoral and cellular immune responses (Doherty *et al.*, 1992). However, if constructed properly, some of these viral vectors may avoid immune recognition. For example, neurons latently infected with HSV are not rejected by the host immune response and appear to function normally (Glorioso *et al.*, 1995). Further, latency does not depend on viral replication as replication-deficient virus can readily establish latency (Glorioso *et al.*, 1995). Even if an immune response is generated toward the vector, it may not always be detrimental. Studies with vaccinia vectors have demonstrated that preexisting or induced immunity to vaccinia does not inhibit *in vivo* tumor transfection (Lee *et al.*, 1994; Lattime *et al.*, 1996). At present, it is unclear how immunogenic these new viral vectors will be and what will be the consequences of a given immunologic response. However, it is clear that viral vectors require greater analysis, and many modifications will need to be made before they will become safe, nonimmunogenic options in the delivery of genes to human patients.

III. Immunity to Gene Transfer Products

Gene therapy has the potential to introduce exogenous proteins, either self or nonself, into specific cells or anatomic regions, alter cellular interactions or physiology, and achieve therapeutic end points. When nonself products are introduced into immunocompetent individuals, it is expected that specific immune responses, both cell mediated and humoral, will be mounted against the foreign antigens. Such immune responses offer a unique method for vaccination, whereas rapid elimination of transduced cells and neutralization of gene transfer products limit the usefulness of gene therapy in some settings, particularly replacement therapy in genetic disease.

Eliciting an immune response by DNA immunization was first reported by Tang and co-workers in 1992 (Tang *et al.*, 1992). DNA vaccination has been utilized to treat a variety of infectious diseases, including viral, bacterial, and parasitic, as well as tumors in animal models. The generation of both antibody- and cell-mediated immune responses has been shown in response to various antigens expressed from different vectors administered by different routes and protocols (Table I). Most of these vaccinations were performed by intramuscular injection of protein-encoding plasmids, as striated muscle cells are unique among mammalian cell types in their capacity to take up and express free DNA in the absence of a viral vector or physical carrier (Wolff *et al.*, 1990; Acsadi *et al.*, 1991; Kitsis *et al.*, 1991; Jiao *et al.*, 1992). The mechanisms by which myocytes preferentially take up and express naked plasmid are still unknown. It is postulated that a unique cytoarchitectural feature present in both skeletal and cardiac myocytes, but not in the other tissues, such as transverse tubules, may play a role in the uptake of naked DNA. Low lysosomal and DNase content of skeletal and cardiac muscle may also enable the plasmid DNA to persist longer than in other tissues, facilitating its prolonged expression (Wolff *et al.*, 1990; Acsadi *et al.*, 1991; Kitsis *et al.*, 1991; Jiao *et al.*, 1992). Although intramuscular injection of plasmid DNA seems to be the major route of DNA vaccination, other approaches under investigation demonstrate, at least in certain situations, superiority to injection intramuscularly (Raz *et al.*, 1994). For example, protection against influenza virus infection could be achieved by intramuscular (Yankauckas *et al.*, 1993), intradermal (Raz *et al.*, 1994), and gene gun (Justewicz and Webster, 1996) administration. Intradermal gene immunization induced higher antibody titers than did direct gene injection into skeletal muscle and did not cause local inflammation and necrosis (Raz *et al.*, 1994). Transferring genes to different anatomic regions, targeting different cell types, and localizing gene products to different subcellular compartments may affect antigen presentation (discussed in detail in a later section) and the subsequent immune response.

TABLE I
DNA VACCINATION FOR INFECTIOUS DISEASES

Disease	Antigen	CMI	Ab	Reference
Influenza	Hemagglutinin	+	+	Robinson *et al.* (1993); Justewicz and Webster (1996)
	Nucleoprotein	+	+	Yankauckas *et al.* (1993); Raz *et al.* (1994)
HIV	gp120	+	+	Shiver *et al.* (1995)
	gp160	+	+	Shiver *et al.* (1995); Wang *et al.* (1993)
	rev	+	−	Shiver *et al.* (1995)
HBV	Surface antigen	+	+	Davis *et al.* (1995); Whalen *et al.* (1995); Michel *et al.* (1995); Bohm *et al.* (1996); Schirmbeck *et al.* (1995)
	Envelope protein	+		Davis *et al.* (1995)
	Core antigen	+	+	Kuhober *et al.* (1996)
Rabies	Glycoprotein	+	+	Xiang *et al.* (1994); Ertl *et al.* (1995)
Measles	Nucleoprotein		+	Fooks *et al.* (1996)
BHV	Bovine herpesvirus-1 glycoprotein	+		Babiuk *et al.* (1995); Gid. Cox *et al.* (1993)
Tuberculosis	65-kDa heat shock protein	+	+	Lowrie *et al.* (1994); Huygen *et al.* (1996)
	Antigen 85	+	+	Huygen *et al.* (1996)
	36-kDa proline-rich antigen	+		Tascon *et al.* (1996)
Malaria	Circumsporozoite protein	+	+	Sedegah *et al.* (1994); Mor *et al.* (1995)
Leishmaniasis	Major surface glycoprotein		+	Xu and Liew (1994)

Whereas immunity to microbes and tumors generally requires successful TH1 immunization (Yankauckas *et al.*, 1993; Raz *et al.*, 1994; Shiver *et al.*, 1995; Davis *et al.*, 1995; Whalen *et al.*, 1995; Bohm *et al.*, 1996; Kuhober *et al.*, 1996; Schirmbeck *et al.*, 1995; Xiang *et al.*, 1994; Lowrie *et al.*, 1994; Huygen *et al.*, 1996; Tascon *et al.*, 1996; Sedegah *et al.*, 1994; Mor *et al.*, 1995; Xu and Liew, 1994; Raz *et al.*, 1996; Corr *et al.*, 1996), treatment of autoimmunity involves preferential induction of a TH2 response. DNA vaccination may offer promise for prevention and/or treatment of autoimmune disease. Waisman and co-workers (Waisman *et al.*, 1996) reported that vaccination of experimental autoimmune encephalomyelitis (EAE) mice with the Vβ8.2 variable region gene of the T-cell receptor, expressed on pathogenic T cells, protected mice from EAE. After intramuscular injection of naked DNA encoding Vβ8.2, antibodies specific for Vβ8.2

were generated. This did not cause depletion of the Vβ8.2+ T cells, but rather they underwent a shift in the pattern of cytokine production. Such "suppressive vaccination" was associated with a reduction in the TH1 cytokines interleukin (IL)-2 and IFNγ and an elevation in the production of IL-4, a TH2 cytokine associated with suppression of disease. A novel feature of DNA immunization for autoimmune disease, reversal of the autoimmune response from TH1 to TH2, may make this approach attractive for treatment of TH1-mediated diseases such as multiple sclerosis, juvenile diabetes, and rheumatoid arthritis. It may be possible to achieve TH1 versus TH2 immunity by altering the route of plasmid DNA administration (Feltquate *et al.*, 1997).

With an increase in the understanding of human inherited disease pathogenesis, gene therapy has become a reality for some disorders and an increased possibility for many others. Different methods for gene addition are being tested, based on the cell type necessary to make the therapy effective. Gene therapy has been considered to correct genetic diseases such as adenosine deaminase deficiency (Ferrari *et al.*, 1992; Hoogerbrugge *et al.*, 1992; Blaese *et al.*, 1995; Bordignon *et al.*, 1995), hemophilia A and B (Kay *et al.*, 1993, 1994; Dwarki *et al.*, 1995; Fang *et al.*, 1995; Hortelano *et al.*, 1996; Connelly *et al.*, 1996; Yao *et al.*, 1996; Hurwitz *et al.*, 1997), α_1-antitrypsin deficiency (Kay *et al.*, 1992; Knoell and Wewers, 1995; Okuyama *et al.*, 1996), cystic fibrosis (CF) (Hyde *et al.*, 1993; Caplen *et al.*, 1995; Rosenfeld and Collins, 1996), low-density lipoprotein (LDL) receptor deficiency (Kobayashi *et al.*, 1996; Kozarsky *et al.*, 1994, 1996a,b; Li *et al.*, 1995), Duchenne's muscular dystrophy (Morgan, 1994; Dunckley *et al.*, 1993; Vincent *et al.*, 1993; Cox *et al.*, 1993), lysosomal storage diseases (Moullier *et al.*, 1993, 1995), and congenital erythropoietic porphyria (Moreau-Gaudry *et al.*, 1995a,b). Although gene therapy is very attractive, preclinical experiments in animal models suggest that it may be difficult to obtain adequate therapeutic levels for long periods of time (Kay *et al.*, 1994; Dwarki *et al.*, 1995; Fang *et al.*, 1995; Hortelano *et al.*, 1996; Connelly *et al.*, 1996; Yao *et al.*, 1996; Hurwitz *et al.*, 1997; Kobayashi *et al.*, 1996; Kozarsky *et al.*, 1994, 1996a,b). One reason is due to host immune responses to the gene transfer products. For example, to develop a potential gene therapy strategy for the treatment of hemophilia A, Connelly and coworkers (1996) reported complete correction of the hemophilia A phenotype by using an adenoviral vector expressing a human FVIII cDNA in a canine model. Within 48 hr after peripheral vein administration of the vector to FVIII-deficient dogs, the hemophilic phenotype was corrected. Direct measurement of human FVIII in the dog plasma showed FVIII expression at amounts well above the human therapeutic level. However, FVIII expression in treated dogs lasted only 1–2 weeks, due to the develop-

ment of a human FVIII-specific inhibitory antibody response. Hortelano and co-workers (1996) reported that antibodies to human factor IX were detected 3 weeks after treatment, which accelerated the clearance of human factor IX from the circulation of mice implanted with gene transduced allogenic myoblasts. Using gene gun treatment of mouse epidermis *in vivo* with human α_1-antitrypsin messenger RNA, Qiu and co-workers (1996) elicited a strong, consistent antibody response to human α_1-antitrypsin, which showed an increased titer with subsequent boosts. Similar antibody responses to gene transfer products have also been reported by many other investigators (Blaese *et al.*, 1995; Riddell *et al.*, 1996; Tripathy *et al.*, 1996; Kay *et al.*, 1994; Dwarki *et al.*, 1995; Fang *et al.*, 1995; Hortelano *et al.*, 1996; Connelly *et al.*, 1996; Yao *et al.*, 1996; Hurwitz *et al.*, 1997; Kobayashi *et al.*, 1996; Kozarsky *et al.*, 1994, 1996a,b).

Neutralizing antibody to gene products has drawn the most attention of researchers. However, cell-mediated immunity is also expected to play a role in eliminating transduced cells and limiting transgene expression. Nabel and co-workers (1992) reported that direct gene transfer of a foreign class I major histocompatibility complex gene, HLA-B7, to specific sites in porcine arterial wall elicited inflammation and cytolytic T cells specific for HLA-B7. Kozarsky and co-workers (1996b) reported that gene replacement therapy of human LDL receptor gene into the murine model of familial hypercholesterolemia transiently corrected the dyslipidaemia; however, both humoral and cellular immune responses to LDL receptor developed, which extinguished transgene expression.

Two approaches have been taken to circumvent the immune response to the gene transfer products. One approach is to suppress immune function by a variety methods. Immunosuppressive agents, such as CTLA4-Ig (Kay *et al.*, 1995) and cyclosporin A (Fang *et al.*, 1995), suppress the specific immune response. Unfortunately, the systemic administration of these immunosuppressants affects systemic immune function and results in a number of complications, such as infection and malignancy, and other undesirable drug-specific toxicities, which make them less attractive or even untenable for clinical application. Intrathymic inoculation (DeMatteo *et al.*, 1995) could induce unresponsiveness to foreign antigens, although the involuted thymus of adult humans makes this approach not feasible for human gene therapy. Finally, local immunosuppression can be generated using gene therapy vectors that express both a gene of interest and a gene encoding an immunosuppressive molecule, such as the 19k glycoprotein (gp 19k) found in the adenovirus E3 region (Lee *et al.*, 1995; Ilan *et al.*, 1997; Efrat *et al.*, 1995), interleukin-1 receptor antagonist (McCoy *et al.*, 1995), or viral interleukin 10 (Qin *et al.*, 1997a). These methods have

met with limited success and can induce local but not systemic immunosuppression.

The second approach to suppress immune function is to use less immunogenic gene products. For example, as an alternative strategy for correcting familial hypercholesterolemia, Kobayashi *et al.* (1996) and Kozarsky *et al.* (1996a,b) expressed the human very low density lipoprotein (VLDL) receptor ectopically in the liver of LDL receptor knockout mice. The VLDL receptor is expressed in peripheral tissues, including muscle, adipose tissue, kidney, and brain, but not liver. Hepatic expression of the VLDL receptor, which binds to apolipoprotein E found in VLDL and intermediate density lipoprotein, has the potential to diminish LDL levels in familial hypercholesterolemia by increasing the catabolism of its precursors. In contrast to the human LDL receptor, which is truly a neoantigen in the LDL receptor knockout mice, the human VLDL receptor protein is >94% homologous to the mouse protein. Infusion of recombinant adenoviruses containing the VLDL receptor gene corrected the dyslipidemia in the familial hypercholesterolemic mouse and circumvented immune responses to the transgene, leading to more prolonged metabolic correction (Kozarsky *et al.*, 1996b).

IV. Cytokine Regulation of Promoter Function

As discussed in the first section, it is widely believed that viral clearance is mediated principally by the destruction of infected cells by CTLs. However, specific T-cell-mediated lysis would likely not be wholly relevant in the following situations: (1) Many investigators have reported that transgene expression declined within 2–5 days after exposure to adenoviral vectors (Yang *et al.*, 1994b,c; Dai *et al.*, 1995; Engelhardt *et al.*, 1994; DeMatteo *et al.*, 1995; 1996; Kass-Eisler *et al.*, 1993; Shaked *et al.*, 1994; Hurwitz *et al.*, 1997), which is too early for naive animals to fully develop primary specific immune responses. (2) After intravenous administration of adenoviral vectors, reporter gene expression was demonstrated in 30–90% of heptocytes and was reduced dramatically within a short period of time (Yang *et al.*, 1994b,c; Dai *et al.*, 1995; Engelhardt *et al.*, 1994; DeMatteo *et al.*, 1995; Qin *et al.*, 1995, 1997a,b; Kass-Eisler *et al.*, 1993; Shaked *et al.*, 1994), yet it is not likely that all these adenoviral-transduced cells were killed and eliminated by adenoviral-specific CTL, as there was no evidence of acute, massive heptocyte necrosis or apoptosis, the animals appeared normal and showed no apparent toxic effects, and there was no mortality associated with the gene transfer. (3) To limit the deleterious antivector immune response, a number of investigators have used a less immunogenic E2 temperature-sensitive adenoviral vector (second-generation adenoviral

vector) (Engelhardt *et al.*, 1994; Yang *et al.*, 1994b) or administered systemic immunosuppression (Yang *et al.*, 1995c; DeMatteo *et al.*, 1995, 1996; Dai *et al.*, 1995; Kay *et al.*, 1995; Yao *et al.*, 1996; Lochmuller *et al.*, 1995). Nonetheless, even in situations where the immune response has been suppressed or is irrelevant (e.g., use of retroviral vector), the expression of vector-transferred genes is still limited *in vivo* and is far less than what is observed in model systems *in vitro*, especially when viral promoters were used (Hurwitz *et al.*, 1997; Rettinger *et al.*, 1994; Scharfmann *et al.*, 1991; Challita and Kohn, 1994; Dwarki *et al.*, 1995. (4) Evidence shows that transgene expression is eliminated even in the presence of substantial amounts of vector DNA in the transduced cells (Yao *et al.*, 1996; Hurwitz *et al.*, 1997; Challita and Kohn, 1994; Dwarki *et al.*, 1995). These results suggest that mechanisms other than the antigen-specific immune lysis may mediate noncytodestructive events that can determine the persistence of transgene expression.

Studies by Chisari and co-workers demonstrated that in transgenic mice which replicate the hepatitis B virus (HBV) genome, CD8+ CTLs can reduce cellular HBV mRNA by a noncytotoxic mechanism (Tsui *et al.*, 1995; Guidotti *et al.*, 1996). Analysis showed that IFNγ and TNFα are secreted by antigen-activated CTL and result in specific posttranscriptional clearance of viral mRNA. Steady-state levels of nuclear and cytoplasmic viral mRNA are markedly and specifically reduced, and the cytokine response elements involved in this effect map to HBV promoters between nucleotides 3157 and 1239 of the genome, but importantly do not include the HBV X promoter. This demonstrates that positive and negative regulatory HBV promoter elements exist that are influenced by TNFα and IFNγ. These results also suggest that a major limitation to current gene therapy is cytokine-initiated signaling events that limit vector gene expression from certain viral promoters. Thus, cytokine-regulated promoter function, rather than specific immune destruction, may limit transgene expression.

