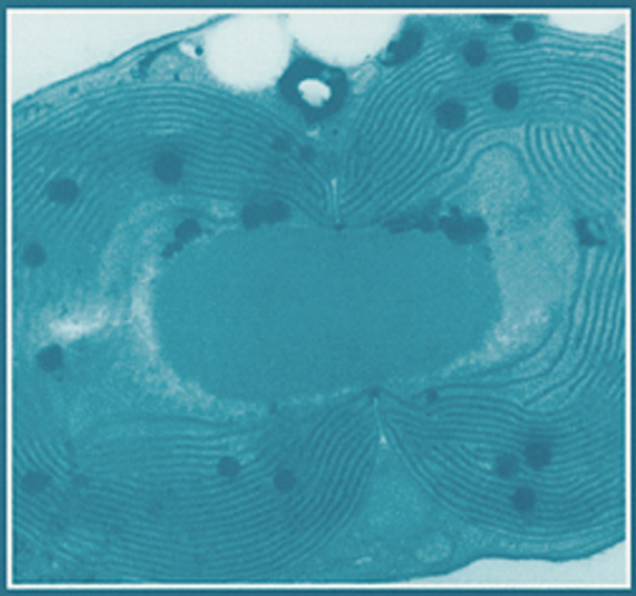
INTERNATIONAL REVIEW OF CYTOLOGY

A SURVEY OF CELL BIOLOGY

Edited by
Kwang W. Jeon



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A Survey of Cell Biology

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Front cover photograph: Electron Micrograph of a cyanelle of Cyanophora Paradoxa.
(A) A divining cyanelle with an ingrowing septum. (For more details, see Chapter 2, figure 2A)

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Glucocorticoid and Thyroid Hormone Receptors in Mitochondria of Animal Cells

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This article concerns the localization of glucocorticoid and thyroid hormone receptors in mitochondria of animal cells. The receptors are discussed in terms of their potential role in the regulation of mitochondrial transcription and energy production by the oxidative phophorylation pathway, realized both by nuclear-encoded and mitochondrially encoded enzymes. A brief survey of the role of glucocorticoid and thyroid hormones on energy metabolism is presented, followed by a description of the molecular mode of action of these hormones and of the central role of the receptors in regulation of transcription. Subsequently, the structure and characteristics of glucocorticoid and thyroid hormone receptors are described, followed by a section on the effects of glucocorticoid and thyroid hormones on the transcription of mitochondrial and nuclear genes encoding subunits of OXPHOS and by an introduction to the mitochondrial genome and its transcription. A comprehensive description of the data demonstrates the localization of glucocorticoid and thyroid hormone receptors in mitochondria as well as the detection of potential hormone response elements that bind to these receptors. This leads to the conclusion that the receptors potentially play a role in the regulation of transcription of mitochondrial genes. The in organello mitochondrial system, which is capable of sustaining transcription in the absence of nuclear participation, is presented, responding to T_3 with increased transcription rates, and the central role of a thyroid receptor isoform in the transcription effect is emphasized. Lastly, possible ways of coordinating nuclear and mitochondrial gene transcription in response to glucocorticoid and thyroid

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hormones are discussed, the hormones acting directly on the genes of the two compartments by way of common hormone response elements and indirectly on mitochondrial genes by stimulation of nuclear-encoded transcription factors.

KEY WORDS: Mitochondria, Glucocorticoid hormone receptor, Thyroid hormone receptor, Hormone response elements, Transcription regulation, DNA–receptor interaction, Oxidative phosphorylation.

I. Introduction

The coordination of cellular functions is a multifactorial, multicomponent process, involving several regulatory agents, acting on many metabolic and developmental processes taking place in various compartments and organelles of the cell. One of the main regulatory systems that has evolved in multicellular organisms to cope with communication of cells over great distances is the hormonal one, steroid and thyroid hormones being prominent members of this class of regulators, affecting cell metabolism, differentiation, and growth. In particular, energy production involving both nuclear and mitochondrial processes is to a great extent affected by steroid and thyroid hormones, and the question arises as to how these hormones regulate processes necessitating the cooperation of two different cell organelles (Mutvei *et al.,* 1989; Soboll, 1993a; Pillar and Seitz, 1997; Enriquez *et al.,* 1999b; Wrutniak *et al.,* 1998).

A major role in cellular energy production is played by the enzymes of the respiratory chain, some of their subunits being encoded by nuclear and some by mitochondrial genes (Attardi and Schatz, 1988) and it is well established that glucocorticoid and thyroid hormones regulate the expression of a number of these genes (see Sections II and III.A.1). In this article we first briefly present the current knowledge on the mode of action of steroid and thyroid hormones, the central role played by the receptors of these hormones on nuclear transcription, their presence in mitochondria, and the possible role of the mitochondrially localized glucocorticoid and thyroid receptors in the transcription of mitochondrial genes encoding respiratory enzyme subunits. We then discuss possible ways of coordination of transcription of the mitochondrial genes with the transcription of nuclear enzymes of oxidative phosphorylation (OXPHOS) genes, also induced by the hormones. Special emphasis will be given to data pointing to a direct effect of glucocorticoids and thyroid hormones on mitochondrial gene transcription. A short review of the physiology of glucocorticoid and thyroid hormones in relationship to energy metabolism will precede the section on molecular mechanisms of steroid and thyroid hormones.

II. Physiology of Glucocorticoid and Thyroid Hormones: Energy Metabolism

A. Glucocorticoid Hormones

Glucocorticoids (Fig. 1) regulate developmental, growth, and metabolic processes and play a major role in survival during stress (De Feo, 1996; Frayn, 1986; Tataranni *et al.,* 1996). All these processes are linked to increased energy demands dependent on the levels of circulating corticosteroids, which increase resting energy expenditure (REE) and metabolic rates, in parallel decreasing the respiratory quotient, suggesting that energy expenditure is fueled by fatty acid oxidation (Brillon *et al.,* 1995). Indeed, glucocorticoids lead to lipolysis in fat tissues and to release of fatty acids, which are oxidized in the liver for energy production (Horber *et al.,* 1991). Furthermore, glucocorticoids stimulate proteolysis in skin, lymphoid tissue, and

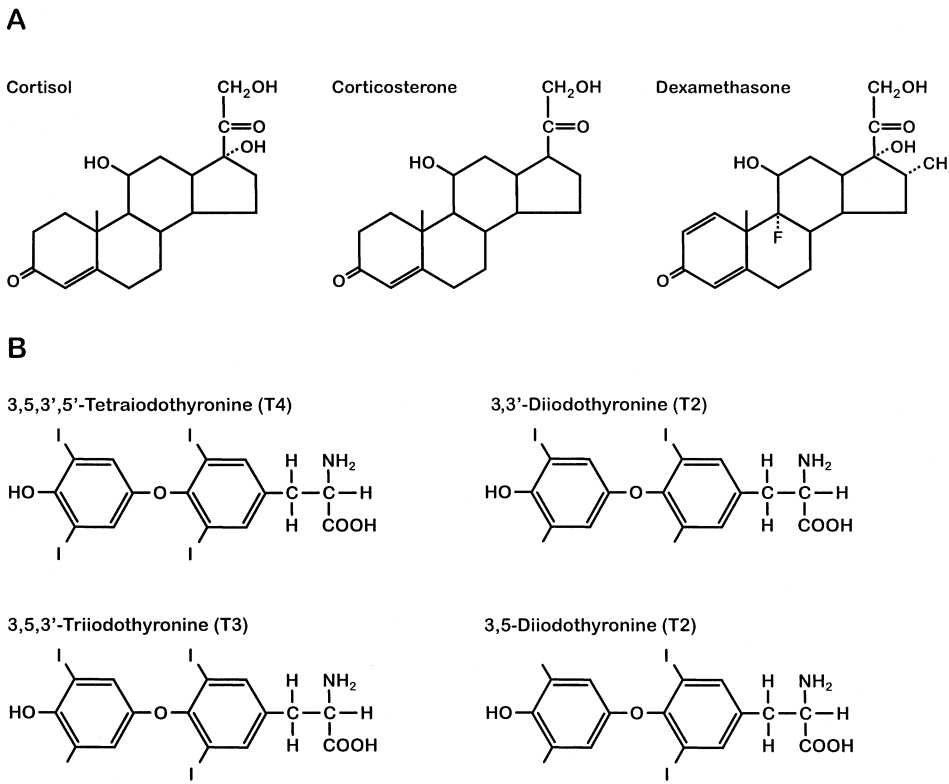

FIG. 1 The principal glucocorticoid (A) and thyroid (B) hormones.

muscle, with the production of amino acids that are used for glucose and glycogen synthesis (Beaufrere *et al.,* 1989; Baxter and Forsham, 1972; Darmaun *et al.,* 1988).

Several studies have shown the involvement of glucocorticoids in the regulation of mitochondrial respiration and oxidative phosphorylation, in part by activating nuclear and mitochondrial genes encoding mitochondrial components (Allan *et al.,* 1983; Monsour and Nass, 1970, 1974; Yu and Feigelson, 1970) (see Section IV.A) and affecting the number and size of the mitochondria (Lowe *et al.,* 1955; Kimberg *et al.,* 1968). Rapid nongenomic hormonal effects can also modulate mitochondrial energy production (see Section III.B). It should be mentioned that 20–30% of the resting metabolic rate is not accounted for by mitochondrial adenosine triphosphate (ATP) synthesis but rather by futile proton cycling across the inner mitochondrial membrane (Rolfe *et al.,* 1999; Stuart *et al.,* 1999).

B. Thyroid Hormones

Thyroid hormones (Fig. 1) are the main regulators of basal metabolic rates, increasing respiration, regulating protein, lipid, and sugar metabolism (Soboll, 1993a; Kadenbach *et al.,* 1995; Pillar and Seitz, 1997; Munoz and Bernal, 1997), and affecting the growth and development of organs, among them the central nervous system (CNS) (Muscat *et al.,* 1995; Koibuchi and Chin, 2000; Yen and Chin, 1994). Thyroid hormones are especially active in liver, heart, kidney, and brain. Experimentally, their effects have been recognized in thyrectomized animals, showing defects in the above mentioned parameters (less oxygen consumption, decelerated growth and development, particularly of the CNS, and derangements in protein, lipid, and sugar metabolism), which can be prevented by hormone administration (Pillar and Seitz, 1997). Thyroid hormones promote the biogenesis of mammalian mitochondria (Gross, 1971), as demonstrated in the developing kidney and liver and changes in basal metabolic rates brought about by thyroid hormones (TH) generally coincide with an increase in the number of respiratory units.

Thyroid hormones have both short- and long-term effects (Soboll, 1993a,b; Mutvei *et al.,* 1989; Nelson, 1990; Goglia *et al.,* 1999; Hörlein *et al.,* 1996). A very rapid stimulation of oxidative phosphorylation, oxygen consumption, and translocator activity can be observed in less than 2 min after addition of 3,5,3′-triiodothyronine (T_3) to isolated mitochondria (Sterling *et al.,* 1986), or 15 min after *in vivo* administration of the hormone (Palacios-Romero and Mowbray, 1979). These effects are exerted in the presence of inhibitors of protein synthesis and, therefore, do not require *de novo* synthesized proteins. The long-term effects of TH involve gene activation and protein synthesis. This was first shown by Tata and co-workers (Tata *et al.,* 1962, 1963; Tata, 1964; Roodyn *et al.,* 1965) who could prevent the TH-induced increase of the basic metabolic rate of hypothyroid rats by inhibitors of RNA and protein synthesis. Subsequently, the effects of TH on mitochondrial RNA and protein synthesis have been demonstrated. A 2- to 8-fold

increase of mitochondrial mRNA levels is induced by TH (Mutvei *et al.,* 1989). The area of the inner membrane is increased (Joste *et al.,* 1989) and alteration of phospholipid composition leading to increased permeability of protons is recorded 10–12 hr after T_3 administration (Brand *et al.,* 1992, 1994). This leads to changes in coupling efficiency between mitochondrial electron transport and phosphorylation. An increased expression of uncoupling proteins UCP_1 and UCP_3 is also demonstrated, as well as a correlation between UCP_3 mRNA levels, mitochondrial coupling, and thyroid state (Barbe, 2000), suggesting that control of UCP_s expression is involved in the T_3 regulation of the proton leak. Thyroid hormones also upregulate precursor protein import rates into mitochondria (Craig *et al.,* 1998). An increase in cardiolipin is observed due to stimulation of cardiolipin synthetase. Increased transcription rates of mitochondrial genes by T_3 has been observed recently in an in organello mitochondrial system (Enriquez *et al.,* 1999a; Wrutniak-Cabello *et al.,* 2001) and these findings will be presented in more detail in Section IV.E.

III. Molecular Mechanisms of Glucocorticoid and Thyroid Hormone Action

A. Effects of Steroid and Thyroid Hormones on the Nucleus

1. Effects on Transcription

Glucocorticoids, like all steroid hormones, and thyroid hormones act on gene transcription by interacting with nuclear receptors, polypeptides, which convert the hormonal stimulus into a transcription response (Yamamoto, 1985; Schütz, 1988; Green and Chambon, 1988; Evans, 1988; Beato *et al.,* 1995; Tsai and O'Malley, 1994; White and Parker, 1998). The now classic scheme of steroid hormone action on genes was first proposed by Karlson (1963) and later completed by Jensen *et al.* (1968), who introduced the hormone receptor concept (Fig. 2).

The first step in the chain of events leading to changes in gene expression is binding of the hormone ligand to its cognate receptor (Parker, 1993), inducing an allosteric change of the protein molecule, which thus is "activated" and as either homodimer, heterodimer, or monomer interacts with cognate DNA elements, the hormone responsive elements (HREs), positioned near, further upstream, or, less frequently, within the structural genes. Once bound to HREs, the receptor interacts with other transcription factors, coactivators, corepressors, and histone-modifying enzymes recruiting the basal transcription machinery, to activate, enhance, attenuate, or repress gene transcription (Torchia *et al.,* 1998; Robyn *et al.,* 2000; Wolffe and Guschin, 2000). Steroid nuclear coactivators interact by way of LXXLL motifs with the AF-2 domain of liganded receptors and to general transcriptional coactivators CBP/p300 and P/CAF (Chakravarti *et al.,*

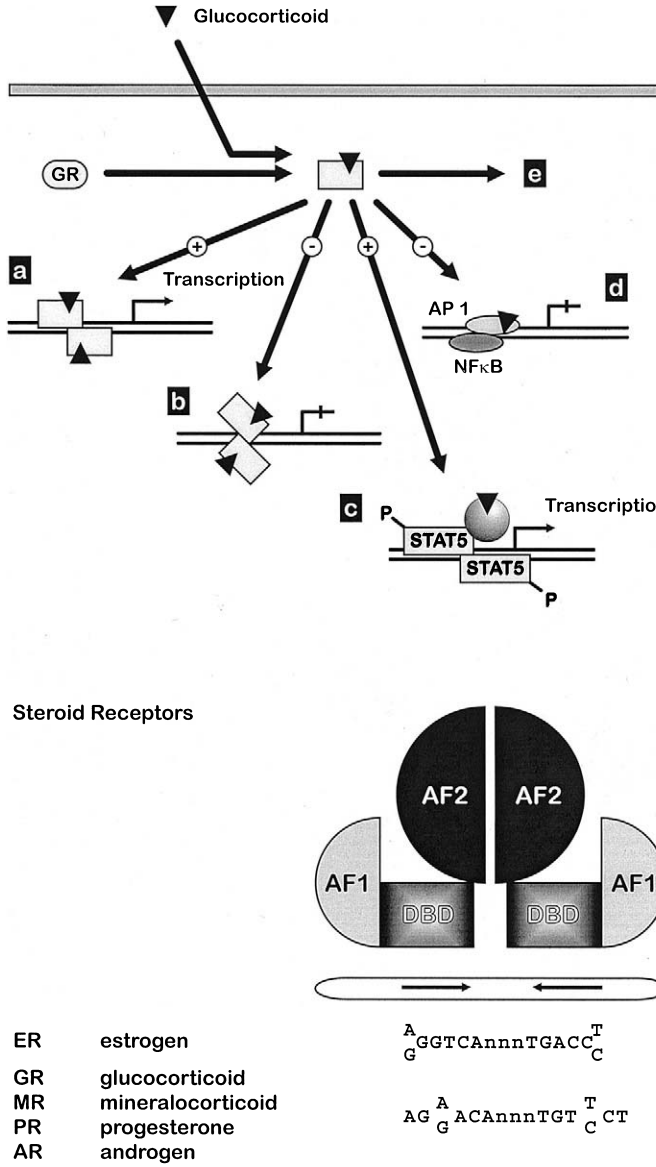

FIG. 2 Mode of actions of the glucocorticoid receptor. The GR, as a dimer, interacts with GREs leading to activation (a) or inhibition (b) of gene transcription, as a monomer interacts directly with proteins (AP1 and NF-κB) activating (c) or inhibiting (d) gene transcription and as a membrane-bound receptor (e) acts in a still undefined way (redrawn from a slide of G. Schütz, with permission). The lower part of the figure shows the consensus sequence of steroid and thyroid receptor response elements (HREs). Homodimers recognize palindromic HREs spaced by three nucleotides in a symmetrical way. Heterodimers (e.g., TR/RXR) recognize diverse HREs, in which half core motifs can be arranged as palindromes, inverse palindromes, or direct repeats. From White and Parker (1998).

1996; Heery *et al.,* 1997), which possess histone acetyltransferase (HAT) activity (Ogryzko *et al.,* 1996; Bannister and Kouzarides, 1996), resulting in acetylation of histone 4-lysine residues, chromatin remodeling, and enhancement of transcriptional activity (Nightingale *et al.,* 1998; Lee *et al.,* 1993). The receptors also interact with coactivator-associated arginine methyltransferase (CARM) resulting in methylation of arginine residues of proteins (Koh *et al.,* 2001).

Among the identified coactivators, well studied is the steroid receptor coactivator (SRC) family, encompassing SRC-1, SRC-2 (TIF2, GRIPI, NCoA-2), and SRC-3 (RAC3, TRAM-1, p/CIP). Although all SRCs interact and activate multiple steroid/nucear receptors, evidence indicates that each SRC coactivator may exhibit a specific mode of function and it is likely that the levels of receptors, coactivators, and other transcriptional regulators may play a role in the control of the level of gene expression in response to specific hormones (Fig. 3). The molecular details of hormonal induction and repression are still not well known. The recruitment of histone acetyltransferases and methytransferases influences histone–DNA interaction, as demonstrated by the appearance of DNase sensitivity at these sites. The nucleosome structure does not seem to be dissolved at the site of receptor binding as a consequence of histone modification, but through unpacking is rendered more accessible to regulatory proteins, enhancing RNA polymerase transcription. The state of receptor phosphorylation (Moudgil, 1990; Weigel, 1996) is crucial for the manifestation of the hormonal effect and for the interaction and cross-talk of the endocrine system with other regulatory agents, such as growth factors, neurotransmitters, immunomodulators, and cytokines (Power *et al.,* 1991; Jones *et al.,* 1994; Rangarajan *et al.,* 1992; Krstic *et al.,* 1997; Migliaccio *et al.,* 1998).