In support of this hypothesis, there are at least three general situations *in vivo* in which gene transfer vectors could be exposed to a cytokine-rich environment: (1) Gene therapy has been considered for the treatment of diseases [including cancer, AIDS, transplantation, atherosclerosis, autoimmunity, ischemia-reperfusion (Crystal, 1995; Hanania *et al.*, 1995; Blau and Springer, 1995] that have in common the ubiquitous production of cytokines through either antigen-specific or nonspecific stimulation of lymphocytes, other leukocytes, vascular endothelial cells, or organ parenchymal cells by infection, ischemia, necrosis, or trauma. (2) Gene therapy protocols frequently involve gene transfer of cytokines, such as IFNγ and TNFα, in order to either elicit immune responses (e.g., cancer and infectious diseases) or inhibit immune responses (e.g., transplantation and autoim-

mune diseases). (3) Specific and nonspecific immune responses to gene transfer vectors or gene products are capable of inducing several cytokines, including TNFα and IFNγ (discussed in other sections). The cellular targets of gene transfer vectors and the other physiologic signals impinging on these cells have previously and tacitly been considered passive participants in the process of gene transfer and expression. In addition, although much is known about the effects of viral components on host cell transcription, there are few studies in the current gene therapy literature that examine the effects of cytokines or other cellular signals on the transcriptional and translational disposition of transferred genes. It is likely that these cytokine effects have not been noted previously in model *in vitro* systems because of the lack of a changing cytokine environment. The effects have also not been noted in model *in vivo* systems because of attention to other important basic variables, such as dosing, specificity, and sufficient vector transfer to the appropriate anatomic site. Thus, there is a significant lack of knowledge in the area of cytokines and their effects on regulating transgene expression. Therefore, a major requirement for improving gene therapy is the elucidation of the effects of cytokine and other ligand-initiated signaling on transferred gene expression.

Harms and Splitter (1995) reported that IFNγ treatment of transiently or stably transfected cell lines representing various tissues and species resulted in the suppression of transgene expression driven by the SV40 or CMV viral promoters, whereas transgene expression driven by the mammalian MHC I promoter was enhanced. These results offer an explanation for the short-term transgene expression observed by many researchers using viral promoters to express transgenes *in vivo* and suggest that for long-term, sustained transgene expression, the mammalian MHC I promoter/enhancer could be a better choice than the commonly used viral promoters.

Qin *et al.* (1997b) demonstrated that IFNγ and TNFα initiate marked inhibitory effects on selected viral promoters (HCMV, RSV), but were markedly less inhibitory on a cellular promoter (β-actin) in adenoviral vectors *in vitro* (Table II). Importantly, the combination of IFNγ plus TNFα had marked synergistic inhibitory effects on the HCMV and RSV promoters but no effect on the β-actin promoter. Inhibition is at the mRNA level and cytokines do not cause vector DNA degradation, inhibit total protein synthesis, or kill infected/transfected cells. Similar effects of cytokine-initiated regulation could be observed with plasmid vectors with CMV, RSV, and SV40 promoters (Table II). IFNγ and TNFα also inhibited reporter gene expression from the retroviral vector MFG-LacZ with the MMLV-LTR promoter. Transgene expression could be restored 2 days after these cytokines were removed from culture, which again indicates that inhibition of gene expression is at the transcriptional and/or transla-

TABLE II
CYTOKINES INHIBIT REPORTER GENE EXPRESSION FROM ADENOVIRAL, RETROVIRAL, AND PLASMID VECTORS

Vector	Promoter	% Inhibition of β-Galactosidase Activity		
		IFNγ (30 U/ml)	TNFα (30 U/ml)	INFγ/TNFα (30 U/ml)
Adenovirus	HCMVie	25.4	52.7	70.6
	RSV-LTR	47.9	36.8	76.8
	B-Actin	22.1	−13.4	10.9
Plasmid	HCMVie	27.0	30.2	70.7
	RSV-LTR	27.5	34.1	50.7
	SV40	19.0	28.4	40.5
Retrovirus	MMLV-LTR			
		I. Cells treated with cytokines for 20 hr:		
		77.8	33.3	96.0
		II. Cells treated with cytokines for 3 days:		
		71.3	63.4	95.5
		III. Cells treated with cytokines for 20 hr, washed, and incubated for an additional 2 days:		
		17.5	7.9	6.1

tional level and that cytokines do not cause vector nucleic acid degradation or kill transduced cells.

It is notable that the CMVie, RSV, SV40, and MMLV-LTR promoters and enhancers, which are cytokine sensitive as shown earlier, are widely used in gene transfer vectors. Thus, vectors in current use may be constructed inappropriately to provide for sustained gene expression, as the cytokine environment or the gene transfer product itself may inhibit its own promoter. For example, IFNγ and TNFα have been transduced into tumor cell lines or tumor-infiltrating lymphocytes by retroviral vectors with the MMLV-LTR promoter (Marincola *et al.*, 1994; Karp *et al.*, 1993). Much work has focused on characterizing short-term gene expression of cells transduced *in vitro* rather than evaluating chronic gene expression in conjunction with potential antitumor activity *in vivo*. In fact, genetically modified, TNFα-secreting, nonimmunogenic murine fibrosarcomas failed to elicit a host immune response, which is in contrast to the effects with an IL-2-secreting tumor (Karp *et al.*, 1993). In another example, an attempt to enhance the efficacy of a plasmid DNA vaccine to rabies virus was made by coinoculating cytokine [IFNγ and the granulocyte macrophage colony stimulatory factor (GM-CSF)] expressing plasmids along with the viral antigen encoding plasmid under the control of the SV40 early promoter. The GM-CSF expressing plasmid increased antiviral immunity by stimulating both antibody and T helper cell responses. Conversely, an IFNγ expressing plasmid

had no such immunostimulatory effects, but rather resulted in a slight decrease in antibody and cytokine production (Xiang and Ertl, 1995). This unanticipated effect of IFNγ could have been due to inhibition of the SV40 early promoter by IFNγ, so that less viral antigen was produced. Finally, T lymphocytes are particularly recalcitrant to attempts at stable gene transfer and expression (Rosenberg, 1991; Chu *et al.*, 1992). Because these cells are immunologically active, and in particular produce IFNγ and TNFα, the results presented here may provide a partial explanation for this resistance.

From a molecular mechanistic standpoint, it has been reported that IFNγ and IFNα can inhibit the transcription of CMVie genes (Gribaudo *et al.*, 1993). In the case of IFNγ, the inhibitory effect on CMVie gene enhancer function is related, at least partially, to cytokine-induced inhibition of NF-κB activity (Gribaudo *et al.*, 1995). It is likely that NF-κB activity is directly inhibited by the p202 protein, which is induced by IFNγ and which directly binds to and inhibits the function of the p50 and p65 subunits of NF-κB (Wang *et al.*, 1996). p202 can also bind to and inhibit the c-Fos and c-Jun subunits of the AP-1 transcription factor and the retinoblastoma protein (pRb). pRb in turn regulates other transcription factors and cell cycle proteins (Wang *et al.*, 1996; Choubey and Lengyel, 1995). Therefore, p202 also halts progression of cells through the cell cycle. Because IFNγ does not inhibit cellular proliferation in the authors' models (Qin *et al.*, 1997b), it is unlikely that p202 mediates the effects on promoter inhibition. IFNγ induces other activities such as 2′-5′ oligoadenylate synthetase, dsRNA-dependent protein kinase, and Mx proteins, which regulate translation from viral mRNA transcripts (Sen and Ransohoff, 1993; Landolfo *et al.*, 1995). From the standpoint of individual viral genetic elements, such as CMVie or RSV, much is known about specific promoter and enhancer sequences (Nelson *et al.*, 1990; Ruddell, 1995) and about some of the transcription factors that regulate them (Ruddell, 1995; Liu *et al.*, 1994; Zhang *et al.*, 1995). However, the regulation of these genetic and protein elements by cytokine signaling in the context of gene transfer vectors remains unexplored.

It is well known that IFNs, produced by viral-infected cells as part of the innate immune system, can signal to neighboring cells to resist viral replication and prevent virus spread (Farrar and Schreiber, 1993; Samuel, 1991; McNair and Kerr, 1992). TNFα is also a potent antiviral cytokine that acts alone or in a synergistic manner with IFNγ to protect cells from viral infection (Wong and Goeddel, 1986; Wietzerbin *et al.*, 1990; Wong *et al.*, 1992). Evidence now shows that Kupfer cells, known producers of IFNγ and TNFα, play a role in eliminating adenoviral vectors in the liver in an antigen-nonspecific manner following *in vivo* administration (Worgall *et al.*, 1997). The inhibition of early viral promoters (Qin *et al.*, 1997b;

Harms and Splitter, 1995) provides yet another mechanism for the antiviral effects of IFNs and TNFα, suggesting that these cytokines represent basic cellular defenses against viral nucleic acids and that cells can distinguish viral from cellular sequences. It is important to note that some endogenous cellular promoters are also likely to be inhibited by these cytokines (Rival *et al.*, 1996) so that this phenomenon is not strictly demarcated between viral and cellular promoters. The determination of the mechanisms involved in cytokine-regulated gene expression and a comprehensive understanding of promoter cytokine sensitivities will fundamentally alter the design of vectors for gene transfer and gene therapy.

V. Immunostimulatory DNA Sequences

Hosts respond rapidly to bacterial infection by producing cytokines that regulate infection and antigen-specific immune responses. A wide variety of bacterial products, such as lipopolysaccharide (LPS), teichoic acid, peptidoglycans, and formyl methionine, can induce immune activation (Marrack and Kappler, 1994), yet, until recently, bacterial DNA, like all foreign DNA, was viewed as immunologically inert. However, studies have demonstrated that bacterial DNA can be antigenic and evoke both cellular and humoral immune responses.

The immunostimulatory effect of bacterial DNA was initially discovered in 1984 when Tokunaga *et al.* (1984) observed tumor resistance in mice inoculated with *Mycobacterium bovis* BCG. To define the immunomodulatory molecule responsible for this activity, extracts were fractionated and screened for *in vivo* antitumor activity, which led to the isolation of MY-1, composed almost entirely of DNA and RNA. Tumor resistance was shown to be sensitive to DNase but not RNase (Tokunaga *et al.*, 1984; Shimada *et al.*, 1985). Thus, the DNA component of MY-1 was considered responsible for the biologic activity. Experiments *in vivo* and *in vitro* demonstrated that MY-1 DNA-induced tumor resistance resulted from enhanced NK cell activity (Shimada *et al.*, 1985; Mashiba *et al.*, 1988). MY-1 stimulated the production of IFNα/β and IFNγ, resulting in increased NK activity (Yamamoto *et al.*, 1988). Lymphocytes from LPS-insensitive C3H/HeJ mice responded to the MY-1 DNA, as well as those from LPS-sensitive BALB/c mice, and because the activities of the DNA fraction were not influenced by the presence of polymixin B, this excluded the possibility of contaminating LPS as an active substance (Yamamoto *et al.*, 1992b). Additional studies showed that DNA from a variety of bacteria, viruses, and invertebrates, but not mammalian sources, could stimulate the NK response, suggesting that base sequence or base modifications, rather than the DNA backbone itself, were critical for activity (Yamamoto

et al., 1992b). Experiments utilizing synthetic oligonucleotides bearing randomly selected DNA sequences from *M. bovis* demonstrated that the activation was due to a series of CpG motifs that generally followed the sequence 5′-purine-purine-C-G-pyrimidine-pyrimidine-3′ (Yamamoto *et al.*, 1992a, 1994a; Tokunaga *et al.*, 1992). These sequences were frequently palindromic, although palindromes were not essential for activity (Ballas *et al.*, 1996). Synthetic oligonucleotides containing CpG motifs have activity in both human and murine systems, suggesting a generalized mammalian response to microbial DNA for cytokine induction and disease resistance (S. Yamamoto *et al.*, 1992a; T. Yamamoto *et al.*, 1994b; Tokunaga *et al.*, 1992).

There are two important differences between bacterial and mammalian DNA. First, mammalian DNA exhibits CpG suppression (Bird, 1987). This refers to the fact that mammalian DNA has a much lower expression of CpG dinucleotides than predicted by random base utilization. In contrast, bacterial DNA has the normal expected CpG distribution. Second, cytosine is commonly methylated in mammalian DNA, whereas this base is not methylated in bacterial DNA (Hergersberg, 1991). Both cytosine methylation or the elimination of the CpG from synthetic oligonucleotides abolishes immunostimulatory activity, suggesting that unmethylated CpG motifs are important for immune recognition of bacterial DNA (Ballas *et al.*, 1996; Krieg *et al.*, 1995). Although methylation of CpG sites is usually considered important for transcriptional regulation, the origin of CpG suppression in mammalian DNA is unclear and has generated much speculation. It has been proposed that CpG suppression and/or methylation was selected during vertebrate evolution so that immune defenses could be triggered preferentially by microbial DNA (Krieg *et al.*, 1995).

Bacterial DNA has multiple effects on cells of the immune system (Table III). Kreig *et al.* reported that bacterial DNA is a potent mitogen for murine B cells (Krieg *et al.*, 1995). DNA from a variety of bacteria can induce proliferation and antibody production by murine B cells under conditions in which mammalian DNA is inactive. DNA analysis demonstrated that the sequences responsible for B-cell stimulation were essentially the same CpG DNA sequences as those that elicited IFN production in studies with MY-1. The demonstration of murine B-cell activation by the same CpG motifs that enhanced NK cell activity heightened interest in the immune potential of bacterial DNA. Several studies have since verified that bacterial-derived CpG sequences elicit interferon production, NK cell activation, Ab production, and B-cell proliferation in both human and murine lymphocytes (Yi *et al.*, 1996a; Yamamoto *et al.*, 1994b; Kuramoto *et al.*, 1992). Klinman *et al.* (1996) evaluated the ability of CpG motifs to induce cytokine production by T cells, B cells, macrophages, and NK cells. Unmethylated CpG DNA motifs directly activate B cells to

TABLE III
RESPONSE OF LEUKOCYTES TO CPG MOTIFS

Cell Type	Response to CpG Motifs	Reference
B cells	Proliferation, Ab production, IL-6 and IL-12 production	Krieg *et al.* (1995); Yi *et al.* (1996a); Klinman *et al.* (1996)
T cells	IL-6, IFNγ production	Klinman *et al.* (1996)
Macrophages	Activation (TNFα, IL-1, PAI-2)	Stacey *et al.* (1996)
NK cells	Activation, IFNγ production	Shimada *et al.*, (1985); Mashiba *et al.*, (1988); S. Yamamoto *et al.* (1988, 1992a,b); T. Yamamoto *et al.* (1994a,b); Yi *et al.* (1996a); Kuramoto *et al.* (1992); Tokunaga *et al.* (1992); Klinman *et al.* (1996)

secrete IL-6 and IL-12 within several hours and, as a result of the IL-6 production, to secrete IgM. CpG acts on NK cells, and, to a lesser degree, on $CD4^+$ T cells to induce IFNγ production. Thus, immune activation induced by CpG DNA includes both humoral immunity through the induction of IL-6 secretion and increased cellular immunity through IFNγ secretion. CpG stimulatory motifs also induced B, T, and NK cells to secrete cytokines even more effectively than LPS. Of interest, the authors were unable to detect any CpG-induced production of IL-2, IL-3, IL-4, IL-5, or IL-10 *in vitro* or *in vivo* by any of the cell types evaluated, and no significant cytokine production was observed by CD8+ T cells or macrophages. However, Stacey *et al.* (1996) demonstrated that macrophages can ingest and are activated by bacterial DNA-containing CpG motifs as evidenced by TNFα, IL-1, plasminogen activator inhibitor-2, and increased NF-κB-binding activity. These data suggest that internalization of CpG DNA by macrophages may activate a signaling cascade leading to activation of NF-κB and inflammatory gene induction.

The precise molecular mechanisms by which bacterial DNA activates immune cells and regulates cytokine expression are unknown. Intracellular trafficking of bacterial DNA has not been defined; however, DNA appears to enter cells by bulk phase endocytosis or podocytosis (Akhtar and Juliano, 1992; Bennett, 1993). In addition, it is known that some cell types express receptors on their surface that can bind DNA (Bennett *et al.*, 1985; Yakubov *et al.*, 1989; Hefeneider *et al.*, 1992; Kimura *et al.*, 1994). Once inside cells, bacterial DNA could induce cellular effects by a number of mechanisms, including binding a transcriptional regulator or blocking its action, forming triple-stranded DNA to alter transcriptional activity, or binding to

mRNA to block translation of a key regulatory protein by an antisense mechanism (Pisetsky, 1996). Another alternative may be that CpG motifs allow entry of DNA into a cell compartment where it can mediate its effects due to its polyanionic structure (Pisetsky, 1996). In parallel with the ability of viral DNA to activate IFNγ-induced cytoplasmic enzymes (Sen and Ransohoff, 1993; Landolfo *et al.*, 1995), bacterial DNA may directly activate or inactivate cytoplasmic or nuclear proteins important in transcriptional regulation.

The implications or consequences of CpG-induced immune responses in regards to gene therapy are only now being realized. Sato *et al.* (1996) demonstrated that plasmids bearing CpG motifs can dramatically enhance the immune response to DNA immunization and limit transgene expression. The authors demonstrated that BALB/c mice injected intradermally with an expression vector encoding β-galactosidase and containing the ampicillin resistance gene produced a strong CTL and antibody response against the β-galactosidase transgene. However, BALB/c mice injected intradermally with a similar vector containing the kanamycin resistance gene did not generate a humoral or cellular response to β-galactosidase. Further analysis showed that the ampicillin resistance gene contained two CpG motifs (5′-AACGTT-3′), whereas the kanamycin resistance gene contained none. Hypothesizing that these sequences in the ampicillin resistance gene may upregulate the immune response to the transgene, the investigators subcloned this hexanucleotide sequence next to sites flanking the kanamycin resistance gene. The addition of one or two hexanucleotide repeats to the noncoding region of the vector backbone dramatically enhanced the immune response to β-galactosidase in a dose-related fashion. Both the localization and the precise sequence of the CpG motif within the plasmid backbone were important for the observed effect. The unmethylated CpG sequence also enhanced the CTL and Th1 type immune response after intradermal gene vaccination, which likely contributed to limiting transgene expression. The authors speculated that IFNα induced by CpG motifs further interfered with gene expression. The addition of neutralizing antibodies to IFNα doubled β-galactosidase expression, whereas the addition of IFNα (50 pg/ml) diminished β-galactosidase expression by 40%. Thus, the presence of immunostimulatory CpG motifs may induce the synthesis of proinflammatory cytokines that further interfere with transgene transcription.