Not all actions of steroid and thyroid hormones are a result of direct interaction of receptor with DNA. As demonstrated in the case of the glucocorticoid receptor (Reichardt *et al.,* 1998), transgenic mice in which the glucocorticoid receptor (GR) has been modified so that it cannot dimerize ($GR^{dim-/dim-}$) are viable and many of the GR effects crucial to survival are exerted by way of its interaction, not directly with DNA, but with other regulatory proteins that bind to DNA (e.g., jun/fos, NF-κB, aa) (Jonat *et al.,* 1990; Yang-Yen *et al.,* 1990). As discussed in see Section III.B, many of the effects of steroid/thyroid hormones are rapid and nongenomic.

Among the receptors, thyroid hormone receptor (TR) [and retinoic acid receptor (RAR)] possess ligand-independent activity (Fig. 4) that leads to the silencing of positively regulated target genes, due to the recruitment of at least two nuclear corepressor proteins, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) (Cohen *et al.,* 2000). These corepressors, in turn, recruit a multiprotein complex with histone deacetylase activity that appears to modify chromatin and prevent transcription. In the presence of their cognate ligands, TR and RAR release the nuclear corepressors and recruit members of the coactivator family [e.g., SRC-1, TIF-II, CREB-binding protein (CBP), p300, pCAF, and other coactivators]. As already mentioned, the coactivator complexes possess histone acetyltransferase activity, contributing to transcription activation.

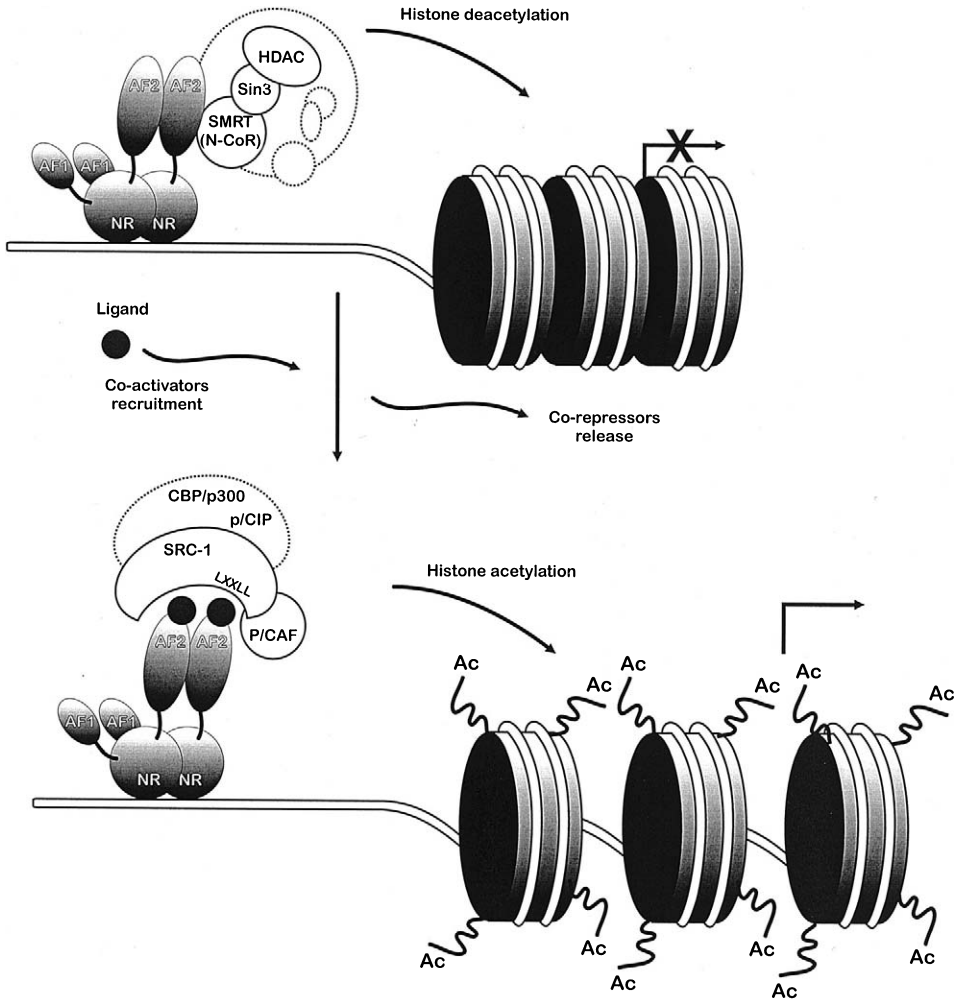

FIG. 3 Model of nuclear receptor-mediated activation of gene transcription. Binding of the hormone to the receptor leads to conformation modification and activation of the molecule, which then tightly interacts with cognate HREs, binding CBP and recruiting the basal transcription apparatus to the gene promoters. The receptor also interacts with the p160 family of steroid receptor coactivators (SRC) complexed with CBP. The histone acetyltransferases and methyltransferases associated with some of the activators play a significant role in chromatin remodeling necessary for the gene activation process. Thyroid hormone receptors act in the unliganded form as transcription repressors (see Fig. 4 and text). From Collingwood *et al.* (1999), reproduced by permission of the Society for Endocrinology.

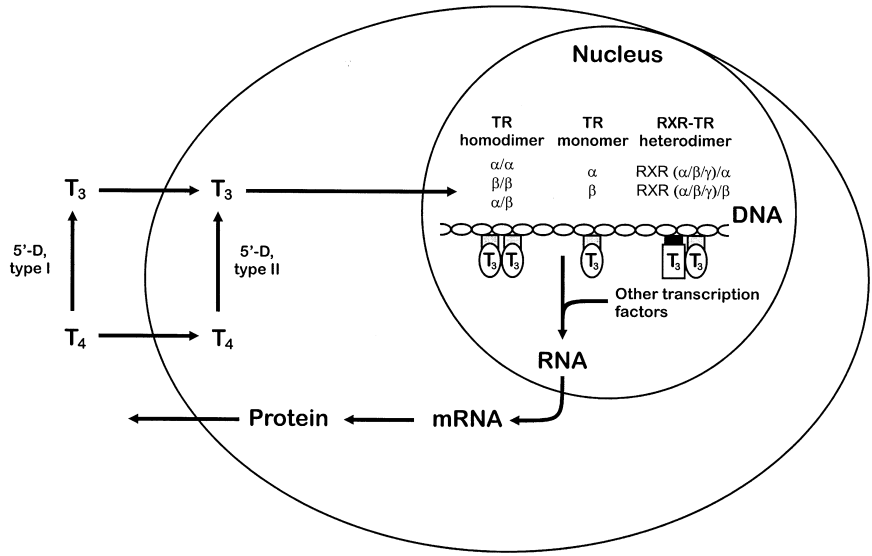

FIG. 4 Model of thyroid hormone receptor action on gene transcription. The active form of thyroid hormone, triiodothyronine (T_3), is produced by deiodination of thyroxine (T_4) by the enzymes $T_4 5'$-deiodinase ($5'$-D) type I and II. Type I $T_4 5'$-deiodinase is found predominantly in the liver and kidneys; its action is responsible for the production of two-thirds of the total T_3 in the body. Type II $T_4 5'$-deiodinase is responsible for most of the T_3 found in the pituitary, the brain, and brown fat. T_3 enters the cell or is produced locally and then transported into the nucleus. Transcriptionally active forms of thyroid hormone receptor (TR) include monomers, homodimers, and heterodimers with nuclear protein partners, such as the retinoid X receptor (RXR). The T_3-receptor complex interacts with specific sequences in DNA regulatory regions and modifies gene expression. In most cases, TR binds in an unliganded form acting as a transcription repressor. Upon binding of T_3 to the receptor, TR dissociates from the TRE alleviating the repressor action of TR. 9-*cis* RA (9-cis-retinoic acid) is the ligand for RXR. Adapted with permission from Larsen and Igbar (1992), as modified by Brent (1994).

TR seems to recruit NcoR preferentially and RAR recruits SMRT preferentially to hormone response elements. The recruited corepressor complex can be both nuclear receptor and receptor complex specific.

2. Glucocorticoid Receptors

GR was first purified from rat liver (Govindan and Sekeris, 1978; Wrange *et al.,* 1979) and rat thymus (Tsawdaroglou *et al.,* 1981) and the gene cloned (Evans, 1989; Govindan *et al.,* 1985; Danielsen *et al.,* 1986). A second GR was detected by Bamberger *et al.* (1995) (now called GRβ to distinguish it from the first isolated receptor, GRα). Both GR species are products of the same gene (Fig. 5) consisting of eight common exons and two different ninth exons (9α and 9β), and result from differential splicing. The last (ninth) exon of GRα is 9α, whereas that of GRβ is 9β).

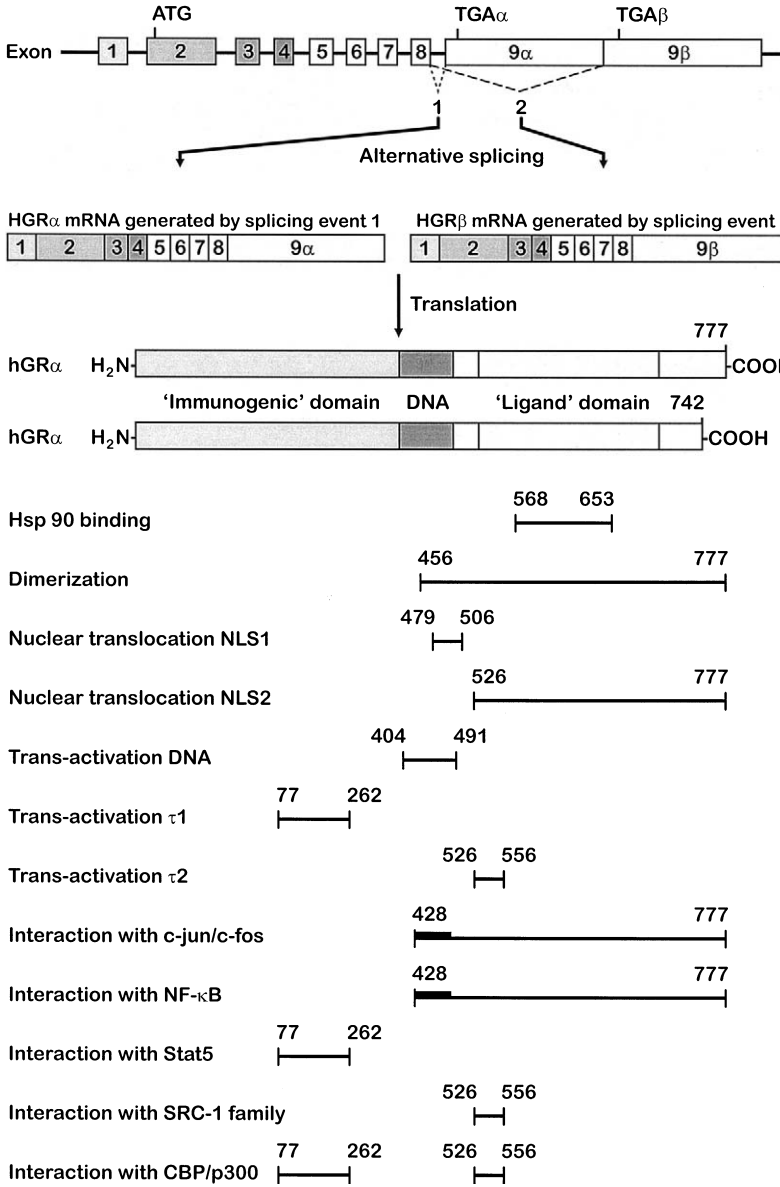

FIG. 5 Structural organization of nuclear receptors: human GR gene and splicing products. The receptor proteins are characterized by polypeptide domains serving the various functions: Shown are the ligand binding domain, the DNA-binding domain, and the two transcription activating domains. Depicted are also regions responsible for dimerization of the receptor, for interaction with heat shock proteins, nuclear regulatory proteins, and nuclear localization (NLS). Alternative splicing events generate two different hGR-mRNAs that differ in size. Translation of the mRNAs produces two isoforms of GR, hGRα and hGRβ, respectively, which have an identical structure up to amino acid 727, but then diverge. hGRα: 727 aa, $M_r = 98$ kDa; hGRβ: 742 aa, $M_r = 94$ kDa. Boxes and lines represent exons and introns, respectively. Reprinted from Vottero and Chroussos (1999). Glucocorticoid receptor β: View I. *TEM* **16,** 333–338, with permission from Elsevier Science.

Exon 1 is not translated, exon 2 encodes the amino-terminal part, exon 3 and 4 encode the two Zn fingers, and exons 5–9α encode the ligand-binding domain (LBD). GRβ has no ligand-binding activity and its biological effects probably are due to transcriptionally inactive dimer formation with GRα, competition with GRα for DNA-binding sites, or competition for limited amounts of cofactors that are required for transcriptional activity of GRα.

As all nuclear receptors (Hurd and Moudgil, 1998), GR is characterized by the presence of various functional domains (Hollenberg and Evans, 1988), an LBD, a DNA-binding domain (DBD), transcription-activating regions (t1, located at the N-terminus and t2 located at the C-terminus), a nuclear localization signal (NLS) between amino acids 477 and 507 (Picard and Yamamoto, 1987), a dimerization domain, phophorylation sites (Ser and Thr), and regions of interaction with heat shock, other cytoplasmic, and also nuclear proteins. Binding of the GR to HREs is accomplished by two finger-like structures, rich in cysteines in the DBD. Each finger is composed of a Zn ion bound to four cysteines in a tetrahedric form. The first Zn finger closer to the amino-terminal part of the receptor is responsible for the recognition of the HREs. Whereas the t2 function is activated upon hormone binding to the receptor, t1 is not dependent on the presence of the ligand: t1 interacts with the basic transcriptional machinery by way of the TAF proteins and through these with the TATA-binding proteins TBP, thus stabilizing the transcription initiation complex. GR is mainly localized in the cytoplasm (see Section IV.C.1) in the form of a nonactivated molecular complex with heat shock protein 90 (hsp90), p53/immunophilin, and p23 (Gehring, 1998; Alexis *et al.,* 1992). Hsp70 is loosely bound to the complex.

These chaperone proteins are important for the correct folding and stabilization of the receptor in hormone accessible conformation. Under normal growth conditions, consecutive cycles of hsp90-driven dissociation and hsp70-driven reassembly maintain the hormone-responsive complex in the absence of hormone. Although most publications conform a predominant cytoplasmic localization of the unliganded GR (Papamichail *et al.,* 1980), some reports are contradictory, probably due to the varying methodology applied (e.g., differing fixation methods in immunolocalization studies), different cell types, or varying cell cycle phases examined (Fejes-Tóth *et al.,* 1998). In these studies, either a predominant nuclear localization or an equal distribution between nucleus and cytoplasms of the receptor has been shown. In any case, upon activation through ligand binding, the glucocorticoid receptor complex dissociates, GR dimerizes, and can bind in the nucleus to its respective HREs. GR has been also localized in the mitochondria of various animal cells. This will be discussed in Section IV.C.

3. Thyroid Hormone Receptors

The thyroid hormone receptors (Fig. 6) are also members of the large superfamily of nuclear receptors (Lazar, 1993; Brent, 1994). However, in contrast to GR and most other receptors of the same family, the unliganded thyroid hormone receptors

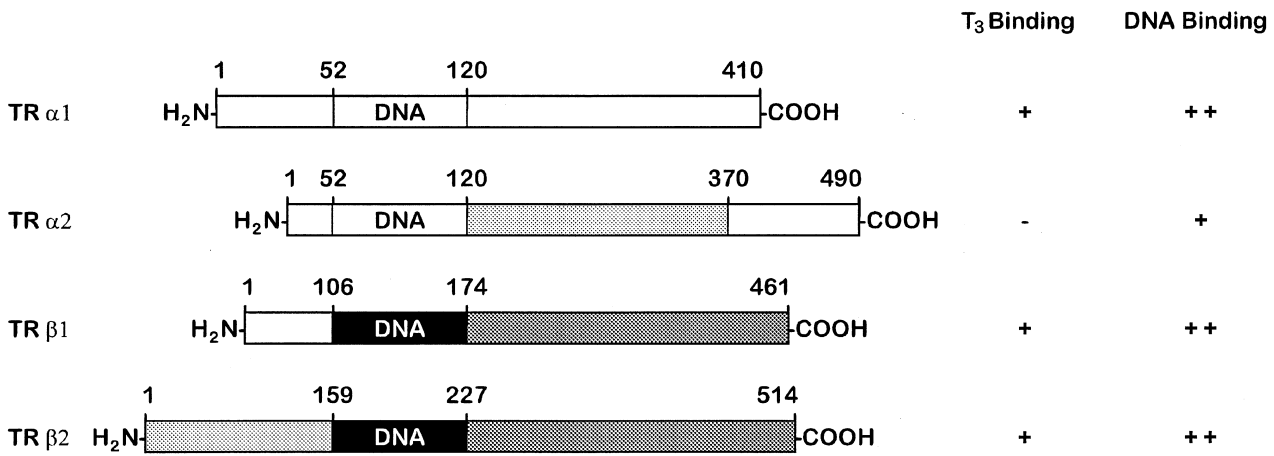

FIG. 6 Deduced protein structure of the major products of the thyroid hormone receptor TRα and β genes. Adapted with permission from Brent (1994).

are biologically active, bind in this form, and silence positively regulated genes, whereas upon hormone binding the repressive effect is abolished and the gene is activated (Sap *et al.,* 1989; Fondell *et al.,* 1993; Helmer *et al.,* 1996; Barettino *et al.,* 1994). Two genes encode triiodothyronine (T_3) receptors, c-*erbAa* localized on chromosome 17 and c-*erbAb* localized on chromosome 3 (Sap *et al.,* 1986; Weinberger *et al.,* 1986). In rats, mice, and humans, due to alternative splicing, the c-*erbAa* gene encodes three proteins, a1, a2, and a3, of which only a1 binds T_3 and acts as a ligand-modulated transcription factor (Brent, 1994; Yang *et al.,* 1996). In the same species, the c-*erbAb* gene encodes two receptors, β_1 and β_2 (Fig. 6), which both bind T_3 with a 10-fold higher affinity than thyroxine. The general architecture of TRs is that of the nuclear receptor family, with similar functional domains. The various thyroid hormone receptor species show identical DNA-binding domains. The receptors form homodimers but also heterodimers, particularly with retinoid X receptor (RXR) (Glass, 1994) and bind to the respective thyroid hormone responsive elements (TREs), which consist of the basic hexameric consensus sequence AGGT/ACA in several different arrangements, such as direct repeats separated by a 4-bp spacer, everted repeats, separated by 4 bp, or other configurations. In addition to RXR, many other molecules are directly or indirectly associated with T_3R. T_3R forms heterodimers with other members of the receptor superfamily, e.g., vitamin D_3 receptor or the peroxisome prolifator-activated receptor (PRAR) (Munoz and Bernal, 1997). The T_3R–RXR heterodimers interact with a variety of activators/corepressors, which have been discussed in Section III.A.1.