The therapeutic use of DNA in gene therapy exposes patients to high concentrations of free DNA that contains bacterial and viral sequences. Plasmid DNA is propagated in bacteria and lacks CpG base methylation. Thus, these plasmids have the potential to induce cytokines, activate immune cells, and serve as adjuvants. A DNA adjuvant effect can be desirable

for gene immunization, but unnecessary and perhaps harmful for gene replacement. Vectors for somatic or stem cell gene replacement therapy should be designed to lack these immunostimulatory CpG motifs, whereas vectors for gene vaccination should probably be engineered to have multiple CpG repeats. In fact, immunostimulatory CpG motifs may be critical for DNA immunization as the magnitude of the response induced by different vectors appears related to the content of these sequences (Sato *et al.*, 1996). However, it should be noted that plasmids containing CpG immunostimulatory motifs may induce adverse effects, such as local inflammation, shifting the Th1/Th2 balance, and the development of antibodies against DNA.

The potent immune activation by CpG oligonucleotides has implications for the use of antisense oligonucleotides. Antisense oligonucleotides targeted to complementary messenger RNAs are now in human clinical trials, yet it is apparent that oligonucleotides have many nonantisense side effects (Lisziewicz *et al.*, 1994; Bayever *et al.*, 1993). A review of the literature by Krieg *et al.* (1995) revealed at least 18 reports in which oligonucleotides that cause *in vitro* or *in vivo* lymphocyte activation or protection from apoptosis contained matches for the CpG DNA motif. This suggests that CpG motifs may supercede antisense effects and that the avoidance of unmethylated CpG motifs in antisense oligonucleotides is desirable.

VI. Alteration of Immune Function by Viral Products

Previous sections detailed specific and innate immune responses against vectors and transgenes and how cytokine products of the immune system affect the function of viral and nonviral vectors. It is important to understand that the interaction between the immune system and gene transfer vectors is not one way but bidirectional. This is evident in the previous section, which reviewed the interaction between naked DNA and the immune system and showed how DNA activates certain components of the immune response. In this context it is noteworthy that viruses also produce a variety of molecules that can directly or indirectly regulate immune responses. In particular, virally encoded proteins may serve to evade or counteract immune defenses (Gooding, 1992; Kotwal, 1996). This section reviews a variety of vectors and the cellular and molecular mechanisms by which they regulate immune responses (Table IV).

A strain of lymphocytic choriomeningitis virus (LCMV) encodes a variant of the viral capsid GP-1 glycoprotein. The variant alters the tropism of the virus so that it more readily infects splenic dendritic antigen-presenting cells (APC) (Borrow *et al.*, 1995). Infection of splenic APC stimulates antiviral immunity and results in a CD8–dependent loss of dendritic cells

TABLE IV
ALTERATION OF IMMUNE FUNCTION BY VIRAL PRODUCTS

Immune Alteration	Virus	Mechanism	Reference
Infection of dendritic APC	LCMV	Altered tropism	Borrow *et al.* (1995)
Interfere with proinflammatory cytokines	Poxviruses	Inhibit ICE	Ray *et al.* (1992)
		Soluble IL-1R	Spriggs *et al.* (1992); Alcamí and Smith (1992)
	Adenovirus	IL-6RE inhibition	Takeda *et al.* (1994)
Interfere with TNF	Poxviruses	Soluble TNFR I and II	Smith *et al.* (1991, 1996); Upton *et al.* (1991); Hu *et al.* (1994); Macen *et al.* (1996a)
	Adenovirus	Inhibit TNF signal cascade	Gooding *et al.* (1988, 1990, 1991a,b); Horton *et al.* (1991); Tufariello *et al.* (1994); White *et al.* (1992); Efrat *et al.* (1995)
Interfere with IFN	Poxviruses	Soluble IFNR	Upton *et al.* (1992); Mossman *et al.* (1995, 1996); Colamonici *et al.* (1995)
		Inhibit eIF-2α kinase	Beattie *et al.* (1991, 1995); Davies *et al.* (1992, 1993); Chang *et al.* (1992)
	Influenza	Inhibit eIF-2α kinase	Lee *et al.* (1990)
	Adenovirus	Inhibit TNF signal cascade	Kalvakolanu *et al.* (1991); Gutch and Reich (1991); Ackrill *et al.* (1991).
Interfere with TCR	HIV-1	Inhibit $p56^{lck}$ and MAPK	Greenway *et al.* (1996)
Cytokine homologs	EBV	vIL-10	Hsu *et al.* (1990); Qin *et al.* (1996); Gawley *et al.* (1996)
	KSHV	IL-6, IRF, MIP	Moore *et al.* (1996)

Interfere with humoral immunity	HSV	FcR	Johnson *et al.* (1988)
	CMV	FcR	Thäle *et al.* (1994)
	HSV	C3bR	Friedman *et al.* (1984)
	Poxviruses	Complement binding proteins	Shchelkunov *et al.* (1993); Isaacs *et al.* (1992)
Inhibit apoptosis	Poxviruses	Inhibit ICE	Tewari *et al.* (1995)
		Inhibit TNF	Macen *et al.* (1996a)
		Inhibit Fas and perforin	Macen *et al.* (1996b)
		Inhibit FLICE	Thome *et al.* (1997)
	Adenovirus	Inhibit TNF	Gooding *et al.* (1988, 1990, 1991a,b); Horton *et al.* (1991); Tufariello *et al.* (1994); White *et al.* (1992)
		Inhibit E1A	Rao *et al.* (1992)
	SV40	Inhibit p53	McCarthy *et al.* (1994)
	Herpesvirus	Diverse mechanisms	Henderson *et al.* (1993); Zhu *et al.* (1995); Leopardi and Roizman (1996); Thome *et al.* (1997)
Inhibit MHC function	Adenovirus	Decrease Transcription	Meijer *et al.* (1992); Routes *et al.* (1993).
		Bind MHC class Iα in ER	Burgert and Kvist (1985); Anderson *et al.* (1985); Beier *et al.* (1994); Feuerbach *et al.* (1994)
	CMV	Retain MHC in ER	Jones *et al.* (1996); Wiertz *et al.* (1996a).
		Retrograde transfer to proteasome	Ahn *et al.* (1996); Wiertz *et al.* (1996b)
		Inhibit NK	Farrell *et al.* (1997); Reyburn *et al.* (1997)
	HSV	Inhibit TAP	A. B. Hill *et al.* (1994); A. Hill *et al.* (1995); Früh *et al.* (1995; York *et al.* (1994); Schust *et al.* (1996)
	EBV	Inhibit processing	Levitskaya *et al.* (1995)
	Poxviruses	Inhibit MHC expression	Boshkov *et al.* (1992)
	HIV-1	Inhibit MHC expression	Scheppler *et al.* (1989)

and subsequent generalized nonspecific immunosuppression and persistent viral infection. Although LCMV or other arenaviruses have not been considered for use as vectors in gene therapy, these results suggest that other vectors with tropisms for APC, or the deliberate targeting of viral or plasmid vectors to APC for the purpose of microbial vaccination or tumor immunization, could lead to immune-mediated destruction of APC. The systemic consequences of this would be mitigated by the fact that microbial or tumor vaccination, tend to involve only localized and restricted numbers of APC. More ominously, the targeting of a significant majority of systemic macrophages or other APC to correct metabolic disorders (e.g., lysosomal storage diseases) could have profound and undesirable immunosuppressive effects if the vector was designed to persist or propagate or if a vector-specific immune response was generated.

Many viruses encode proteins that interfere with proinflammatory cytokine function (Gooding, 1992; Kotwal, 1996; Alcamí and Smith, 1995). The cowpox virus *crmA* gene encodes a serpin inhibitor of interleukin-1β converting enzyme (ICE), which prevents the functional maturation of IL-1 (Ray *et al.*, 1992). *crmA* also inhibits the cellular apoptotic response to certain death-initiating signals that rely on ICE function (Tewari *et al.*, 1995). Thus, *crmA* inhibits several aspects of immune function. Cowpox virus and the related vaccinia virus also encode secreted, soluble IL-1-binding proteins that affect the pathogenesis of viral infection (Spriggs *et al.*, 1992; Alcamí and Smith, 1992). Therefore, orthopox viruses interfere with IL-1 function at different steps, including protein processing and receptor binding. The vaccinia virus has been used in many experimental gene therapy protocols, yet the immunoregulatory effects of these viral products have not been evaluated with respect to their effect on vector or immune function (Ramsay and Kohonen-Corish, 1993; Ramsay *et al.*, 1994).

Other inhibitors of proinflammatory cytokines include the 12S splice product of the E1A gene of adenovirus, which blocks the assembly of IL-6 response element-binding complexes and thus IL-6 function (Takeda *et al.*, 1994). Although adenoviruses considered for use in gene transfer are E1-deleted, this shows that viruses are capable of interfering with cytokine function by regulating downstream transcription factor assembly and function. Further evidence for regulation of transcriptional activity comes from reports that papillomavirus (Werness *et al.*, 1990; Lechner *et al.*, 1992; Mietz *et al.*, 1992), SV40 (Mietz *et al.*, 1992; Farmer *et al.*, 1992), and adenovirus (Renee and Berk, 1992) produce proteins that bind and inactivate the p53 transcriptional repressor. These viruses have all been considered for use as gene transfer vectors, although the p53-binding activities have transforming potential (Werness *et al.*, 1990; Lechner *et al.*, 1992;

Mietz *et al.*, 1992; Farmer *et al.*, 1992; Renee and Berk, 1992) and would require deletion prior to consideration for use as therapeutic vectors.

Multiple viruses encode inhibitors of TNF function. The poxviruses Shope fibroma virus, myxoma virus, malignant rabbit fibroma virus, variola, and cowpox virus encode soluble, secreted homologs of TNF receptor types I and II (Smith *et al.*, 1991, 1996; Upton *et al.*, 1991; Hu *et al.*, 1994). These proteins bind TNFα, TNFβ, and/or lymphotoxin (Smith *et al.*, 1991, 1996; Upton *et al.*, 1991; Hu *et al.*, 1994); contribute to the pathogenesis of viral disease (Upton *et al.*, 1991); and can inhibit TNF-driven apoptosis (Macen *et al.*, 1996a). The adenovirus E3-14.7K protein inhibits TNF-driven apoptosis, presumably by inhibiting some aspect of TNF-initiated signal transduction (Gooding *et al.*, 1988, 1990; Horton *et al.*, 1991), and may contribute to virulence (Tufariello *et al.*, 1994). Other adenoviral proteins, including E3-10.4K, E3-14.5K, and E1B-19K products, also inhibit TNF-driven apoptosis, presumably by altering intracellular signaling (Gooding *et al.*, 1991a,b; White *et al.*, 1992). Although all adenoviral vectors are E1 deleted, many express E3 gene products and may be capable of altering immune effector function. Inhibition of TNF function by E3 products suggests that E3 products may have immunosuppressive properties. In fact, E3 products can enhance vector persistence and expression (Efrat *et al.*, 1995; Lee *et al.*, 1995; Ilan *et al.*, 1997). These examples suggest that other classes of viruses and vectors likely encode additional regulators of TNF activity that do not necessarily bear close sequence homology to known members of the TNF signaling pathway so that their function would not be readily apparent by data base homology testing alone.

Inhibition of IFN function and signaling is another common mechanism of viral immune perturbation (reviewed in Liu *et al.*, 1994; McNair and Kerr, 1992). The orthopox viruses myxoma virus, ectromelia, and vaccinia encode soluble proteins that bind IFNγ or IFNα, block signal transduction, and contribute to viral pathogenesis (Upton *et al.*, 1992; Mossman *et al.*, 1995, 1996; Colamonici *et al.*, 1995). One of the antiviral effects of IFNγ is the induction of a kinase that phosphorylates elongation initiation factor-2 (eIF-2α) and prevents cellular and viral protein synthesis (Samuel, 1991). Vaccinia virus encodes two different inhibitors of the kinase that act at different molecular loci and affect the sensitivity of virus-infected cells to IFN (Beattie *et al.*, 1991, 1995; Davies *et al.*, 1992, 1993; Chang *et al.*, 1992). Influenza viruses also encode an inhibitor of the kinase (Lee *et al.*, 1990). Adenoviral E1A proteins block IFNα, β, and γ driven induction of the ISGF3 transcriptional complex and possibly also other undefined transcriptional regulators (Kalvakolanu *et al.*, 1991; Gutch and Reich, 1991; Ackrill *et al.*, 1991). These reports show that IFN signaling and function can be perturbed at multiple levels by many different viruses, suggesting

that other mechanisms of virus-related IFN inhibition remain to be elucidated and that a variety of gene transfer vectors may perturb IFN signaling.

The *Nef* gene product of HIV-1 is important for viral replication and pathogenesis and acts at least in part by perturbing T-cell function. It has been demonstrated that Nef binds directly to the $p56^{lck}$ and MAP kinases and inhibits their kinase activity (Greenway *et al.*, 1996). Therefore, Nef likely interferes with a wide variety of second signaling pathways, particularly those emanating from the T-cell antigen receptor, and may contribute to virally induced immunosuppression. HIV-based vectors are currently being developed for use in gene transfer, and attention to the role of *Nef*, or its deletion, will need to be considered.

Viruses also encode cytokine homologs. The *BCRF1* open reading frame of Epstein–Barr virus (EBV) encodes a homolog of cellular IL-10, termed viral IL-10 (Hsu *et al.*, 1990). Viral IL-10 may be more immunosuppressive than cellular IL-10, as it possesses the immunosuppressive properties of cellular IL-10 without initiating some of the immunostimulatory events that are characteristic of cellular IL-10 (Qin *et al.*, 1996; Crawley *et al.*, 1996). Kaposi's sarcoma-associated herpesvirus (KSHV) encodes homologs for IL-6 and interferon regulatory factor and two homologs for human macrophage inflammatory protein (Moore *et al.*, 1996). The poxviruses encode soluble inhibitors of chemokines (Graham *et al.*, 1997). Presumably, these soluble gene products are important for viral pathogenesis. Although EBV and KSHV have not yet been considered for use as gene vectors, the related HSV I has been used as a vector (Kaplitt *et al.*, 1994a). Although most viral genes are deleted in HSV vectors, these reports demonstrate the principle that herpesviruses, and likely other human viruses, encode cytokine homologs that could have significant immunomodulatory effects if incorporated into transfer vectors.

Viral products may also interfere with humoral immunity. HSV gE and gI glycoproteins form a complex that acts as an IgG Fc receptor (Johnson *et al.*, 1988), and murine CMV also produces an Fc receptor (Thäle *et al.*, 1994). These reports postulated that these Fc receptors interfere with the normal antiviral function of immunoglobulins and contribute to viral pathogenesis. HSV gC glycoprotein acts as a complement C3b receptor that may further inhibit antibody function and enhance viral pathogenicity (Friedman *et al.*, 1984). Variola and vaccinia poxviruses also encode complement-binding proteins (Shchelkunov *et al.*, 1993) that inhibit the complement cascade and viral neutralization and contribute to virulence (Isaacs *et al.*, 1992).

Apoptosis can regulate both lymphocyte responses and the responses of target cells to cytotoxic immune effectors. As mentioned earlier, the cowpox *crmA* gene inhibits ICE-dependent apoptosis (Tewari *et al.*, 1995), and

TNF-dependent apoptosis can be inhibited by a variety of poxvirus and adenovirus proteins (Macen *et al.*, 1996a; Gooding *et al.*, 1988, 1990, 1991a,b; Horton *et al.*, 1991; Tufariello *et al.*, 1994; White *et al.*, 1992). Adenovirus E1A-driven apoptosis can be inhibited by adenoviral E1B-19K (Rao *et al.*, 1992); p53–dependent apoptosis is inhibited by the binding activity of SV40 large T antigen (McCarthy *et al.*, 1994); Fas- and perforin/granzyme-dependent apoptosis are inhibited by both *crmA* and a related serpin proteinase inhibitor, SPI-1, encoded by poxvirus (Macen *et al.*, 1996b); and the herpesviruses code for a variety of proteins that inhibit apoptosis driven by several different stimuli (Henderson *et al.*, 1993; Zhu *et al.*, 1995; Leopardi and Roizman, 1996; Thome *et al.*, 1997). These reports demonstrate that viral gene products regulate multiple levels of a variety of different signaling pathways that induce apoptotic cell death. Although adenoviral E1 proteins are deleted in transfer vectors, apoptotic regulatory genes have not been consistently deleted in poxvirus or herpesvirus vectors. Thus, cellular transduction with these vectors may alter immune effector function if tumor or parenchymal cells are the target of the vector. Conversely, transduction may alter immune regulation if lymphocytes are the target of the transfer vector. The occurrence of so many forms of apoptotic regulation also suggests that there are likely to be additional molecular mechanisms of regulation that remain to be elucidated in other viruses and viral vectors.