T_3 receptors are expressed in a developmentally specific pattern (Chatterjee and Tata, 1992). The tissue distribution of the various forms of the receptors varies, both quantitatively and qualitatively: whereas $A\beta_1$ is expressed in a wide variety of tissues, $A\beta_2$ is found almost exclusively in the pituitary, acting there as inhibitor of thyroid-stimulating hormone (TSH)-α- and β-subunit gene transcription, by binding to negative T_3 response elements present on these genes (Burnside *et al.,* 1989). Thyroid status influences the expression of T_3R mRNA. In rats, injection of T_3 increases $T_3R\beta_1$ mRNA and decreases $T_3R\beta_2$ mRNA in pituitary and decreases $T_3R\alpha_1$ and $T_3R\alpha_2$ mRNA in heart, kidneys, and pituitary (Burnside *et al.,* 1989). In addition to the localization of TR in cytoplasm and nuclei, a specific TR form has been found in mitochondria. This will be discussed in Section IV.C.

B. Nongenomic Effects of Steroid and Thyroid Hormones

In the classic model of steroid action described above, the effector mechanism involves binding of the hormones to receptors present in the nucleus or/and in the cytoplasm, modulation of transcription, and initiation of various physiological processes dependent on RNA transcription and protein biosynthesis. A considerable latency of these steroid effects (>30 min) is the consequence of these time-consuming actions. Considerable data have accumulated within the past years that

provide evidence for rapid actions of steroid and thyroid hormones. Some responses to hormones occur within a few minutes and therefore are not compatible with the classic scheme of steroid genomic action. The main evidence for the classification of an effect as nongenomic is its rapid time course and its insensitivity to inhibitors of transcription and translation (Kanazir, 1990; Wehling, 1997; Falkenstein *et al.,* 2000). Presumably, the hormone ligand in these cases interacts with membrane receptors. The nature of the protein(s) interacting with the ligand to induce the rapid effects is unknown. It could be a classic membrane-bound receptor, a pore protein, or a new type of receptor.

Two examples of nongenomic effects of glucocorticoids are the rapid prevention of c-jun phosphorylation by blocking of the JNK signaling cascade in HeLa cells (Caelles *et al.,* 1997) and the rapid effect on actin assembly in human endometrial cells (Koukouritaki *et al.,* 1997). Prompt changes in the actin cytoskeleton leading to secretion, exocytosis, and volume regulation are considered to be part of an early cellular response to glucocorticoids independent of gene transcription.

Several "nongenomic" effects of thyroid homones have been reported (Bronk and Bronk, 1962; Mutvei and Nelson, 1989; Martens *et al.,* 1991; Segal, 1989; Martino *et al.,* 1986; Hardy and Mowbray, 1992). T_3 injection in hypothyroid rats increases oxygen consumption and oxidative phosphorylation measured in isolated liver mitochondria within 30 min (Palacios-Romero and Mowbray, 1979). Inhibition of protein synthesis did not abolish this effect. With isolated mitochondria, the same effects were observed within 2 min after hormone addition. Stimulation of the mitochondrial carrier adenine nucleotide translocase (ANT) was also seen (Sterling, 1986). Thyroid hormones also affect calcium signaling within minutes, independent of new protein synthesis.

T_3 treatment *in vivo* results in activation of mitochondrial Ca^{2+} influx and temperature-dependent swelling of Ca^{2+}-loaded rat liver mitochondria (Davis *et al.,* 1989). Treatment with TH similarly results in a dramatic decrease in membrane potential, proton gradient, and proton motive force measured in Ca^{2+}-loaded mitochondria (Kalderon *et al.,* 1995). Thyroid hormones influence thermogenesis and the lipolytic activity of catecholamines less than 30 min after administration.

The possibility that some of the rapid effects of thyroid hormones could be exerted by 3,5-diiodo-L-thyronine (3,5-T_2) and 3,3′-T_2 (Fig. 1) has been supported experimentally (Goglia *et al.,* 1994a,b, 1999). 3,5-T_2 stimulates oxygen consumption in isolated perfused rat liver (Horst *et al.,* 1989), a rapid stimulation of rat liver cytochrome activity by T_2s has been demonstrated in a nuclei free system, and metabolic effects have been shown in rats and humans. Goglia *et al.* (1999) showed that administration of T_2s enhance mitochondrial respiratory rate and cytochrome oxidase activity, similar to the effects of T_3. However, T_2s act 1 hr after administration, whereas the effects of T_3 were significant only after 24 hr. 3,5-T_2 was found to bind to the Va subunit of cytochrome *c* oxidase, leading to conformational changes of the enzyme, completely abolishing the allosteric inhibition of ATP on respiration (Arnold and Kadenbach, 1997; Arnold *et al.,* 1998), thus

activating the respiratory chain. The presence of a 5′-deiodinase activity in mitochondria converting T_3 to T_2 could explain in this context some of the T_3-induced effects in isolated mitochondria.

IV. Effects of Glucocorticoid and Thyroid Hormones in Mitochondria

A. Glucocorticoids, Thyroid Hormones, and Mitochondrial Gene Activation

Mitochondria are the powerhouses of the eukaryotic cell. Under aerobic conditions, more than 95% of the cell energy is produced in mitochondria by the oxidation in the respiratory chain of the NADH derived from the tricarboxylic cycle. In this process, the major role is played by the respiratory chain complexes I to IV, composed of several proteins, some subunits of which are encoded by the mitochondrial genome (see Section IV.B) and synthesized in the mitochondria and some encoded by the nuclear genome and synthesized on cytoplasmic ribosomes.

Under steady-state conditions, there is a close relationship between levels of mRNAs for OXPHOS enzymes encoded in the nucleus and in mitochondria and between these mRNAs and enzyme activity. Gagnon *et al.* (1991) reported such a correlation in six rat tissues (ventricle, liver, m. soleris, m. plantaris, and white and red portions of m. gastrocnemicus) and similar results have been described by Hood *et al.* (1989) in rat heart, brain, liver, kidney, soleus muscle, and superficial white vastus muscle. The conclusion of the authors is that although tissue-specific regulatory processes also operate, the steady-state expression of nuclear and mitochondria respiratory subunit genes is coordinately regulated and these data suggest that the regulation is achieved by pretranslational mechanisms.

As already mentioned (Section II), glucocorticoids and, in particular, thyroid hormones have profound effects on the biogenesis (number and size) and the function of mitochondria, best studied in liver and muscle. These effects are dependent on the developmental stage of the animal and the organ examined. Mitochondrial respiration, oxidative phosphorylation, and ion translocation are processes regulated by these hormones (Wakat and Haynes, 1977; Allan *et al.,* 1983). The effects on energy metabolism are in part the result of induction of subunits of respiratory enzymes encoded both by the mitochondrial and the nuclear genome. Rachamin *et al.* (1995) found that dexamethasone induces several mitochondrial genes in the distal colon of rats, four encoding subunits of cytochrome *c* oxidase (COXI, II, and III) and one the 16 S mitochondrial ribosomal RNA. These authors observed that dexamethasone also increases the abundance of COXIV, a nuclear-encoded subunit of cytochrome oxidase, to a similar extent as to that of the mitochondrial-encoded subunits. Although the hormone leads to increased levels of COXI mRNA

in colon and skeletal muscle, no increase was detected in brain and heart. The effects on the mitochondrial enzymes of oxidative phosphorylation are correlated with the effects of dexamethasone on Na^+ reabsorption and the resulting increased energy needs of the colon epithelium. Djouadi *et al.* (1994, 1996) reported an effect of glucocorticoids on mitochondrial biogenesis of developing kidney and a coordinate transcriptional regulation by glucocorticoids of the nuclear-encoded medium chain acyl-coenzyme A (CoA) dehydrogenase and mitochondrial malate dehydrogenase in this organ. In hepatoma H-35 cells, 12 hr after addition of dexamethasone, there is an increased transcription of cytochrome *b*, COXI, and of 16 S genes (Kadowski and Kitagawa, 1988). van Itallie (1992) observed in cell cultures an increased COXIII and cytochrome *b* mRNA 3 days after dexamethasone administration, but no stimulation of the nuclear-encoded $COXV_a$ subunit. Recently, Weber *et al.* (2002) demonstrated an effect of dexamethasone on mitochondrial biogenesis in skeletal muscle of rats as well as on differentiated mouse C2C12 muscle cells, i.e., marked stimulation of mitochondrial DNA transcription and increased levels of cytochrome *c* oxidase subunit II and III mRNA, and of the activity of the cytochrome *c* oxidase. That the elevated respiratory chain activity is used for enhanced aerobic metabolism was demonstrated by the decreased lactate production of the cells. The effect of dexamethasone on cytochrome *c* oxidase subunit III mRNA was not seen in colon and kidney, whereas decreased levels of this mRNA in liver and H_4-II-E hepatoma cells was observed. This is in contrast to the already mentioned findings of van Itallie (1992) and Kadowski and Kitagawa (1988) in cell cultures; among other factors, the discrepancy could be the result of strain differences, different serum lots, and different cell confluence. The stimulatory effect of dexamethasone was abolished by RU486, implying glucocorticoid receptor mediation. Although the effect is receptor dependent, the varying responses of the different tissues to dexamethasone regarding mitochondrial transcription imply that additional permissive factors are needed for the full expression of the hormonal effect.

Induction of several liver mitochondrial genes has been shown by Mutvei *et al.,* (1989) after thyroid hormone administration to hypothyroid rats. The steady-state level of all mitochondrial mRNAs is higher after TH application (Gadaleta *et al.,* 1975, 1986, 1989; Mutvei *et al.,* 1989; van Itallie, 1990). In neonatal rats, 12 S, 16 S, and mRNAs for COXIII, COXIV, and ND 1, 4, 5, and 6, are induced by T_3 (Mutvei *et al.,* 1989; van Itallie, 1990; Wiesner *et al.,* 1992, 1994; Iglesias *et al.,* 1995).

Using a quantitative fluorescent cDNA microarray to identify novel hepatic genes regulated by thyroid hormone, Feng *et al.* (2000) prepared fluorescent-labeled cDNA from hepatic RNA of T_3-treated and hypothyroid mice and hybridized it to a cDNA microassay representing 2225 different genes. They showed that 55 genes are regulated by the hormone, 45 of which were not previously known to be regulated by T_3. Fourteen genes were regulated positively and 41 negatively. The genes affected encoded for proteins with diverse functions,

e.g., gluconeogenesis, lipogenesis, signaling by adenylate cyclase and insulin, cell proliferation, and apoptosis.

Several nuclear genes encoding subunits of respiratory enzymes are induced by thyroid hormones, such as those for cytochrome *c* and c_i, β-FiATPase, COXIV, COXV, COXVI c_1, ATPase, and succinate reductase (Scarpulla *et al.,* 1986; Luciakova and Nelson, 1992; Dümmler *et al.,* 1996; Müller and Seitz, 1994; Izquierdo *et al.,* 1995; Joste *et al.,* 1989). T_3 induction of mRNA for COXIV, Va, VIc, cytochrome *c*, cytochrome c_1, and dicyclohexylcarbodiimide (DCCD) binding subunits of ATPase reaches a maximum at 12–36 hr. However, two genes are rapidly induced by T_3, i.e., rat liver mitochondrial glycerol-3-phosphate dehydrogenase, 4–6 hr after hormone administration to hypothyroid neonatal rats (Dümmler *et al.,* 1996), and the β-F_1-ATPase subunit, 1 hr after hormonal induction (Izquierdo and Cuezva, 1993).

B. The Mitochondrial Genome of Mammals

An exemplary eukaryotic cell contains 1000–2000 mitochondria. The mammalian mt genome is a circular double-stranded molecule of about 16 kb (human: 16.5 kb, rat 16.2 kb) (Fig. 7) (Anderson *et al.,* 1981; Bibb *et al.,* 1981). Each cell contains 10^3–10^4 mitochondrial genome copies compared with two copies of the nuclear-encoded single copy genes. The two strands of the circular mtDNA chromosome

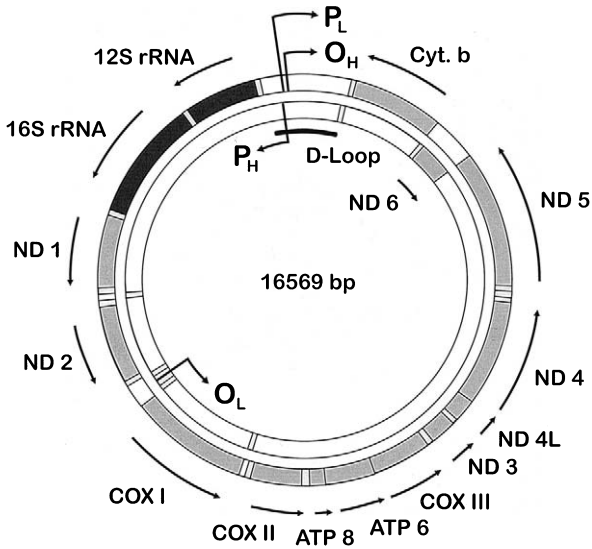

FIG. 7 The mitochondrial genome.

have an asymmetric distribution of purines and pyrimidines generating heavy (H) and light (L) strands. Each strand is transcribed from one predominant promoter, PL and PH, located on the control region, which includes a displacement (D) loop (Montoya *et al.,* 1982; Chang and Clayton, 1984; Clayton, 1991). The D-loop region is the only mitochondrial DNA segment that does not encode RNA or proteins and the only region of transcription initiation. There is basically no available intergenic space that allows regulation of gene expression, so that the modulation of gene expression is apparently confined to the D-loop. However, the possible presence of intragenic regulatory sequences offer possibilities for regulation of other transcription steps, i.e., elongation, termination, and release (Christianson and Clayton, 1986). The genome is transcribed by a specific mitochondrial RNA polymerase in large 16-kb polycistronic mRNA molecules that are subsequently processed at the sites of transfer RNA (tRNA) coding genes to give mitochondrial messenger RNAs (mRNAs), ribosomal RNAs (rRNAs), and tRNAs (Ojala *et al.,* 1981). The mRNA encodes 13 subunits of the mitochondrial energy-generating pathway, oxidative phosphorylation (OXPHOS), i.e., three subunits of cytochrome oxidase (COXI, II, and III), seven subunits of NADH-CoQ reductase (ND 1–7), one subunit of cytochrome *b* and two subunits of ATP synthase (ATP synthase 6 and 8), two ribosomal RNAs (12 S and 16 S rRNA), and 22 tRNAs. With the exception of ND 6 and Glu, Ala, Cys, Tyr, and Pro-tRNAs, which are generated from the L-strand transcripts, all other RNAs are processed molecules from the H-strand. Transcription is bidirectional and proceeds around the circle. A transcription factor (mt-TFA) required for high levels of transcription initiation was isolated, able to form transcription-competent preinitiation complexes, and was purified to homogeneity (Fisher and Clayton, 1988; Larsson *et al.,* 1996, 1998; Shadel and Clayton, 1993). Very probably another factor exists (mTFB), in analogy to the one that has been identified in *Xenopus* mitochondria (Antosheckin and Bogenhagen, 1995). The mitochondrial transcription factor TFA is a high mobility group DNA-binding protein with two DNA-binding domains and a carboxy-terminal tail essential for transcription. The fact that the rRNA genes, which are situated in the promoter-proximal region of the H-strand, are transcribed at a rate 15–60 times higher than the most distally located H-strand protein-coding genes (Gelfand and Attardi, 1981) suggested the occurrence of transcription attenuation at the 16 S rRNA/leucyl-tRNA border (Montoya *et al.,* 1983). Indeed, a mitochondrial transcription termination factor (mt-TERF) has been isolated, involved in the termination of the smaller strand transcription unit responsible for the synthesis of the two rRNAs. This factor interacts with the 3′-end of the 16 S rRNA (Kruse *et al.,* 1989). In footprinting assays, the factor protects a 28-bp segment immediately adjacent and downstream of the 16 S rRNA/leucyl-tRNA boundary. This termination factor has been cloned and expressed *in vitro,* the expressed protein binds as a monomer to DNA, has the expected specific binding capacity for the double-stranded oligonucleotide containing the tridecamer sequence, required for directing termination, and produces a DNase I footprint similar to that produced

by the natural protein. However, it has no transcription termination-promoting activity in an *in vitro* system, suggesting the need for additional factor(s) in this process (Fermandez-Silva *et al.,* 1997).

Genome comparison between α-proteobacteria and mitochondrial genomes of protists and yeast supports the endosymbiont hypothesis, i.e., that mt genomes are descendants of α-proteobacteria (Margulis, 1970; Kurland and Andersson, 2000). It seems that the ancestral mt genome lost massive amounts of genes, not essential to life, within the eukaryotic cytosol and that many mt genes have been duplicated in the nucleus. The circular nature of the mt genome, the absence of histones, the polycistronic transcripts, the 12 S and 16 S rRNAs, among others, suggest a bacterial type of transcription mechanism, possibly also of transcription regulation (see Section IV.E). It seems that mt transcription is not as efficient as nuclear transcription, therefore the larger number of mt genes per cells. In some developmental physiological conditions, the number of mitochondria increases (reflecting the need for higher respiration, as in oocytes); thus, interpretation of mRNA levels should consider both gene dosage and transcriptional activation (see Section IV.F).

Animal mt-mRNAs lack significant 5′- and 3′-untranslated nucleotides. The start codon is generally located three nucleotides from the 5′-end of the mRNA. No poly(A) tail is posttranscriptionally added.

C. Glucocorticoid and Thyroid Hormone Receptors in Mitochondria

The possible presence of glucocorticoid- (Ulrich, 1959; Venuto *et al.,* 1962) and thyroid hormone-binding sites (Sterling and Milch, 1975) in mitochondria was first reported at a period prior to the isolation of the receptors and the sequencing of their genes. After cloning of the genes and characterization of the receptor proteins and with the advent of more sensitive molecular and cell biological techniques for the detection of receptors, e.g., polymerase chain reaction (PCR), immunoblotting, immunocytochemistry, confocal laser scanning microscopy, and immunogold electron microscopy, definite proof for the presence of GR and TR in mitochondria has been provided.