One of the most extensively studied mechanisms by which viruses alter immune function is through changes in the expression and function of major histocompatibility complex molecules (McFadden and Kane, 1994). Adenovirus E1 proteins decrease transcriptional activation of class I MHC genes (Meijer *et al.*, 1992; Routes *et al.*, 1993), whereas the adenovirus E3-19K product binds to nascent class I MHC α chains in the endoplasmic reticulum (ER), resulting in chain retention and decreased cell surface expression (Burgert and Kvist, 1985; Anderson *et al.*, 1985; Beier *et al.*, 1994; Feuerbach *et al.*, 1994). These effects reduce cytotoxic T-cell responses in some (Efrat *et al.*, 1995; Lee *et al.*, 1995), but not all, cases (Cox *et al.*, 1994; Flomenberg *et al.*, 1996).

Human CMV causes decreased synthesis of class I MHC, retention of nascent MHC class I in the ER, and early and rapid degradation of newly synthesized MHC polypeptide chains (del Val *et al.*, 1992; Yamashita *et al.*, 1993, 1994; Beersma *et al.*, 1993; Campbell and Slater, 1994; Warren *et al.*, 1994; Jones *et al.*, 1995). These activities contribute to viral pathogenesis and escape from immune recognition (Warren *et al.*, 1994; Gilbert *et al.*, 1996). Detailed analysis of CMV proteins demonstrates that the *US3* gene product retains MHC complexes within the ER and prevents intracellular transport and cell surface expression (Jones *et al.*, 1996; Wiertz *et*

al., 1996a). The *US2* and *US11* gene products cause retrograde transfer of MHC chains from the ER to the cytosol, with subsequent delivery to the proteasome and proteolysis (Ahn *et al.*, 1996; Wiertz *et al.*, 1996b). These CMV proteins interfere not only with T-cell antigen recognition, but also NK cell function (Reyburn *et al.*, 1997).

The HSV ICP47 immediate early protein inhibits the transporter associated with antigen processing (TAP) (A. Hill *et al.*, 1995; A. B. Hill *et al.*, 1994; York *et al.*, 1994; Früh *et al.*, 1995; Schust *et al.*, 1996). This results in the failure of TAP to load antigenic peptides into nascent MHC class I molecules, which then remain in the ER. This inhibition of antigen presentation or MHC expression may be associated with viral immune evasion (York *et al.*, 1994) or pathogenesis (Schust *et al.*, 1996). The EBV EBNA1 protein has a specific internal Gly-Ala repetitive sequence that interferes with antigen processing and MHC class I-restricted presentation and likely contributes to viral persistence (Levitskaya *et al.*, 1995). Additional reports show that poxviruses (Boshkov *et al.*, 1992) and HIV-1 (Scheppler *et al.*, 1989) can also inhibit MHC class I expression, although the precise viral gene products and molecular mechanisms have not been elucidated. In summary, these reports demonstrate that many classes of virus have subverted a number of molecular mechanisms to inhibit antigen processing or transport and MHC function. Many of these proteins are likely to be expressed by currently available transfer vectors. These proteins could contribute to decreased immunogenicity of vector and transgene components and result in prolonged transgene expression. Alternatively, they could contribute to deleterious vector persistence or interfere with tumor immunity for cancer treatment or microbial immunity for vaccination.

VII. Transfer Vectors as Enhancers of Antigen Presentation

The nature of gene transfer and expression requires that viral or nonviral vectors be constructed, manipulated, and delivered so that nucleic acids are taken up in endosomal compartments, translocated to the cytoplasm, and transported to the nucleus for transcriptional activation. This sequence of intracellular transport and compartmentalization suggests that transfer vectors, and manipulations to improve their association with and entry into cells, will necessarily introduce vectors into antigen processing and presentation pathways, thereby increasing their immunogenicity. In the case of gene therapy for microbial or tumor immunity, this may enhance vector efficacy, although an exuberant immune response may be detrimental by causing too rapid a clearance of cells containing or expressing vector. In the case of gene therapy for metabolic disorders or inflammatory dis-

eases, increased immunogenicity is clearly detrimental to the goals of the therapy and may even induce autoimmunity. Thus, an understanding of how nucleic acid transfer pathways intersect with antigen-presenting pathways will be important to improve gene expression without inciting unwanted immune responses (Table V).

It is first important to consider the types of APC that are susceptible to gene transduction. DNA immunization can be achieved by multiple routes. Ballistic transfer of the DNA markedly enhances immunogenicity (Fynan *et al.*, 1993), and cellular analysis of antigen presentation reveals that cutaneous, ballistic delivery of DNA results in transfection of dermal dendritic cells, which migrate to draining lymph nodes where they express and present antigen to T and B cells (Condon *et al.*, 1996). DNA immunization by intramuscular injection of naked plasmid DNA effectively generates class I-restricted, $CD8^+$ CTL. Adoptive transfer experiments show that the CTL are restricted to the MHC haplotype of bone marrow-derived APC and *not* to the MHC of the transfected myocytes (Ulmer *et al.*, 1996; Doe *et al.*, 1996; Corr *et al.*, 1996). Further experiments show that antigen presentation is due to transfer of proteins from transfected myocytes to APC and not to the direct plasmid transduction of the APC (Ulmer *et al.*, 1996; Doe *et al.*, 1996). How antigen transfer occurs is not known; however, the immune responses generated by muscle gene therapy are sufficient to completely abrogate the utility of gene transfer for metabolic disease (Shull *et al.*, 1996). It is also possible to transfect or infect macrophages or monocytes directly with DNA or viral vectors and achieve gene expression (Erbacher *et al.*, 1996; Weir *et al.*, 1996). In fact, virtually any cell type that can be transduced can also serve as an effective APC (Cayeux *et al.*, 1996; Riddell *et al.*, 1996). These results demonstrate that viral and nonviral vectors can transduce cells so that transferred gene products directly or indirectly enter cellular antigen processing and presenting pathways in professional or nonprofessional APC. These results mean that even if the immunogenicity of viral vectors is abrogated or sufficiently diminished to preclude meaningful antivector immunity, alloimmune or even autoimmune responses to the transgene product are a significant possibility. Furthermore, the rate of immunization and the cell type targeted will further determine the type and magnitude of the immune response (Bronte *et al.*, 1997; Feltquate *et al.*, 1997).

Gene vectors may further enhance immunity toward vector or transgene components by enhancing costimulatory signals for lymphocyte or neutrophil activation, homing, or effector function. For example, ICAM-1 expression is increased on bronchial epithelium by adenoviral vectors (Pilewski *et al.*, 1995; Stark *et al.*, 1996). Adenoviral vectors also cause IL-8 expression by airway epithelial cells (Amin *et al.*, 1995). Likewise, respiratory syncytial

TABLE V
TRANSFER VECTORS AS ENHANCERS OF ANTIGEN PRESENTATION

Effect on Antigen Presentation	Vector	Mechanism	Reference
Transduction of APC	Ballistic DNA	Dendritic cell transduction	Fynan *et al.* (1993); Condon *et al.* (1996)
	Intramuscular DNA	Antigen transfer to APC	Ulmer *et al.* (1996); Doe *et al.* (1996); Corr *et al.* (1996)
	Ex vivo transduction	Transfection of APC directly	Erbacher *et al.* (1996)
		Infection of APC directly	Weir *et al.* (1996)
		Nonprofessional APC	Cayeux *et al.* (1996); Riddell *et al.* (1996)
Cellular activation by vectors, enhancement of costimulatory activity	Adenovirus	Increased ICAM-1	Pilewskiet *et al.* (1995); Stark *et al.* (1996)
		IL-8 expression	Amin *et al.* (1995)
	RSV	IL-6, IL-8, TNFR 1	Arnold *et al.* (1994)
	Flavivirus	Increased ICAM-1	Shen *et al.* (1995)
	SIV	Increased $\alpha^E\beta_7$	Gummuluru *et al.* (1996)
	Rotavirus	IL-8	Sheth *et al.* (1996)

	Rhinovirus	IFNγ	Gern *et al.* (1996)
	Plasmid DNA	IL-1, IL-6, IL-12, IFNs	S. Yamamoto *et al.* (1992b); Tokunaga *et al.* (1992); Ballas *et al.* (1996); Kreig *et al.* (1995); Yi *et al.* (1996a,b); T. Yamamoto *et al.* (1994a); Stacey *et al.* (1996); Kimura *et al.* (1994); Sato *et al.* (1996)
Enhance entry into antigen processing and presentation pathways	Liposomes	Delivery to class I MHC	Lopes (1992); Nair *et al.* (1992a,b); Collins *et al.* (1992); van Binnendijk *et al.* (1992); Zhou *et al.* (1992a,b); Miller *et al.* (1992); Huang *et al.* (1992); Chen *et al.* (1993); Jondal *et al.* (1996)
		Delivery to class II MHC	van Binnendijk *et al.* (1992)
	Electroporation	Delivery to class I MHC	Chen *et al.* (1993)
	Sendai	Delivery to class I MHC	Liu *et al.* (1995)
	Dendrimers	Delivery to class II MHC	Kukowska-Latallo *et al.* (1996)
	Tat	Delivery to class II MHC	Frankel and Pabo (1988); Mann and Frankel (1991); Fawell *et al.* (1994); Goldstein (1996)
	Antennapedia homeodomain	Delivery to class I MHC	Derossi *et al.* (1994); Allinquant *et al.* (1995); Troy *et al.* (1996)

virus induces IL-6, IL-8, and soluble TNFR I from pulmonary epithelial cells (Arnold *et al.*, 1994). The flavivirus West Nile virus can induce ICAM-1 expression on fibroblasts (Shen *et al.*, 1995), and the SIV lentivirus can induce $\alpha^E\beta_7$ integrin expression on T cells (Gummuluru *et al.*, 1996). Rotavirus stimulates IL-8 secretion by intestinal epithelial cells (Sheth *et al.*, 1996), and rhinovirus activates T cells to produce IFNγ (Gern *et al.*, 1996). Thus multiple different families of virus, some of which have been considered for use as vectors, can activate many different target cell types to produce adhesion molecules or cytokines that are anticipated to enhance immunity and inflammation. Although these effects may be desirable for gene therapy vaccination schemes for microbial or tumor immunity, they would likely impede efforts to treat metabolic or inflammatory diseases.

Proinflammatory effects are not limited to viral vectors since, as detailed earlier, plasmid DNA can directly enhance immunogenicity, proinflammatory cytokine production, macrophage activation, NK activity, and B-cell activation (S. Yamamoto *et al.*, 1992a; Tokunaga *et al.*, 1992; Ballas *et al.*, 1996; Krieg *et al.*, 1995; Yi *et al.*, 1996a,b; T. Yamamoto *et al.*, 1994b; Stacey *et al.*, 1996; Kimura *et al.*, 1994; Sato *et al.*, 1996). In addition, because plasmid DNA is derived from bacterial sources, it is frequently contaminated with small quantities of LPS, which can further induce costimulatory activity (Wicks *et al.*, 1995). It was also detailed earlier that several cytokines can impede viral or nonviral vector function by negative regulation of promoter activity. This means that immune responses can directly clear transduced cells or indirectly turn off transduced gene expression by transcriptional silencing. In sum, virtually all transfer vectors have the potential to stimulate proinflammatory cytokine or receptor expression or target cell activation. To date, these issues have not been widely addressed in evaluating and redesigning vectors to improve gene expression. These results suggest that detailed immune analysis of target cell responses to vector transduction is required to further the development of safe and effective transfer vectors.

The precise intracellular location of transfer vectors and the relation to class I and class II MHC processing and presentation pathways is far from elucidated at this point. Nonetheless, various lines of investigation suggest that transfer vectors enhance antigen presentation. Liposomal delivery of peptides and proteins to T, B, APC, or fibroblasts efficiently primes for class I MHC-restricted $CD8^+$ CTL *in vivo* and *in vitro* (Lopes and Chain, 1992; Nair *et al.*, 1992a,b; Collins *et al.*, 1992; van Binnendijk *et al.*, 1992; Zhou *et al.*, 1992a,b; Miller *et al.*, 1992; Huang *et al.*, 1992; Chen *et al.*, 1993; Jondal *et al.*, 1996). The same result may also be obtained by electroporation (Chen *et al.*, 1993). Class II MHC-restricted CTL may also be generated by liposomal protein delivery, yet this pathway is chloroquine insensitive (van Binnendijk *et al.*, 1992), suggesting that the peptide is

delivered directly to the cytoplasm, bypassing endocytic vesicles so that endolysozomes are not involved in antigen trafficking. An electron microscopic study of cellular uptake of DNA–cationic lipid complexes shows rapid endocytosis followed by release into the cytoplasm (Labat-Moleur *et al.*, 1996). Sendai virus, which is a membrane encapsulated virus, is able to enter class I MHC presentation pathways that are independent of TAP, proteasome subunits, and brefeldin A sensitive transport (Liu *et al.*, 1995). This antigen-presenting activity is also independent of viral fusion and hemagglutinin-neuraminidase activities, which suggests that direct liposomal fusion to the cell membrane is involved in antigen transport and presentation. These results imply that both liposomal plasmid DNA delivery and encapsulated viral nucleic acid delivery can efficiently direct antigen to class I and probably class II pathways. Therefore, gene transfer vectors by their nature enhance the immunogenicity of their delivered load. The implication for gene therapy is that all vectors will potentially be immunogenic unless only self-components are incorporated in the vector or the recipient can be made tolerant to foreign molecules. A corollary is that the enhanced immunogenicity of vectors could induce autoimmune responses.

Other methods of transduction also intersect conventional antigen processing and trafficking pathways. Polyamidoamine dendrimers deliver DNA to a chloroquine-sensitive compartment (Kukowska-Lattallo *et al.*, 1996), which suggests that class II MHC presentation could ensue. The tat protein of HIV-1 can translocate across cell membranes and, as a result of this property, has been considered for use as a vehicle in gene transfer. Tat adsorbs to the cell membrane and is taken up in a chloroquine-sensitive endocytic compartment, which suggests it intersects the class II presentation pathway (Frankel and Pabo, 1988; Mann and Frankel, 1991; Fawell *et al.*, 1994; Goldstein, 1996). The third α-helix of the *Drosophila* antennapedia homeodomain can also translocate through cell membranes in an energy-independent fashion and can be used to deliver nucleic acids to the cytoplasm (Derossi *et al.*, 1994; Allinquant *et al.*, 1995; Troy *et al.*, 1996). The homeodomain can deliver peptides to a brefeldin A-sensitive, chloroquine-insensitive, conventional class I processing pathway to generate $CD8^+$ CTL (Schultze-Redelmeier *et al.*, 1996). Receptor-mediated endocytosis can deliver antigens to the class II pathway, yet bypass the need for HLA-DM enzymatic activity for removal of the invariant chain from class II (Ma and Blum, 1997). These examples further support the contention that most, if not all, transfer vectors intersect class I and class II MHC-associated antigen-processing pathways.

VIII. Conclusions

The interaction of the immune system with gene therapy vectors, their components, and their products is bidirectional. Each can regulate and

affect the other in either positive or negative ways. There is no simple immune response to a vector, but rather a variety of specific and innate responses that interact with each other and with vector-initiated regulatory events that determine the final level and consequences of transgene expression. It is likely that many of these events can be predicted with enough fidelity such that they can be manipulated to enhance the effect of gene transfer for microbial or tumor immunization. However, it is clear that before gene transfer for gene replacement or inflammatory disease can be successful, many of these immune processes must be understood in considerably more detail before they can be circumvented. Precisely how antivector and antitransgene immune responses can be eliminated, the molecular nature of cytokine regulation of promoter activity, the molecular locus of CpG binding to transcriptional regulators, and the role of antigen-presenting pathways in gene expression are major topics requiring further dissection and elucidation.

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How Do Major Histocompatibility Complex Genes Influence Odor and Mating Preferences?

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Man's best friend might be used to sniff out histocompatible donors
Lewis Thomas (1974)

I. Introduction

The highly polymorphic genes of the major histocompatibility complex (MHC) influence odor and mating preferences. House mice (*Mus musculus*) prefer to mate with individuals carrying dissimilar MHC genes, and three studies have implicated MHC disassortative odor and mating preferences in humans (Table I). There are two potential reasons why females prefer to mate with MHC dissimilar males. MHC-dependent mating preferences may enhance the immunological resistance of a female's progeny to pathogens and parasites (e.g., heterozygote advantage) (Apanius *et al.*, 1997). MHC disassortative mating preferences may also reduce inbreeding, which would increase the MHC and overall genetic heterozygosity of an individual's progeny (Potts and Wakeland, 1993; Potts *et al.*, 1994). Whatever the advantages are for individuals, MHC disassortative mating preferences provide a potentially important selective factor driving the polymorphisms of MHC genes in vertebrate populations (Potts and Wakeland, 1990; Potts *et al.*, 1991; Hedrick, 1992).

Most of the work on MHC-dependent mating preferences has focused on determining how females recognize the MHC identity of potential mates. Evidence accumulated since the mid-1970s indicates that MHC genes influence individual odors (Table II): mice can distinguish the odors of mice that differ genetically only at classical MHC loci (Yamazaki *et al.*, 1983a; Penn and Potts, 1998), rats can discriminate the odor of rats that differ genetically only in the MHC region (MHC-congenic strains) (Brown *et al.*, 1989), and women prefer the odor of men who have dissimilar genes (Wedekind *et al.*, 1995; Wedekind and Füri, 1997). It is unclear, however, how MHC genes influence individual odor.

There are several ways that MHC genes can influence odor. First, because MHC molecules occur in urine and sweat, it has been suggested that MHC

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TABLE I
MHC-DEPENDENT ODOR AND MATING PREFERENCES

	Mating Preferences		Odor Preferences		
	Male	Female	Male	Female	Reference[a]
House mice	+[b]				(1)
	+				(2)
	+				(3)
	+	−			(4)
	−				(5)
		+		+	(6)
		−			(7)
		+			(8)
				+	(9)
		+			(10)
		−			(11)
		+			(12)
Sheep	−	−			(13)
Humans				+	(14)
		+			(15)
	−	−			(16)
			+	+	(17)

[a] (1) MHC-congenic strains of male mice show disassortative mating preferences in the laboratory (Yamazaki *et al.*, 1976). (2) Rederived strains of homozygous F_2 segregant mice showed MHC disassortative mating preferences (Yamazaki *et al.*, 1978). (3) Male mice cross-fostered onto MHC dissimilar parents showed MHC assortative mating preferences, whereas in-fostered controls showed MHC disassortative preferences in the laboratory (Yamazaki *et al.*, 1988; Beauchamp *et al.*, 1988). (4) Although male mating preferences were reversed by cross-fostering, no female mating preferences were detected (Beauchamp *et al.*, 1988). (5) No male mating preferences were found in a laboratory study of mice (Eklund *et al.*, 1991). (6) Female MHC disassortative odor and mating preferences were found in one laboratory study that controlled male–male interactions (Egid and Brown, 1989), but (7) not in another (Manning *et al.*, 1992a). (8) A study on wild-derived mice in seminatural enclosures found female MHC disassortative mating preferences (Potts *et al.*, 1991). (9) A laboratory study found female mice prefer the odors of MHC dissimilar males (Ninomiya and Brown, 1995). (10) Cross-fostering mice altered MHC-dependent mating (first mount) preferences in a laboratory study of mice (Eklund, 1997a). (11) No evidence for MHC-dependent mating preferences were found in a laboratory study using wild mice (Eklund, 1997b). (12) Cross-fostered female mice in seminatural conditions preferred to mate with MHC similar males (Penn and Potts, in press). (13) Paterson and Pemberton (1998) found no evidence for MHC-dependent mating preferences in a population of feral sheep. (14) Women prefer the odors of MHC dissimilar men (Wedekind *et al.*, 1995). (15) Hutterite men and women show MHC disassortative mating patterns (Ober *et al.*, 1997). (16) No MHC-dependent mating preferences were detected in Amerindians (Hedrick and Black, 1997). (17) Wedekind and Füri (1997).

[b] A plus indicates positive evidence for MHC-dependent odor or mating preferences.

molecules or fragments are the odorants (the MHC molecule hypothesis) (Singh *et al.*, 1987, 1988b; Roser *et al.*, 1991). Second, the unique pool of peptides bound by MHC molecules may be the precursors for the volatile odorants (the peptide hypothesis) (Singer *et al.*, 1997). Third, MHC genes may influence odor indirectly by shaping an individual's particular popula-

TABLE II
MHC GENES INFLUENCE INDIVIDUAL ODOR

Source of Genetic Variation among Odor Donors	Smellers (Subjects)				
	House Mice		Norway Rats		
	Trained	Untrained	Trained	Untrained	Humans
Entire MHC region					
MHC congenic mice	(1)	(2)	(3)	(4)	(5)
MHC congenic rats			(6)	(7)	
Humans			(8)		(9)
Subregions within MHC					
MHC recombinant mice	(10)				
MHC recombinant rats				(11)	
Class I and II MHC loci					
bm mutants	(12)		(13)		
dm mutants	(14)	(2)			

(1) Yamazaki *et al.*, 1979, 1982; Yamaguchi *et al.*, 1981, (2) Penn and Potts, 1998, (3) Beauchamp *et al.*, 1985; Brown *et al.*, 1990; Singh *et al.*, 1988a, (4) Schellinck *et al.*, 1995, (5) Gilbert *et al.*, 1986, (6) Schellinck *et al.*, 1991, (7) Brown *et al.*, 1987, (8) Ferstl *et al.*, 1992, (9) Wedekind *et al.*, 1995; Wedekind and Füri, 1997, (10) Yamazaki *et al.*, 1982, (11) Brown *et al.*, 1987, 1990, (12) Yamazaki *et al.*, 1983a, 1986, 1990a; Boyse *et al.*, 1987, (13) Beauchamp *et al.*, 1990, (14) Yamazaki *et al.*, 1991.

tions of commensal microflora (the microflora hypothesis) (Howard, 1977). Fourth, it has been suggested that MHC molecules are converted during degradation from peptide-presenting molecules into transporters that bind to aromatic molecules produced by commensal gut microbes (the carrier hypothesis) (Pearse-Pratt *et al.*, 1992). Finally, a synthetic explanation is offered in which MHC molecules alter the available pool of peptides, and their metabolic products are made volatile by commensal microflora (the peptide–microflora hypothesis). This hypothesis, which combines peptide and microflora hypotheses, is the most consistent with available data and may help explain the disparities among different studies.

This review provides an overview of the studies on MHC-dependent odor and mating preferences in mice, rats, and humans. First, a critical review of the studies on MHC-dependent mating preferences in mice and humans is provided. Second, studies that indicate that MHC genes influence individual odors in mice, rats, and humans are reviewed. Third, the mechanisms that have been proposed to explain how MHC genes control odor are reviewed. Fourth, how MHC-determined odors influence the development of MHC-dependent mating preferences through chemosensory imprinting is examined. Although there is increasing evidence for MHC-dependent mating preferences in mice and humans, the underlying mechanisms and evolutionary significance remain elusive. Moreover, more species need to be examined to

make any generalizations about MHC-dependent mating preferences and their importance in maintaining MHC polymorphisms.

II. Major Histocompatibility Complex (MHC)-Dependent Mating Preferences

MHC-dependent mating preferences have been found in house mice in both laboratory and seminatural conditions. Two studies have also found evidence for MHC-dependent odor and mating preferences in humans (Table I).

A. Laboratory Studies with Mice

The first experiments on MHC-based mating preferences were prompted by a serendipitous observation by an animal care technician who noted that mice that differ genetically only at the MHC (MHC congenic strains) appeared to associate with dissimilar strains (Boyse *et al.*, 1987). Subsequent studies by Yamazaki and colleagues (1976, 1978) found that in four of six congenic strains, male mice preferred to mate with MHC dissimilar females. The reason for the variation among strains is unclear, but it may be due to inadvertant selection against inbreeding avoidance mechanisms during the derivation of the strains (Manning *et al.*, 1992a). It is also unclear if males or females were doing the choosing in these experiments. The mate preference assay used by Yamazaki and colleagues consisted of placing a male into an arena with two females, one MHC similar and one dissimilar, and then noting which one of the females mated with the male. Thus, males may have tended to mate with dissimilar females because females were more receptive to dissimilar males.

Yamazaki and colleagues (1988) found direct experimental evidence for MHC-dependent mating preferences in male house mice. They found that the MHC disassortative mating preferences of a male can be reversed by cross-fostering males at birth onto MHC dissimilar parents. This cross-fostering experiment suggests that mice learn the MHC identity of their family and then avoid mating with individuals carrying familial MHC haplotypes (familial imprinting). However, a subsequent laboratory experiment failed to detect MHC-dependent mating preferences in male house mice (Eklund *et al.*, 1991). The reason for the disparate results in these studies is unclear, but one possible explanation is that MHC-dependent mating preferences are weak in males and therefore difficult to detect. Mating preferences are usually more pronounced in females because of their greater parental investment; therefore, MHC-dependent mating preferences should be more easily detected in females.

MHC-dependent mating preferences have been tested in female mice in several laboratory tests with mixed results. One laboratory study found

no evidence for MHC-dependent mating preferences in female mice (Beauchamp *et al.*, 1988). This study, however, failed to control for interactions among males, which is crucial as female preferences for MHC dissimilar males appear to be secondary to preferences for dominant males (Potts *et al.*, 1991). A second laboratory study found MHC disassortative mating preferences in female mice once they controlled male–male interactions (Egid and Brown, 1989). Yet two additional laboratory experiments found no evidence for MHC-dependent mating preferences in female mice, even though male–male interactions were controlled (Manning *et al.*, 1992a; Eklund, 1997b).

Counterintuitively, laboratory experiments with inbred animals do not provide definitive assays for MHC disassortative mating preferences. First, laboratory mice are not as choosy as wild, outbred mice (Manning *et al.*, 1992a). Second, inbreeding avoidance behaviors may have been eliminated in inbred lines due to artificial selection (Manning *et al.*, 1992a). Third, laboratory conditions can create artifacts that mask or alter mating preferences. Tethering males, for example, will control male–male interactions but females may simply prefer the male that is the least stressed from being tethered. The best evidence for MHC disassortative mating preferences therefore comes from observations of animals in natural conditions. The diversity of MHC genes, however, makes it difficult to detect MHC-based mating patterns in wild populations (i.e., it requires enormous sample sizes and genotyping all MHC loci accurately). Thus, one compromise is to conduct mate choice experiments in seminatural conditions with wild-derived animals.

B. Mice in Seminatural Enclosures

The most convincing evidence that mice in the wild mate disassortatively for MHC genes is a study on wild-derived mice in large, seminatural enclosures (Potts *et al.*, 1991, 1992). In an attempt to determine the selective factors that maintain MHC diversity, the authors of this study placed semiwild mice carrying four well-characterized MHC haplotypes into large, seminatural enclosures. The mice were derived from crossing wild mice with four inbred strains carrying known MHC haplotypes. Genotyping the progeny revealed fewer MHC homozygotes than expected in all nine populations (27% fewer MHC homozygotes on average). This deficiency of MHC homozygotes was not due to mortality from pathogens because when unborn pups were genotyped, there was still a deficiency of MHC homozygotes. The deficiency may have been due to abortional selection, but experimental matings in this study and others found no evidence that MHC similar fetuses were at a significant risk of being aborted or reabsorbed. Genetic and behavioral data indicated that the

deficiency of MHC homozygotes was because females tended to mate with MHC dissimilar males. A recent study found that a female's MHC-dependent mating preferences in seminatural conditions can be experimentally reversed by cross-fostering (Penn and Potts, in press). Taken together, studies from the laboratory and seminatural conditions provide strong evidence for MHC disassortative mating preferences in house mice.

C. Humans

Evidence also exists for MHC disassortative odor (Wedekind *et al.*, 1995; Wedekind and Füri, 1997) and mating preferences in humans (Ober *et al.*, 1997). Wedekind *et al.* (1995) found that women (not using oral contraceptives) prefer the odor of shirts worn by MHC dissimilar men to those sharing MHC alleles. This finding has recently been replicated (Wedekind and Füri, 1997). At the time of these studies it was unclear if odor plays a role in human mating preferences, but a survey found that women rely on odor cues for choosing their mates more than other sensory cues (Herz and Cahill, 1997). The main question now is does an MHC-dependent odor preference affect mating preferences?

Studies have examined MHC-dependent mating preferences in two groups of humans that show deficiencies of MHC homozygotes and have found mixed results. Ober (1995) found that Hutterite couples that share common MHC haplotypes have longer interbirth intervals than other couples. Although this finding was interpreted as abortional selection against MHC similar fetuses, the decreased reproductive rate may have been due to lower copulation rates among MHC similar couples. Ober *et al.* (1997) later examined 411 Hutterite couples and found that they were less likely to share MHC haplotypes than random mating expectations—even after statistically controlling for cultural incest taboos.

In contrast, a survey study on 194 couples from 11 South American tribes found no evidence for MHC-dependent mating preferences (Hedrick and Black, 1997). It is difficult to understand these disparate results. One possible explanation is that Hedrick and Black (1997) did not use a large enough population to detect mating preferences. Another possible explanation is that actual mating preferences were missed because they are based on familial rather than self-MHC haplotypes, as has been found in mice (i.e., familial imprinting) (Yamazaki *et al.*, 1988; Eklund, 1997a; Penn and Potts, in press). For example, in a population with four MHC alleles per locus, most (51/64) families will have alleles that are different from self. If individuals are avoiding familial alleles, then researchers should genotype each couples' parents to adequately test for MHC-dependent mating preferences. Interestingly, Ober *et al.* (1997) found evidence that people avoid mating with individuals carrying maternal MHC haplotypes.

III. MHC Genes Influence Odor

How do individuals recognize the MHC identity of potential mates? When MHC-based mating preferences were initially discovered, Thomas (1975) suggested that the enormous diversity of MHC genes might influence individual variation in body odor. Since then, numerous experiments have demonstrated that MHC genes influence individual odors in mice, rats, and humans (Table II). The vast majority of this work has come from the pioneering studies of Yamazaki and colleagues at the Monell Chemical Senses Center.

A. Y-Maze Training Experiments with Mice

Yamazaki and colleagues have performed numerous experiments to determine if genes in the MHC region and single MHC loci affect odor. They found that laboratory mice can be trained to distinguish the body and urine odors of MHC congenic strains (Yamazaki *et al.*, 1979). The mice are trained in a Y-maze olfactometer in which odors from two different MHC congenic strains are blown into two separate arms of the maze. Water-deprived mice are rewarded with water for entering the arm containing one of the two odors. The odors are randomly alternated between arms before trials to prevent the mice from associating the reward with a particular side. Once the mice have learned to discriminate between two odors, the trained mice are then tested for their ability to discriminate the odors of these same two strains using the odors of individuals that they have never encountered. This "generalization" test is particularly convincing because it eliminates the possibility that the mice have simply learned the individual odor donors (Boyse *et al.*, 1987). Yamazaki and colleagues found that laboratory mice can be trained to discriminate the odors of all of the MHC congenics tested (Boyse *et al.*, 1990). This work has been extended and shows that wild-derived mice can be trained to distinguish the odors of MHC congenic strains of mice in a Y maze (Penn and Potts, 1998).

To eliminate other non-MHC factors that may affect odor, such as possible genetic divergence between strains, maternal effects, or accumulated differences in microflora, Yamazaki and his colleagues typically perform an additional "segregant" experiment in their studies. Mice from two different MHC congenic strains are crossed to create heterozygotes that are then crossed to produce MHC homozygotes, such as *aa* and *bb* (F_2 segregants). These F_2 segregants can be dissimilar at the MHC, even though they have the same parents. Yamazaki *et al.* (1978; Yamaguchi *et al.*, 1981) found that the odor of the MHC dissimilar F_2 segregants can be distinguished in a Y maze, whereas the odor of MHC identical segregants cannot.

Yamazaki *et al.* (1985, 1992) also performed experiments with radiation chimeras to demonstrate that the source of the odor variation among MHC congenic strains originates in the lymphatic system. When the hemopoietic system of mice is destroyed by radiation and replaced with bone marrow and spleen cells from MHC dissimilar congenic donors (radiation chimeras), the chimeric mice acquire the donor's MHC distinctive odor.

There are many genes within the MHC besides the highly polymorphic antigen-binding "classical" MHC (class I and II) loci that potentially affect odor. Yamazaki and colleagues have conducted several experiments to determine if classical, antigen-binding MHC loci affect odor. They have found that the odor of MHC recombinant strains that differ in subregions of the MHC containing class I or II loci can be distinguished in a Y maze (Yamazaki *et al.*, 1982). They found that mutant mice that have two deleted class I loci can be discriminated from their normal, parental strain (Yamazaki *et al.*, 1991). Moreover, the odors of mutant mice (*bm* mutants) that differ from their parental strain only by a few amino acid substitutions in the antigen-binding site of a class I locus can be distinguished by mice in a Y maze (Yamazaki *et al.*, 1983a, 1990a). Rats can also be trained to discriminate these mutants from their parental strain in an automated, operant-conditioning olfactometer (Beauchamp *et al.*, 1990; Schellinck *et al.*, 1993). Mice that differ from their parental strain due to mutations at class II loci can also be distinguished in a Y maze (Yamazaki *et al.*, 1990a). It is puzzling, however, that although mice can be trained to distinguish the odor of the *bm* mutants from their parental strain, and yet they failed to distinguish the odor *among* the various *bm* mutants (Yamazaki *et al.*, 1990a). This anomaly needs to be explained.

B. Some Caveats Concerning Inbred Mice and Training Assays

There are two general problems with interpreting the results of studies on MHC genes and individual odors. The first potential problem has to do with the use of inbred strains as odor donors. It is possible that trained mice are able to detect odor differences among inbred strains only because of the homozygosity of the odor donors. The MHC is the only genetic factor that varies among individuals in these inbred strains, whereas mice in the wild are largely heterozygous, and this "noise" from background genes may overwhelm or mask MHC-determined odors. Yamazaki and colleagues (1994) considered this possibility and found that mice can be trained to discriminate MHC identity even among individuals whose background genes are heterozygous, bred by crossing two inbred strains.

A related concern is that most mice in the wild, unlike laboratory strains, are heterozygous at MHC loci. Perhaps mice can distinguish MHC identity among laboratory strains because the mice are homozygous at MHC loci.

Yamazaki and colleagues (1984) conducted an experiment that indicates that mice can be trained to detect MHC haplotypes, i.e., *aa* versus *ab* individuals. They concluded that MHC haplotypes influence unique odors, but that genotypes also have unique odors because combining urine samples from two different genotypes (*aa* + *bb*) can be distinguished from heterozygotes (*ab*). This later interpretation assumes that MHC genes are equally codominant, which is probably not the case. The haplotypic recognition question certainly deserves further attention.