1. Glucocorticoid Receptors

Mitochondrial uptake of radioactivity after incubation of rat liver and muscle mitochondrial homogenates with radioactively labeled glucocorticoids was observed by Ulrich (1959) and Venuto *et al.* (1962), respectively. Beato *et al.* (1969), after administering [^{3}H]cortisol to adrenalectomized rats, recovered in liver most of the radioactivity in the cytosol, with smaller amounts in nuclei and mitochondria. Although most of the radioactivity present in the cytosol could be accounted for by cortisol metabolites, the majority of the nuclear as well as the mitochondrial radioactivity was in the form of nonmetabolized cortisol, as demonstrated by

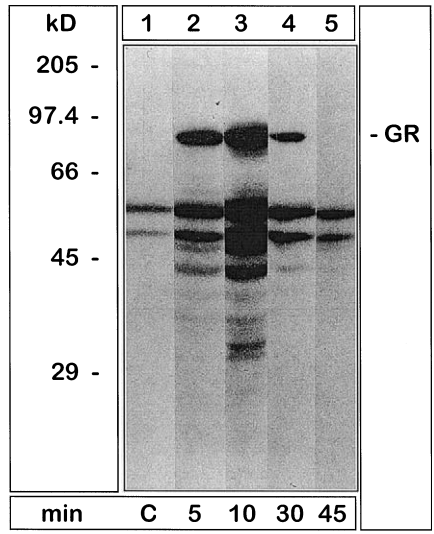

FIG. 8 Presence of glucocorticoid receptor in rat liver mitochondria and time-dependent translocation from the cytoplasm. Adrenalectomized rats were injected with dexamethasone (10 μg/100 g) and sacrificed 5, 10, 30, and 45 min thereafter (lanes 2–5, respectively; lane1, control animal not treated with dexamethasone). Following isolation, trypsinization (10 ng trypsin/μg protein) and SDS treatment of mitochondria, aliquots of 150 μg of mitochondrial proteins were analyzed by 9% SDS–PAGE, Western blotted with anti-GR polyclonal antibody, and autoradiographed. The position of molecular weight markers is shown. Reprinted from Demonakos *et al.* (1993). Import of the glucocorticoid receptor into rat liver mitochondria in vivo and in vitro. *J. Steroid. Biochem. Mol. Biol.* **46,** 401–413, with permission from Elsevier Science.

thin-layer chromatographic analysis, suggesting that in addition to the nucleus, the mitochondria could also be a site of primary action of glucocorticoids. Later, Demonakos *et al.* (1993), applying cell fractionation, immunoblotting, and electron microscopy immunolocalization techniques, demonstrated the presence of glucocorticoid receptor in liver mitochondria of adrenalectomized rats subjected to dexamethasone treatment. In the absence of hormone, most of the GR was found in the cytosol, with only traces in the mitochondria. Within minutes after administration of inducing doses of dexamethasone, the receptor appeared in the mitochondria, with maximal accumulation after 10 min, returning to preinduction levels after 45 min (Fig. 8). In addition to the intact 95-kDa GR, smaller molecular weight polypeptides, reacting with a GR antibody, were present in the mitochondria, possibly representing either artifactual degradation products, physiological turnover products, or intramitochondrially active GR species.

The transport of GR into the mitochondria was studied in an in organello system (Demonakos *et al.,* 1993). GR was synthesized in a reticulocyte lysate programmed by rat GR-mRNA in the presence of [^{35}S]methionine. Purified rat liver mitochondria were assayed for the presence of GR by immunoblotting mitochondrial

extracts subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Within 2 min, a substantial amount of GR was recovered in the mitochondria, in a temperature-dependent process. Mitochondrial import of proteins involves a multicomponent, energy-dependent transport machinery (Murakami *et al.,* 1990; Hartl and Neupert, 1990; Pfanner, 1998; Hoehler *et al.,* 1999; Bauer *et al.,* 2000). Most of the mitochondrially targeted proteins are characterized by an amino-terminal amphipatic helix sequence, which is cleaved by matrix proteinases. No such classic sequence in the NH_2-terminal part of GR has been detected and apparently the imported GR is not cleaved in the translocation process. A deletion GR mutant, lacking 20 amino acids from the amino-terminal part, as well as GR mutants lacking the NLS are efficiently transported (Demonakos, 1995). Mitochondrial localization of GR has also been demonstrated by Moutsatsou *et al.* (2001) in rat brain using a methodology similar to that of Demonakos *et al.* (1993). These authors detected the intact 95-kDa GR in brain cortex mitochondria, synaptosomes, and synaptosomal mitochondria. In addition, smaller molecular weight polypeptides reacting with GR antibodies were found, which they interpreted in a way similar to that of Demonakos *et al.* (1993) for the findings with rat liver mitochondria, described above.

a. Localization of Glucocorticoid Receptors in Mitochondria Although the localization of steroid receptors, particularly of GR, has been analyzed in numerous immunofluorescence studies (Papamichail *et al.,* 1980; Picard and Yamamoto, 1987; Akner *et al.,* 1990, 1995; Brink *et al.,* 1992; Hsu *et al.,* 1992; Jewell *et al.,* 1995; van Steensel *et al.,* 1995; Htun *et al.,* 1996) their presence in mitochondria so far escaped detection. The failure to detect steroid receptors in mitochondria in these earlier studies might be due to the fact that conventional immunofluorescence techniques do not have the necessary power of resolution to clearly identify individual mitochondria. Futhermore, it was uncertain whether the structure of the mitochondria was maintained applying the paraformaldehyde Triton X-100 fixation generally used. Scheller *et al.* (2000) tested the effect of different fixation and permeabilization conditions on the structural integrity of mitochondria, as well as their accessibility to antibodies. Mitochondria were labeled by exposure of HeLa cells to the vital dye Mitotracker CMXRose (CMX), which is taken up by the cells and specifically incorporated into mitochondria (Whitaker *et al.,* 1991). After methanol/acetone fixation, the mitochondria were clearly visible as red fluorescent dot-like or slightly elongated structures, often enriched in juxtanuclear regions (Fig. 9A–C). Cell nuclei were not stained by the drug. Double labeling with mouse monoclonal antibodies against human cytochrome oxidase subunit I corroborated the specificity of the CMX staining and, furthermore, demonstrated the accessibility of the mitochondria to antibodies. (The cytochrome oxidase complex is embedded in the inner mitochondrial membrane.)

In striking contrast, when the cells were fixed with formaldehyde followed by permeabilization with Triton X-100, mitochondria could not be visualized, either by CMX staining or by labeling with COXI antibodies. The mitochondria were no