It is difficult to understand why mice require many training trials to distinguish MHC-determined odors. This implies that MHC-determined odors are either not very salient or the unnaturalness of the assay makes the discrimination difficult. Perhaps MHC-determined odors are easier to discriminate among wild animals that have a "noisy" heterozygous background. Although it is widely assumed that background noise reduces the ability of an animal to detect a signal, evidence indicates that background noise boosts weak signals (stochastic resonance) and that the sensory systems of animals have evolved to exploit this effect (Douglass *et al.*, 1993; Moss and Wiesenfeld, 1995). Thus, using inbred strains of mice as odor donors may decrease the ability of mice to detect MHC-determined odors that normally occur in the context of a complex background.

The second potential problem and most important concern has to do with training assays. The training experiments assume that if animals can be trained to discriminate MHC-determined odors in the laboratory, then mice can make these discriminations in the wild *without training*. Yet showing that mice can be trained to distinguish MHC-determined odors does not necessarily mean that untrained mice in the wild can distinguish these odors. This is an important caveat because the training process may condition mice to discriminate signals that they do not normally attend to in the wild. Indeed, the training procedure may actually invoke sensitivity to MHC-determined odorants. Some mice cannot detect the pheromone androstenone, for example, yet insensitivity to this and other odorants can be reversed by repeated exposure, resulting in the induction of olfactory receptor sensitivity (Wang *et al.*, 1993). By increasing the motivation of a subject, training assays may be able to find discriminations that nontraining assays fail to detect, but they also are more likely to find discriminations that are irrelevant.

Moreover, showing that mice cannot be trained to associate a particular odor with a water reward does not necessarily mean that the odor is indiscriminable. Associability is not equivalent to distinguishability. Mice may be capable of discriminating between two odors and still not be able to make the association with a particular reward. Comparative psychologists have long known that "sometimes an animal can easily discriminate a

certain stimulus and still have considerable difficulty learning to respond to it in a particular manner" (Bolles, 1973). Contrary to what is often assumed, learning mechanisms are not general purpose or equipotential. For example, approximately 100 trials are required to train mice to distinguish between black and white rectangles or between circles and triangles, which seems to suggest that mice are very poor at distinguishing geometric shapes (Kalkowski, 1968). Yet other experiments indicate that mice are surprisingly adept at distinguishing slight visual cues in more natural contexts (Mackintosh, 1973). Offering a reward may not increase the motivation of an animal if the reward is out of context to the stimulus. Thus, there are reasons to be cautious about interpreting both positive and negative results of training studies.

C. Untrained Mice and Rats Discriminate MHC-Determined Odors

Two experiments indicate that untrained mice are capable of distinguishing MHC-determined odors. First, Yamazaki and colleagues performed an experiment using the "Bruce effect" as a biological assay for odor discrimination. The Bruce effect refers to the finding that up to 90% of pregnant female mice block implantation when they are exposed to an unfamiliar male or his odor (Bruce, 1959). Females are more likely to block implantation if the unfamiliar male and stud male are from different strains, perhaps because their odors are more distinguishable. Yamazaki and colleagues used this Bruce effect to test if MHC genes affect odor. They found that females are more likely to block pregnancy if the odors of unfamiliar and stud males are from different MHC congenic strains than when they are from the same strain (Yamazaki *et al.*, 1983b). Moreover, females are also more likely to block pregnancy if the stud and sire males differ by a mutation (*bm1*) at one class I locus than when they are from the same strain (Yamazaki *et al.*, 1986).

Second, a "habituation–dishabituation" experiment has been performed to test if house mice can discriminate odors determined by classical MHC genes without training (Penn and Potts, 1998). The habituation–dishabituation assay consists of two stages: the habituation phase, in which one odor is presented repeatedly until the subject habituates to that stimulus, and the dishabituation phase, in which an odor from a different strain is introduced. The change in investigatory behavior that occurs on presentation of the second odor is used as a criterion for determining olfactory discrimination. It was found that untrained, wild-derived mice are able to discriminate the urine odor of mice that differ genetically only at a class I MHC locus. Taken together, these studies indicate that house mice can detect MHC-determined odors without training.

Studies by R. Brown and colleagues (1987) have found that laboratory rats can distinguish odors of MHC recombinant strains of rats that differ only in class I and II regions. Their results are particularly convincing as they used a habituation–dishabituation assay that does not rely on training the rats. All of the recombinant MHC strains that they tested had distinguishable odors (Brown *et al.*, 1989, 1990). Although rats can distinguish MHC-determined odors, it is unknown if they have MHC disassortative mating preferences or any other MHC-mediated behaviors.

D. Humans Detect MHC-Determined Odors

Several sources of evidence indicate that humans produce and can detect MHC-mediated odors. First, humans can distinguish the odors of MHC congenic strains of mice (Gilbert *et al.*, 1986). Second, rats can be trained to discriminate the odors of humans with different MHC haplotypes (Ferstl *et al.*, 1992). Third, both found that men and women preferred the odor of shirts worn by MHC dissimilar over MHC similar individuals (Wedekind *et al.*, 1995; Wedekind and Füri, 1997). Interestingly, the women described the odor of MHC dissimilar men as reminding them of their mate's odor. This was just a correlational study, of course, so the effect may have been due to non-MHC genes. The most surprising result from the studies by Wedekind and his colleagues was that women taking oral contraceptives preferred the odor of MHC *similar* men. Because birth control pills simulate pregnancy hormonally, the authors suggested that women might reverse their odor preferences during pregnancy to prefer close kin as helpers, just as female mice tend to rear their young cooperatively with MHC similar females (Manning *et al.*, 1992b). Given the widespread interest in human mating preferences, it is puzzling that more work has not been done in this area (Stoddart, 1990).

IV. How Do MHC Genes Influence Odor?

Although it has been shown that MHC genes influence odor, it is still unclear how. MHC molecules bind to small peptides, 5–20 amino acids in length, and the variation among alleles occurs in the antigen-binding site. Therefore, it must be the peptide-binding properties of MHC molecules that influence odor. There are many ways that the antigen-binding site could control odor variation, and various methods have been employed to determine how this could occur. One approach is to use gas chromatography to identify the odorants controlled by the MHC. Among these studies, two have reported that MHC congenic mice produce qualitatively different odor profiles (Schwende *et al.*, 1984; Eggert *et al.*, 1996), whereas another study did not (Singer *et al.*, 1997). One of the problems with this approach

is that gas chromatography is not as sensitive as chemosensory organs and may fail to detect actual odor differences. The odor of an individual is so complex that without a specific hypothesis of the nature of the odorants one is looking for, the hunt is the proverbial needle in a haystack problem. Therefore, it is necessary to know what kind of odor differences MHC genes are likely to control. This section reviews the four hypotheses that have been suggested to explain how MHC genes affect odor, and a fifth, synthetic hypothesis is provided (Fig. 1).

A. The MHC Molecule Hypothesis

It has been suggested that MHC molecules or fragments themselves provide the odorants, as they occur in the urine of rats and human sweat (Singh *et al.*, 1987, 1988b; Roser *et al.*, 1991). Although this hypothesis is attractive in its simplicity, it is unlikely for several reasons. First, MHC molecules are proteins that are nonvolatile, whereas MHC-determined odors are apparently volatile. Fractions of urine that are depleted in protein are still discriminable by mice in a Y maze (Singer *et al.*, 1993). Eliminating the volatile fraction of urine by purging it with nitrogen gas abolishes the ability of rats to distinguish the urine odor of MHC congenic strains (Singh *et al.*, 1988b; Roser *et al.*, 1991). Although this is direct evidence that MHC-determined odorants are volatile, conflicting evidence suggests that these odorants are relatively nonvolatile. MHC-determined odorants are sufficient to induce the Bruce effect, which is mediated by nonvolatile peptides detected by the vomeronasal organ (Dominic, 1966). The relative volatility of the MHC-determined odorants has not been quantified, but if MHC-determined odorants are more volatile than the fragments of MHC molecules, then this would rule out the MHC molecule hypothesis. Second, rats to not discriminate the odor of intact class I molecules purified from the urine, whereas they do discriminate the odor of the remainder of the urine that still contains class I molecules (Brown *et al.*, 1987). Third, rats do not distinguish the odor of blood sera between MHC congenic strains (Brown *et al.*, 1987). Fourth, when large proteins, greater than 10,000 molecular weight, are removed from the urine (digested with proteinases, ultrafiltration, heat, and percholoric acid), the odor of MHC congenic mice is still distinguishable by mice in a Y maze (Singer *et al.*, 1993). This last finding poses a particular problem for the MHC molecule hypothesis.

B. The Peptide Hypothesis

MHC molecules bind to allele-specific subsets of peptides (Falk *et al.*, 1991) and their volatile metabolites may provide the odorants (Singer *et al.*, 1997). This hypothesis holds that MHC molecules function as odorant

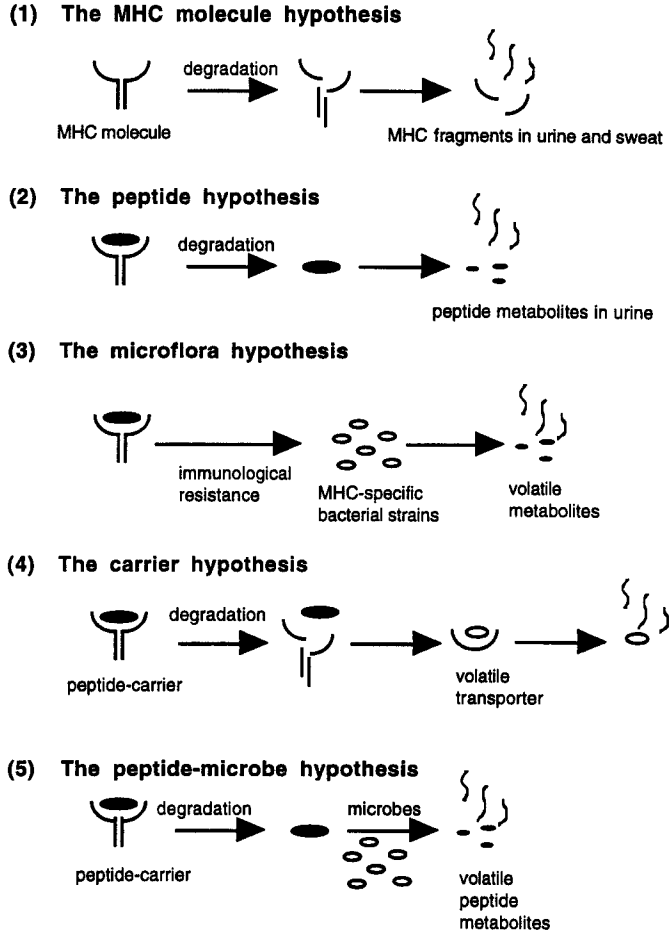

FIG. 1. Five proposed mechanisms to explain how MHC genes influence individual odor. (1) Fragments of MHC molecules in urine and sweat provide the odorants (the MHC molecule hypothesis) (Singh *et al.*, 1987). (2) MHC molecules may alter the pool of peptides in urine whose metabolites provide the odorants (the peptide hypothesis). (Singer *et al.*, 1997). (3) MHC genes may alter odor by shaping allele-specific populations of commensal microbes (the microflora hypothesis) (Howard, 1977). (4) MHC molecules could be altered to carry volatile aromatics (the carrier hypothesis) (Pearse-Pratt *et al.*, 1992). (5) We suggest that MHC molecules alter odor by changing the peptides that are available to commensal microbes (the peptide-microflora hypothesis).

carriers and that peptides provide the precursors of the odorants. It is attractive because it implicates the antigen-binding site of MHC molecules in determining an individual's allele-specific odor. Moreover, it is consistent with the evidence that mammalian pheromones are transported by carrier proteins to scent glands (Albone, 1984; Spielman *et al.*, 1995).

It has been reported that the odor differences among MHC congenic strains are due to changes in the relative amounts of the components of urinary acids, (Fig. 2) which are potentially the metabolities of MHC-bound peptides (Fig. 3) (Singer *et al.*, 1997). Mouse urine contains abundant acidic metabolites, many of which are derived from amino acids (each cell expresses 100,000 MHC molecules so that MHC-bound peptides are probably more common than one might assume), and these volatile acids in urine are widely used as chemosensory signals in mammals. Short-chain aliphatic acids have been referred to as "copulins" because they are sexual attractants in mammals (Michael *et al.*, 1974, 1975); however, there is some controversy about these findings (Albone, 1984). Thus, one study indicates that MHC genes shape the profiles of a mouse's urinary acids, and other studies indicate that these acids act as sexual odorants in mammals. More work clearly needs to be done in this area.

C. The Microflora Hypothesis

MHC genes might control odor indirectly by shaping an individual's specific populations of commensal microbial flora that produce odorants (Howard, 1977). A normal mouse harbors around 10^{12} bacteria on its skin, 10^{10} in its mouth, and 10^{14} in its digestive tract (Mims, 1977), and the variation in microflora is influenced by genetic variation among hosts (Holdeman *et al.*, 1976; Van de Merwe *et al.*, 1983). Urine odor in rodents is influenced by microbes harbored in the preputial gland, a cavity near the genital opening. It has been found that the MHC haplotype of a male mouse influences the odor of his preputial gland and that females are no longer attracted to the odor of MHC dissimilar males when their preputial gland is removed (Ninomiya and Brown, 1995). It is feasible that MHC genes influence odor by shaping microbial flora in an allele-specific fashion,

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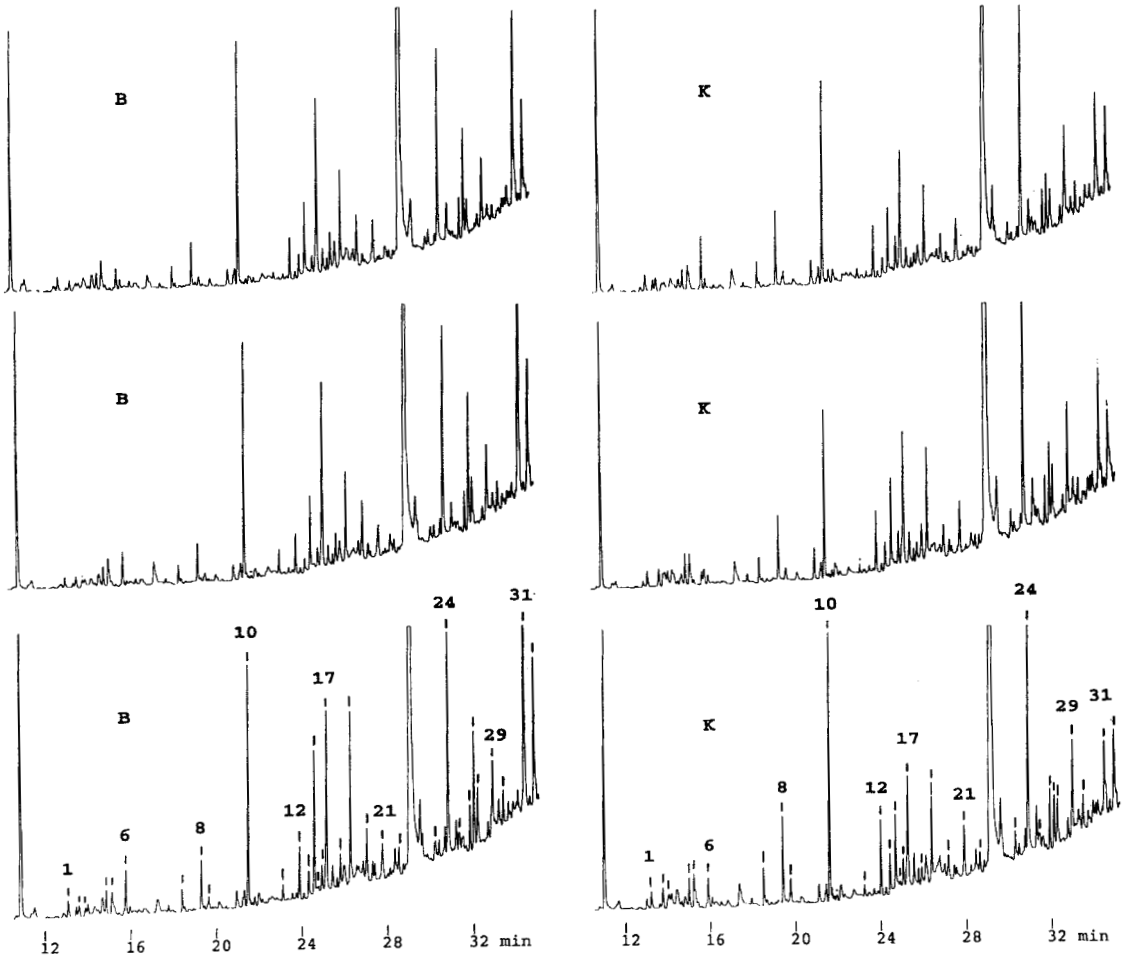
Fig. 2. Gas chromatograms from mouse urine collected from six mice belonging to two different MHC-congenic strains of mice, *bb* and *kk* (Singer *et al.*, 1997). Each component of the urine emerges from the chromatography column at different rates (horizontal axis). The size of the signal (vertical axis) corresponds to the relative amount of each component. This study found no qualitative differences among the chromatograms of MHC-congenic strains, but found statistical differences in 8 of the 32 peak height concentration values which were volatile acids.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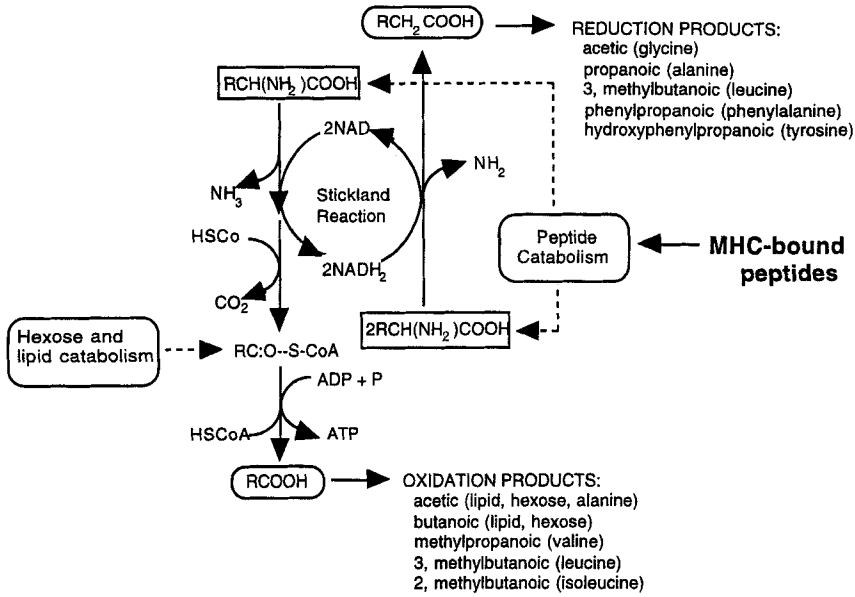

FIG. 3. The proposed biosynthetic pathway for volatile carboxylic acids or "copulins" by the Stickland reaction (from Michael *et al.*, 1975). MHC genes alter concentrations of different volatile carboxylic acids in the urine (Singer *et al.*, 1997) perhaps by controlling the pool of available peptides used in this pathway (NAD = nicotinamide adenine dinucleotide, HSCoA = coenzyme A).

as experimental infection studies indicate that MHC alleles differ in their susceptibility to different pathogens (Apanius *et al.*, 1997).

The microflora hypothesis predicts that animals reared in germ-free conditions will not have MHC distinctive odors. Experiments that use untrained animals support this prediction. For example, urine collected from MHC congenic rats reared in germ-free environments was not discriminated in a habituation–dishabituation assay by other rats until the rats were inoculated with various microbes (Singh *et al.*, 1990; Roser *et al.*, 1991). Another study indicates that the urine odors of germ-free MHC congenic mice are not discriminated by rats in a habituation assay (Schellinck *et al.*, 1995). Experiments that use trained animals as subjects, however, do not support the germ-free prediction. Both mice and rats can be trained to distinguish the urine odor of germ-free congenic animals (Yamazaki *et al.*, 1990b, 1992; Schellinck *et al.*, 1991), although rats have more difficulty learning the odors of germ-free than conventionally housed animals (Schellinck *et al.*, 1991; Schellinck and Brown, 1992). It is possible that trained animals are utilizing cues, such as MHC-associated differences

in hormone metabolite concentrations (Ivanyi, 1978), that they can only detect under intensive training. Other experiments by Yamazaki and colleagues are inconsistent with the microbial flora hypothesis: (1) Mice can distinguish the odor of MHC congenic mice using urine collected from the bladder (K. Yamazaki, personal communication) and (2) mice can detect the MHC odor types of fetal mice (after 9–12 days of gestation) in utero (Beauchamp *et al.*, 1994). However, these experiments were conducted with trained mice that may be attending to incidental signals, i.e., artificially induced by the training procedure.

Although the study showing that MHC controls the concentrations of urinary carboxylic acids has been interpreted as evidence for the peptide hypothesis (Singer *et al.*, 1997), this finding is also consistent with the microflora hypothesis. This is because carboxylic acids are used in biochemical assays for identifying microbes (Jenkins *et al.*, 1992). Therefore, MHC-associated differences in urinary carboxylic acids may be due to variation in microbial flora populations.

D. The Carrier Hypothesis

It has been suggested that MHC molecules influence odor by carrying a unique cocktail of volatile aromatics to the urine (Pearse-Pratt *et al.*, 1992). This hypothesis proposes that when MHC molecules are degraded, they are converted from peptide-presenting structures into "volatile-transporting molecules" in which volatile odorants are bound in the peptide-binding region. The proponents of this hypothesis suggest that the volatile odorants originate from commensal gut bacteria (Brown, 1995) (although this is an unnecessary restriction). The problem with the carrier hypothesis is that it is difficult to imagine how the binding properties of MHC molecules might be converted from being hydrophilic peptide-binding molecules to hydrophobic aromatic-binding molecules.

E. The Peptide–Microflora Hypothesis

Combining the features of the two most compelling mechanisms, the peptide and the microflora hypotheses, suggests a fifth hypothesis: MHC molecules influence odor by binding unique subsets by peptides, which are then carried to the preputial, coagulating, axillary region, or other microbe-harboring glands where their metabolites are made volatile by commensal microflora (Fig. 3). This hypothesis is compelling for several reasons. First, like the peptide hypothesis, it offers a mechanism through which an individual's odor can be shaped by the peptide-binding properties of MHC molecules Second, like the microbial flora hypothesis, it recognizes that microbes play an important role in volatilizing mammalian odorants that are transported to scent glands by protein carriers (Albone, 1984;

Spielman *et al.*, 1995). Third, this hypothesis would explain the disparate results between training and habituation studies (Schellinck and Brown, 1992). For example, the microbial volatilization step may not be necessary for trained mice to recognize odor differences, whereas it would be more important for untrained animals. Fourth, it is consistent with the idea that odorants are urinary acids (Singer *et al.*, 1997), as microbes produce acidic secondary metabolites (Michael and Bonsall, 1977; Jenkins *et al.*, 1992). Although it is likely that microbes play a role in the *production* or volatilization of MHC-mediated odors, they may not be necessary for conferring "individuality." Further experiments need to be conducted using bladder urine from rodents.

V. Chemosensory Mechanisms

It is also unclear how individuals *detect* MHC-determined odors. MHC-determined odors may be detected by the main olfactory system or the vomeronasal organ (VNO). The VNO is a chemosensory system, separate from the main olfactory system, that is involved primarily in the control of reproductive behavior (Wysocki and Meredith, 1987). Yamazaki and colleagues (personal communication) found that mice can still be trained to distinguish odors from MHC congenic strains even after the VNO is disrupted surgically. However, many studies have found that naive animals require a VNO to detect odor cues, but through repeated exposure they can learn to compensate with their main olfactory system (Wysocki and Meredith, 1987). Until recently, it has been impossible to completely disrupt the main olfactory system. Knockout mice (targeted gene disruptions) have now been developed that cannot smell because their olfactory neurons do not express an ion channel that is necessary for depolarization of olfactory neurons (Barinaga, 1996; Brunet *et al.*, 1996). Such genetic knockouts provide an excellent opportunity for examining how MHC genes affect odor (MHC knockouts) and how MHC-determined odors are detected.

Tremendous progress has recently been made in understanding olfaction (Buck, 1996). Olfactory receptors are encoded by a large multigene family (Buck and Axel, 1991) and, interestingly, a set of olfactory-like receptor genes has been found closely linked to MHC loci (Fan *et al.*, 1995). It is unknown if MHC-linked olfactory-like genes are functional, polymorphic, or if they are involved in MHC-mediated odor or mating preferences. When Yamazaki *et al.* (1976) first found evidence for MHC-dependent mating preferences, they postulated that this behavior might be controlled by two linked genes: one for the signal and one for the receptor (also see Goldstein and Cagan, 1981). Theoretical work argues that genetic kin

recognition systems require "detecting," "matching," and "using" loci, although these loci need not be linked (Grafen, 1990). The possibility that MHC-linked olfactory receptor genes play a role in detecting seems less likely, however, since mice learn the MHC-determined odors of their close kin (i.e., familial imprinting).

VI. Familial Imprinting

To understand how MHC-dependent mating preferences occur it is not enough to show that females can recognize the MHC identity of potential mates. For females to mate with MHC dissimilar males, they must also "know" their own MHC identity. They must have a referent, either themselves (self-inspection) or close kin (familial imprinting), with which to compare the odor of potential mates. Although it is often assumed that individuals use a self-inspection mechanism, the cross-fostering experiment by Yamazaki *et al.* (1988) indicates that male mice use familial imprinting. When males were fostered unto MHC dissimilar parents at birth, they preferred to mate with females carrying MHC similar genes than females with the MHC genes of their foster parents. This indicates that the males learn the MHC identity of their family and then avoid mating with individuals carrying familial MHC genes (this may be due to increased familiarity rather than an imprinting mechanism with a sensitive period in early ontogeny). The first attempt to test the familial imprinting hypothesis in female mice failed to find any mating preferences (Beauchamp *et al.*, 1988). A second laboratory study recently found that cross-fostering altered the mating (first mount) preferences of one of two strains of mice (Eklund, 1997a). However, these were laboratory assays with inbred strains. A third study recently found wild-derived female mice in seminatural conditions use a familial imprinting mechanism to avoid mating with males carrying familial MHC genes (Penn and Potts, in press). These studies imply that MHC-determined odors influence the ontogeny of MHC-based mating preferences by altering the development of either the olfactory bulb (Harvey and Cowley, 1984) or other neural mechanisms of infant mice.

VII. Abortional Selection

Several studies have found evidence that couples that share MHC haplotypes have an increased risk of recurrent spontaneous abortion (reviewed by Ober, 1992, 1995; Apanius *et al.*, 1997). Such an abortional mechanism may represent a postcopulatory mate choice mechanism (Eberhard and Cordero, 1995) to produce MHC heterozygous young. Abortional mechanisms may be controlled by maternal–fetal interactions, paternal MHC-

mediated odors (Yamazaki *et al.*, 1986), or both. The main problem with this hypothesis is that the evidence for abortional selection on MHC homozygotes is so mixed from both human (Ober, 1992) and rodent studies (Palm, 1969, 1970, 1974; Hamilton and Hamilton, 1978; Hings and Billingham, 1981, 1985).

Interestingly, it has been suggested that abortional mechanisms may be contingent on a female's infection status: females may abort MHC homozygous offspring when they are heavily infected with pathogens or parasites (Wedekind *et al.*, 1995). There is now experimental evidence that supports this hypothesis (Rülicke *et al.*, in press). This finding provides a potential explanation for inconsistent results among the various studies on abortional selection (e.g., attempts to replicate earlier findings of abortional selection in rodents may have failed because of improvements in controlling infections in animal colonies). The infection-dependent hypothesis should stimulate further research into MHC-mediated abortions.

VIII. Conclusions

There is increasing evidence that house mice and humans prefer to mate with MHC dissimilar individuals. There is much evidence that MHC genes influence odor cues; however, more work needs to be conducted on outbred animals under natural conditions. The underlying mechanisms through which MHC genes influence odor remain elusive. Because the variation among MHC alleles occurs in the peptide-binding region, it must be the binding properties of MHC molecules that control individual odors. Four specific mechanisms have been proposed, and a fifth synthetic hypothesis has been suggested: MHC molecules bind allele-specific subsets of peptides whose metabolites are made volatile by commensal microflora. More work also needs to be conducted on understanding the chemosensory mechanisms through which MHC-determined odors are detected and the development of MHC-based mating preferences (familial imprinting). Finally, more work should be done to determine if MHC homozygotes are at risk of being aborted. Whatever mechanisms are controlling MHC-based mating preferences, this behavior can be important for maintaining and shaping the enormous diversity of the antigen-binding region of classical MHC genes (Potts and Wakeland, 1990; Potts *et al.*, 1991; Hedrick, 1992). Thus, MHC-based mating preferences have important implications for unraveling the evolution of the vertebrate immune system.

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Olfactory Receptor Gene Regulation

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I. Overview

The mammalian olfactory system recognizes and discriminates a vast array of odorant ligands. The number of odors that can be distinguished is on the order of tens of thousands. Recognition is accomplished, initially, by members of a family of 1000 olfactory receptors. Thus the olfactory system rivals the immune system in its ability to recognize a large number of molecules. Although the immune system faces a complex problem in discriminating the universe of antigens, the olfactory system faces a further challenge. The olfactory system must convey to the brain a conscious appreciation of the fine distinctions between odor molecules.

In the immune system, a variety of genetic regulatory mechanisms, including somatic DNA recombination, are involved in the generation of the repertoire of antigen receptors. These mechanisms allow for the selection of individual germline-encoded V genes and increase the diversity of receptors beyond germline-encoded sequences. The olfactory system does not generate protein-coding sequence diversity through somatic DNA recombination. However, the mechanisms used to generate expression and diversity in the immune system may play a role in allowing a given olfactory neuron to choose which of the 1000 olfactory receptor genes to express.

Little is known about the actual molecular events underlying the transcriptional regulation of the olfactory receptor genes. However, a number of interesting observations have emerged that constrain potential models for the transcriptional regulation of the olfactory receptor genes. Before launching into a discussion of transcriptional regulation, a brief introduction to the olfactory system is given. More detailed descriptions of the olfactory system can be found in a variety of excellent reviews (Lancet, 1986; Ressler *et al.*, 1994b; Shepherd, 1994, and references therein).

II. Introduction to the Olfactory System

A. Olfactory Receptor Genes

Olfactory discrimination initially involves the interaction of odorants with receptors on olfactory sensory neurons. Buck and Axel (1991) identified a

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multigene family of seven transmembrane domain proteins likely to encode the odorant receptors. Screens of genomic libraries indicated that the family of odorant receptors may include as many as 1000 genes in mammals, whereas in fish this family is far smaller, reflecting a more limited repertoire of perceived odors (Buck and Axel, 1991; Chess *et al.*, 1992; Ngai *et al.*, 1993a). The mammalian olfactory receptor genes reside in 10 or more large arrays in the genome (Sullivan *et al.*, 1996). The largest array characterized to date is a 350-kb array in the human genome that contains at least 16 olfactory receptor genes (Ben-Arie *et al.*, 1994).

The presence of a large number of olfactory receptor genes with diverse sequences suggests that the recognition of odors in vertebrates is accomplished using receptors, each of which is capable of interacting with a relatively small number of odorants. Precise definition of the ligand specificity of individual receptors awaits functional studies. With one exception, functional studies have not been possible as cloned olfactory receptor genes expressed in heterologous cells have failed to reach the cell membrane (Raming *et al.*, 1993). The specific binding of odorants causes a rise in cAMP, eventually leading to depolarization of the neuron via the cyclic nucleotide-gated channel (Nakamura and Gold, 1987; Pace *et al.*, 1985; Sklar *et al.*, 1986; Dhallan *et al.*, 1990; Goulding *et al.*, 1992). Action potentials then result in the transmission of the signal by the primary neurons in the neuroepithelium directly to the olfactory bulb in the brain.

B. Expression of the Olfactory Receptors in Individual Neurons

RNA *in situ* hybridization experiments with olfactory receptor gene probes suggest that individual olfactory sensory neurons likely express only one or a few receptor genes. In the catfish, which has a repertoire of about 100 receptor genes, a given receptor subfamily, containing between 1 and 6 highly homologous genes, is expressed in 0.5–2% of the olfactory neurons (Chess *et al.*, 1992; Ngai *et al.*, 1993b). In rodents, which have a repertoire of perhaps 1000 genes, a given receptor identifies about 0.1% of the sensory neurons (Raming *et al.*, 1993; Ressler *et al.*, 1993; Strotmann *et al.*, 1992; Taylor, 1960; Vassar *et al.*, 1993). Moreover, different receptor probes anneal with distinct, largely nonoverlapping subpopulations of neurons. The clearest demonstration of the lack of overlap comes from an experiment done in the catfish system in which two receptor genes were analyzed either individually or as a mixture. The results indicated that the number of neurons hybridizing with the mixed probe roughly equaled the sum of the number of neurons hybridizing with the two individual probes. This observation is consistent with the idea that the neurons expressing one receptor do not express the other receptor (Chess *et al.*, 1992). Other experiments (in catfish and rodents) looked at larger numbers of genes,

allowing the generalization of the observation of nonoverlap (Ngai *et al.*, 1993b; Ressler *et al.*, 1993). Taken together, these observations suggest that each neuron expresses only one or a small number of receptor genes, such that individual olfactory neurons are functionally distinct. The recognition of odors may therefore be accomplished by determining which neurons have been activated. Although it is not formally proven, the expression of one olfactory receptor gene per neuron will be assumed in the rest of this review.

C. Spatial Distribution of Neurons Expressing a Given Receptor and the Generation of a Topographic Map

How does the brain determine which neurons have been activated in order to decode olfactory information from peripheral sensory neurons? It turns out that spatial information is used by the olfactory system to encode the nature of the stimulus. The neurons expressing a given receptor are randomly distributed among neurons expressing different receptors (Chess *et al.*, 1992; Ngai *et al.*, 1993b; Ressler *et al.*, 1993; Vassar *et al.*, 1993). This allows the neurons expressing a given receptor to sample a larger fraction of the air present in the nasal cavity than if these neurons were all clustered in one area of the neuroepithelium. Mammals have an additional layer of complexity in the distribution of the neurons expressing a given receptor in the olfactory epithelium. In mammals, there are a handful of zones; each receptor gene is restricted to neurons residing in one of the zones (Ressler *et al.*, 1993; Vassar *et al.*, 1993). The zones may, play an important role in the discriminatory power of the olfactory system. As mentioned earlier, spatial information is used by the olfactory system to encode the identity of an odorant stimulus: How is this accomplished? It turns out that the topography that allows the brain to determine which receptor has been activated is established in the projections of the primary olfactory neurons to the olfactory bulb. There is convergence of the projections of neurons expressing a given receptor (Ressler *et al.*, 1994a; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). Thus, the brain can identify which receptors have been activated by examining the spatial pattern of electrical activity in the olfactory bulb; individual odorants are associated with specific spatial patterns. This raises a number of interesting mechanistic questions: (1) how does each olfactory neuron choose to express a single olfactory receptor from a family of 1000 genes and (2) how is the choice of receptor linked to the precise projection of olfactory neurons?

III. Transcriptional Regulation Problem

Current data on the olfactory receptor gene family suggest that there may be similarities between the mechanisms controlling olfactory receptor

gene expression and the mechanisms controlling the expression of the immunoglobulin genes and the T-cell receptor genes.

For a given neuron to choose one gene from the thousand or so available probably requires three distinct levels of control:

1. There must be a choice of one olfactory receptor gene array from among the dozen or so arrays in the genome. This choice is analogous to the λ/κ choice in immunoglobulin gene regulation.
2. A mechanism must exist to restrict the available olfactory receptor genes in this array to those whose expression is allowed in the zone in which the neuron resides. This restriction of the choice of genes could easily be mediated by a transcription factor or by a combination of transcription factors specific for each zone.
3. There must exist a mechanism to choose a single gene in the array for expression. This step is analogous to the choice of one V region for expression in the case of lymphocyte antigen receptors.

One exciting possibility that current data on olfactory receptor gene expression raise is that rearrangement of olfactory receptor genes is involved in the regulation of their expression. A point mentioned earlier needs to be emphasized: there is no evidence for the generation of olfactory receptor diversity via a rearrangement mechanism. To date, the coding regions deduced from cDNA clones are all colinear with the genome. However, rearrangement could be the mode by which one olfactory receptor gene is chosen from others in a given array in the genome. In this regard, it is useful to think of the rearrangement mechanism in the case of the immunoglobulin genes as a mechanism for choosing one of the variable region genes for expression. One can then regard the diversity generated in immunoglobulin genes through the incorporation of D and J segments and through junctional diversity as secondary to the choice of a given variable region gene by the rearrangement. The following sections focus on data pertaining to the regulation of olfactory receptor genes. Then, Section V presents a model for olfactory receptor gene expression that happens to contain parallels to immunoglobulin gene regulatory mechanisms.

IV. Existing Data Pertaining to Olfactory Receptor Gene Regulation

A. Only One Receptor is Expressed per Neuron

As discussed earlier, current data suggest that only one receptor gene is expressed per neuron. Moreover, the neurons expressing a given receptor gene appear to be distributed randomly among neurons expressing other

receptor genes. The random pattern suggests a stochastic aspect to the mechanisms regulating olfactory receptor gene expression. Another line of evidence supporting a stochastic process derives from an analysis of the olfactory epithelium in developing mice (Sullivan *et al.*, 1995). The developmental study demonstrated that olfactory neurons choose and express olfactory receptor genes earlier in development than the earliest projections to the olfactory bulb. Moreover, in mutant animals lacking an olfactory bulb, the pattern of olfactory receptor gene expression was not perturbed. These data argue that the olfactory receptor neurons are making a receptor gene choice without instruction from the olfactory bulb. It is interesting to note that a study of the developmental time course of olfactory receptor genes in zebrafish indicated that there is asynchrony of the onset of expression of certain genes (Barth *et al.*, 1996). This asynchrony may reflect an additional layer of complexity in the control of fish olfactory receptor genes that is not present in mammals.

B. Olfactory Receptor Genes Reside in a Few Large Arrays in the Genome

The largest olfactory receptor gene array described is a 350-kb array on human chromosome 17 that contains at least 16 genes (Ben-Arie *et al.*, 1994). This array was analyzed by examining a cosmid contig that covered the entire locus. The average spacing of the genes was 20 kb. It is possible that the array is even larger than what was described. In the mouse, a number of studies have identified about a dozen arrays in the genome (Sullivan *et al.*, 1996; Chess *et al.*, 1994; and Chess *et al.*, unpublished observations). One study used an analysis of multilocus crosses between related mouse species to identify a number of loci encoding olfactory receptors (Sullivan *et al.*, 1996). Another series of experiments identified a number of distinct loci using the technique of two-color fluorescence *in situ* hybridization (FISH) (Chess *et al.*, 1994, and Chess *et al.*, unpublished observations). Unlike the human study, studies in mouse have not specified the number of genes present at the various loci.

C. Only One Olfactory Receptor Allele is Expressed in Each Neuron

Given the prominent place that allelic exclusion occupies in the minds of immunologists, a discussion of why expression from one of two alleles may be an important clue to mechanisms underlying olfactory receptor gene regulation will be deferred to later in this review. This section describes the basic experimental observations for olfactory receptor genes (Chess *et al.*, 1994). To determine whether neurons expressing a given receptor transcribe this receptor gene from both paternal and maternal

alleles or from only one of the two alleles required solving two problems: (1) distinguishing the transcripts from the maternal and paternal alleles of a given gene and (2) isolating individual neurons expressing a given gene, as such neurons represent 0.1% of the total neurons in the olfactory neuroepithelium. The first problem was readily solved by analyzing the progeny of an F1 cross between *Mus spretus* and *M. musculus* mice, which had been previously demonstrated to contain polymorphisms in an olfactory receptor gene, I7.

The problem of analyzing individual neurons expressing the 17 olfactory receptor gene was solved using limiting dilution, which allowed the examination of individual olfactory neurons without isolating them. Limiting dilution was performed on dissociated olfactory neurons to obtain pools of cells containing either large numbers of cells or small numbers of cells (200 cells). RNA was extracted from multiple pools at each cell density and analyzed for expression of the I7 olfactory receptor gene by reverse transcription followed by polymerase chain reaction amplification (RT-PCR) with I7-specific intron-spanning primers. All pools containing large numbers of cells generated a PCR product of appropriate size, indicating that each of these pools contains at least one cell expressing the I7 gene. Only 1 in 10 pools containing 200 cells expressed the I7 gene. Statistical analyses indicated that these rare 200 cell pools were likely to contain only a single I7-expressing cell. Only 1 in 10 I7-positive pools should contain two I7-expessing cells. This limiting dilution approach therefore allowed the examination of expression of the I7 gene from a single cell without isolating the individual cell. Each RT-PCR product from a rare positive pool represents the I7 mRNA present in an individual cell.

The analysis demonstrated that olfactory receptor expression derives exclusively from one of two alleles. Equal numbers of cells expressed the maternal as the paternal allele. The random expression of either the maternal or the paternal allele in individual neurons demonstrates that this phenomenon is not the consequence of parental imprinting. (Imprinting by definition would require that all olfactory neurons express the maternal allele or all olfactory neurons express the paternal allele). Analyses of another olfactory receptor gene, located on a different chromosome, revealed similar results. These data demonstrate that in an individual neuron expressing a given olfactory receptor, expression derives exclusively from either the maternal or the paternal allele, but not both. These data are therefore consistent with *cis*-regulatory models in which alleles encoding odorant receptors are regulated independently.

D. Olfactory Receptor Genes Are Asynchronously Replicating: Implications for Allelic Inactivation Early in Development

The observations just presented clearly indicate that an individual neuron expresses only one of two alleles encoding a given olfactory receptor. This

could reflect the stochastic activation of a different receptor gene from the maternal and paternal (allelic) arrays. Alternatively, there could be silencing of not only the second allele of the expressed gene, but of all the genes linked to this second allele in a given olfactory receptor array. A distinct line of study, presented in Section V, suggests that indeed one of the two allelic arrays (for all olfactory gene loci in the genome) is silence prior to development of the olfactory epithelium. Thus, an individual neuron, when it chooses a receptor gene for expression, chooses only a single olfactory receptor from a single allelic array. Evidence that such allelic inactivation is occurring derives from an analysis of the replication timing of members of the olfactory receptor gene family (Chess *et al.*, 1994).

Before the olfactory receptor genes were studied, asynchrony of replication had been observed previously in imprinted genes and X-inactivated genes (Kitsberg *et al.*, 1993; Knoll *et al.*, 1994; Taylor, 1960). Most other genes are replicated synchronously (Holmquist, 1987; Kitsberg *et al.*, 1993). Therefore, an observation of asynchronous replication of a given gene suggests that this gene will be monoallelically expressed. Replicative asynchrony can be observed in all cells, even cells that do not transcribe a given gene. Various olfactory receptor genes were examined using FISH and found to be asynchronously replicating (Chess *et al.*, 1994). Even primary embryo fibroblasts demonstrate this asynchrony of replication, indicating that the asynchrony is established early in development. The asynchrony is random with respect to parental allele, consistent with the transcriptional studies. Thus these data provide indirect evidence for allelic inactivation of olfactory receptor genes early in development.

V. A Model for Olfactory Receptor Gene Regulation

The presence of large arrays of receptor genes supports the idea that the mechanism for choosing a given olfactory receptor involves the stochastic choice of one gene from a linked array by a single enhancer element that can only activate one gene. Inherent in any model of stochastic *cis* regulation is that the two alleles are independently regulated. Thus, the observation that expression of a given gene derives from either the maternal allele or the paternal allele, but not from both, supports a model of stochastic *cis* regulation of the olfactory receptor genes. The first step (Fig. 1A) in this model is the random inactivation of either the maternal or the paternal array of each olfactory receptor locus in all cells early in development. The second step (Fig. 1B), which occurs during olfactory neurogenesis, is the choice of one chromosomal locus by each olfactory neuron. In the third step (Fig. 1C), a *cis*-regulatory element directs the stochastic expression of only one gene from the one active allelic array. [For simplicity, the restriction of a given neuron to receptors appropriate to its epithelial

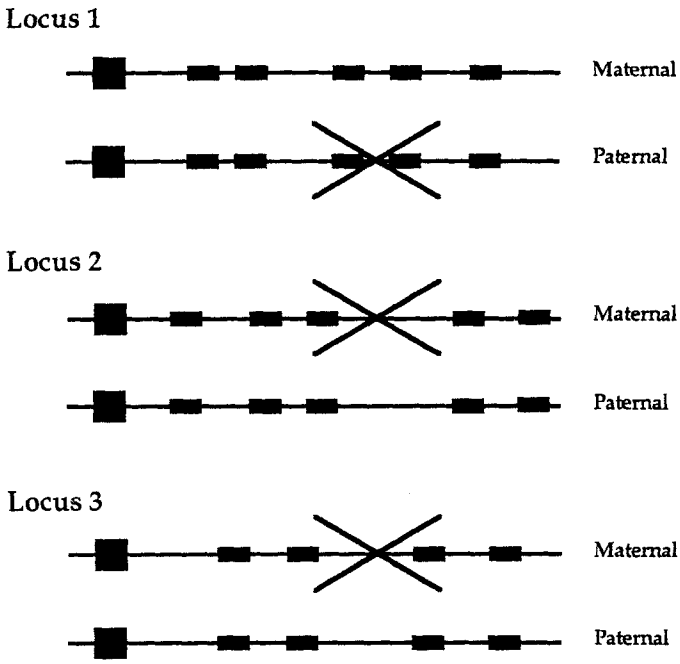

FIG. 1. A model for olfactory receptor gene regulation. (A) Allelic inactivation. Rectangles represent receptor genes, squares represent *cis*-regulatory elements that choose one receptor from a linked array, and X's represent inactivation of the entire allelic array. Only three chromosomal loci encoding olfactory receptors are depicted. Only receptors expressed in one of the four zones are depicted in this model; other receptors expressed in different zones are not depicted.

zone is not depicted in the model. A simple model for how the zonal restriction of expression is accomplished invokes a zone-specific transcription factor(s) that dictates which receptor genes are available within a linked array.]

Why is this model, which invokes a *cis* stochastic mechanism, different from other potential models of olfactory receptor gene expression? This *cis* stochastic model accounts for all olfactory receptor expression data that have been accumulated.

Other models require one or another observation to be set aside. For example, consider for a moment a model in which there is a specific combination of transcription factors that activate each individual gene to the exclusion of all other genes. This model is similar to the mode of regulation of many genes. If this type of model were correct, the specific

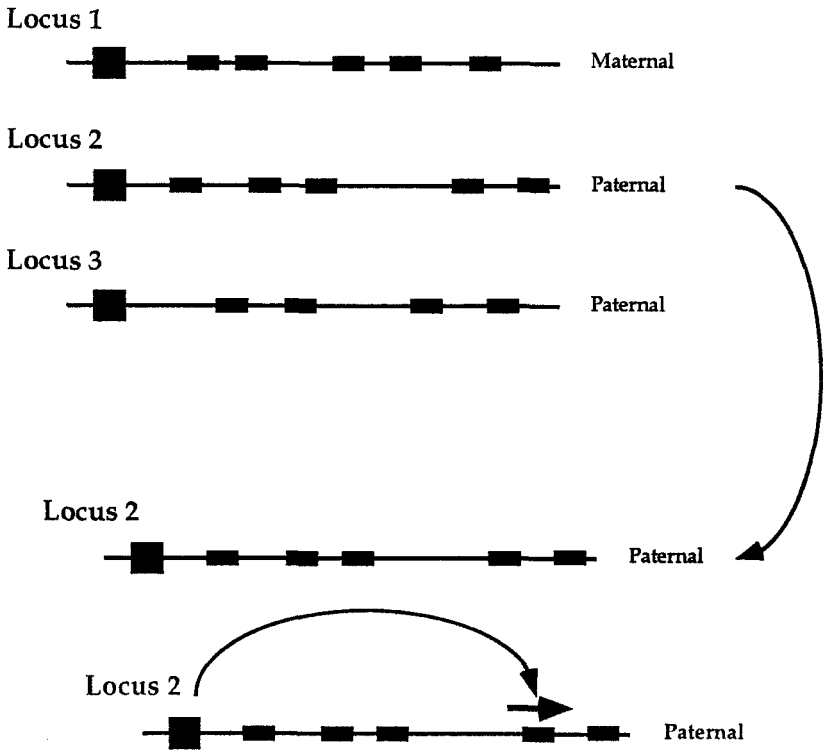

FIG. 1 (*Continued*). (B) Choice of genomic locus. During olfactory neurogenesis, each neuron chooses one of the chromosomal arrays, rendering it available for the activation of one receptor in C. (C) Choice of receptor. Rearrangement or enhancer looping mediates the choice of one receptor gene from the linked array. The *cis*-regulatory element, depicted by the square box, mediates the choice. If rearrangement is the mechanism, either gene conversion moves the receptor gene to a new location adjacent to the *cis* element or the intervening DNA is removed in a manner similar to VDJ recombination in lymphocytes.

combination of transcription factors should activate both alleles. However, only one of the two alleles is expressed. Thus a standard transcription factor model is less satisfying.

Another model is one in which a limiting transcription factor complex, perhaps even one complex per cell, is capable of activating any of the 1000 olfactory receptor genes. Only one allele of one gene is activated by virtue of the fact that only one such complex exists in a given neuron. If this model were correct, there would be no reason to have allelic inactivation at all olfactory receptor gene loci. The asynchrony of replication observed at all examined olfactory receptor gene loci thus argues against a limiting transcription factor complex model.

A. Evolution and the Regulation of Genes

One attractive aspect of the stochastic *cis*-regulation model is that it allows for the expansion (and contraction) of the gene family over evolutionary time. For example, if there is a mechanism in place to select one array in the genome that leaves the others silent, then it does not matter how many arrays the olfactory receptor genes reside in. One array can always be chosen. Similarly, if there is a mechanism in place that allows only one gene from a given array to be chosen, then expansion of the number of genes at this array does not perturb the mechanism, assuring that only a single receptor gene is transcribed in each cell. Expansion and contraction of the olfactory receptor gene family are clearly important evolutionarily (Ngai *et al.*, 1993a). Thus the ability of the stochastic *cis*-regulation model to accommodate such changes in gene number is very important.

B. How is the Choice of Receptor Linked to Axon Guidance?

A full discussion of the interconnection between olfactory receptor gene choice and axon guidance is beyond the scope of this review. There is no doubt that the choice of a receptor is closely linked to the axon projection of the neuron. However, the nature of the linkage is still unclear. Elegant experiments by Axel and colleagues suggest that the receptor may play an instructive role, but may not be the only determinant (Mombaerts *et al.*, 1996). They replaced the coding region of one olfactory receptor gene with a distinct receptor gene and analyzed the axonal projections of the neurons expressing this "swapped in" gene. What they observed was that the axons now projected to a site distinct from either the "donor" or the "recipient" normal projection site. The authors suggest that the receptor itself is therefore playing some instructive role, but is not the only determinant. However, it is also possible that the receptor is merely permissive and that a change in the level of olfactory receptor gene expression can cause subtle changes in axon targeting. Further swap experiments will surely lead to a clearer understanding of the determinants of axon guidance in primary olfactory neurons.

VI. Conclusion

Elucidation of the molecular mechanisms underlying olfactory receptor gene expression should provide insight into the logic of olfactory discrimination and may yield insights applicable to the control of gene expression in other systems. As details of the regulation of olfactory receptor genes emerge, it will be interesting to see if parallels between the regulation of olfactory receptor genes and antigen receptor genes extend beyond monoallelic expression.

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