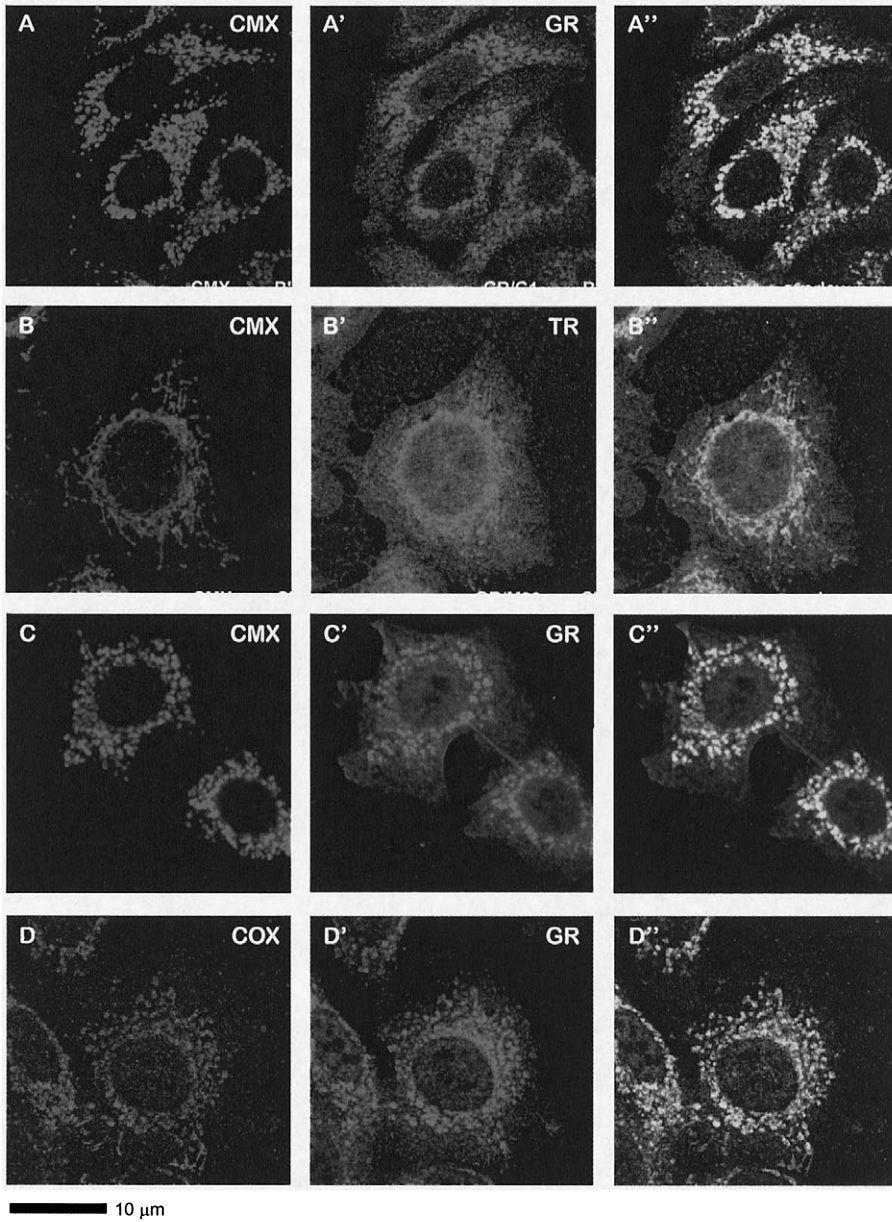

FIG. 9 Localization of glucocorticoid and thyroid hormone receptors in mitochondria of human cells. (A, B, D) HeLa cells. (C) Hep-2 cells. Cells were treated with CMX. Subsequently, the methanol-acetone-fixed specimens were incubated with an anti-GR antibody (A′, C′, D′) or an anti-TR antibody (B′) followed by FITC-conjugated secondary antibodies. (A″ and C″) Colocalization of GR with CMX. (B″) Colocalization of TR with CMX. (D″) Colocalization of GR with COXI. M20 was visualized by Texas-red-conjugated anti-rabbit Ig and COXI by anti-mouse IgG coupled to FITC. (A″–D″) Merged images. Scale bar = 10 μm. From Scheller *et al.* (2000). Localization of glucocorticoid hormone receptor in mitochondria of human cells. *Eur. J. Cell Biol.* **79,** 299–307, with permission from Urban & Fischer Verlag. (See also color insert.)

longer seen as distinct entities and the CMX fluorescence signal was almost uniformly distributed throughout the cytoplasm, most likely due to the disintegration of the mitochondria. The loss of distinct mitochondrial labeling was due to Triton treatment and not to formaldehyde fixation. Formaldehyde fixation followed by permeabilization with acetone gave results similar to methanol-acetone treatment. When HeLa cells were treated in the same way and incubated with GR antibodies, the fluorescence was concentrated in the nucleus. The nucleoplasm but not the nucleoli fluoresced, whereas the cytoplasm was hardly stained above background level.

For localization of GR and TR, Scheller *et al.* (1998, 2000) used methanol/acetone-treated HeLa and Hep-2 cells and monoclonal GR antibodies, which have been described in detail (Westphal *et al.,* 1982, 1984). Further a commercially available rabbit polyclonal antibody, GR-M20 (Santa Cruz, Heidelberg), was used that recognizes an epitope corresponding to amino acids 5–20 at the N-terminus and is known to react with human and other mammalian GR (Rogatzki *et al*, 1998; Sheppard *et al.,* 1998). For localization of TR a commercially available TRα1 antibody (Santa Cruz, Heidelberg) was used that has been shown to react with human TRα1 and TRβ1.

All GR antibodies and the TR antibody tested stained both the nuclei and the cytoplasm (Fig. 9A′–D′). Optical sections revealed a distinct fluorescence of the nucleoplasm, often with several brighter foci surrounding the nonstained nucleoli. In addition to a general cytoplasmatic labeling there was a striking concentration of the fluorescence signal in numerous perinuclear dot-like structures in a pattern resembling the distribution of mitochondria (Fig. 9A′–D′). In fact, the mitochondrial marker CMX produced a nearly coincident staining pattern (Fig. 9A–C, merged images A″–C″). Occasionally, the mitochondria in the cell periphery fluoresced less or not at all with the GR antibodies as compared with the juxtanuclear mitochondria. The specificity of the GR antibody M20 was tested by preincubation with specific blocking peptides. Under such conditions, the fluorescence signal was reduced to background levels and the mitochondria no longer stained. When the cytoplasmic localization of GR with the mitochondrial enzyme cytochrome oxidase subunit I (COX) was compared, an essentially coincident punctate labeling pattern of the cytoplasm in both cell types (Fig. 9A″–D″) was observed. Together, these results indicate that a substantial fraction of the cytoplasmic GR is concentrated in the mitochondria of HeLa and Hep-2 cells. From the results obtained with GR-M20, which recognizes an epitope at amino acids 5–20 at the N-terminus, it is concluded that the GR is imported uncleaved.

Essentially identical results were obtained with other cell lines, such as Chinese hamster ovary (CHO), mouse 3T3 and LTK$^-$, rat RV, and rat kangaroo PTK2 cells. In striking contrast, however, COS-7 cells were almost negative after staining with GR antibodies. This result is consistent with the known low GR content of these cells (Torchia *et al.,* 1997) and, furthermore, underscored the specificity of the antibodies used.

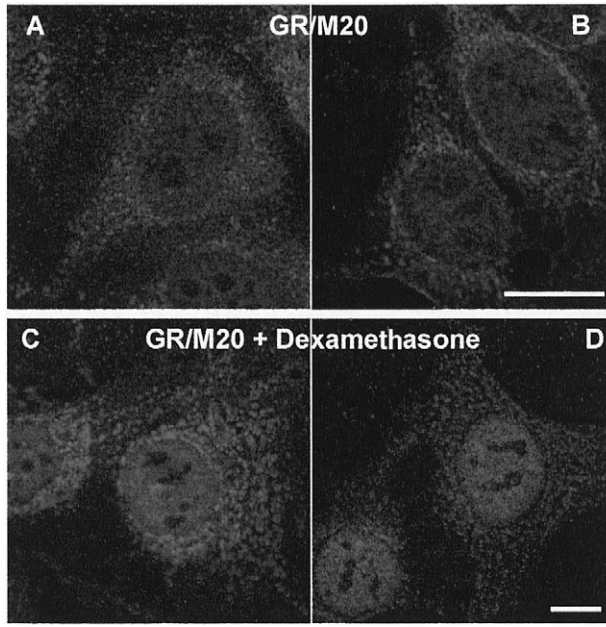

FIG. 10 Effect of dexamethasone on the distribution of GR in HeLa cells. Cells were treated with 1 μM dexamethasone (final concentration) for 15 min and then subjected to immunofluorescence with GR-M20 antibodies. (A, B) Nontreated; (C, D) dexamethasone-treated cells. Hormone treatment leads to nuclear accumulation of GR. Scale bar = 10 μm. From Scheller *et al.* (2000). Localization of glucocorticoid hormone receptor in mitochondria of human cells. *Eur. J. Cell Biol.* **79,** 299–307, with permission from Urban & Fischer Verlag. (See also color insert.)

Upon administration of dexamethasone to HeLa cells grown in serum-free medium, the nucleoplasmic fluorescence increased substantially after staining with GR-M20, indicating that the hormone caused a translocation of receptor proteins into the nuclei (Fig. 10) (Papamichail *et al.,* 1980; DeFranco *et al.,* 1995; Guichon-Mantel *et al.,* 1990; van Itallie, 1992). Interestingly, the mitochondria remained positive after staining with the GR antibodies and now stood out with special clarity against the almost nonfluorescent cytosolic space. As far as can be concluded from these experiments there is no one-way shift of GR from the mitochondria into the cytoplasm.

Most cytological studies have focused on the translocation of the steroid receptors from the cytoplasm to the nucleus and their intranuclear distribution. Cytoplasmatic "clusters" of GR were described by van Steensel *et al.* (1995) but not further characterized. In view of the results of Scheller *et al.* (2000) these "clusters" could represent GR localized in mitochondria. Furthermore, these results now offer an explanation for the reported enrichment of GR in perinuclear regions (Akner *et al.,* 1995; Hsu *et al.,* 1992) that mirrors the high density of mitochondria in this zone. The presence of the GR in perinuclear mitochondria might

reflect the role of glucocorticoids in energy production in the mitochondria and energy provision to the nucleus. As stated by Scheller *et al.* (2000), the percentage of the immunostained mitochondria varied from cell to cell and preliminary results suggest a correlation between GR immunostaining and growth conditions, as well as with the cell cycle phase (Fejes-Tóth *et al.,* 1998).

The presence of GR in mitochondria could be verified by immunogold electron microscopy on ultrathin cryosections of shock-frozen cells. Gold particles were found throughout the cytoplasm but also in the inner space of the mitochondria. The labeling density was higher over mitochondria than in the cytoplasm (Scheller *et al.,* 2000).

b. Green Fluorescent Protein-Tagged Glucocorticoid Receptor To analyze the distribution of GR in living cells, GFP-GR fusion proteins were expressed in HeLa cells using the vector pK7-GFP containing GR-cDNA (Carey *et al.,* 1996). After dexamethasone treatment, a substantial amount of the GFP-tagged receptor accumulated in the nucleoplasm of the HeLa or Hep-2 cells used. The GFP-GR remaining in the cytoplasm was not uniformly distributed throughout the available space but rather was concentrated in the mitochondria, which were visualized by CMX. When GFP was expressed alone as a control, the mitochondria appeared as "black holes" within the uniformely fluorescent cytoplasm. Thus, GFP per se is excluded from the mitochondria and it is the GR moiety that targets the fusion protein to the mitochondria of the living cells (Scheller *et al.,* 2000).

Using localization techniques as described above, Koufali (2002) and Koufali *et al.* (2002) and Psarra *et al.* (2001, 2002) demonstrated the mitochondrial localization of GR in C_6 glioblastoma cells as well as in Müller cells of the lizard retina, which have mitochondria tightly packed in distinct cell compartments, respectively. Koufali *et al.* (2002) treated C_6 glioblastoma cells cultured as monolayers, with 3.5% paraformaldehyde and 0.1% saponin and incubated the fixed and permeabilized cells with antibodies to the NH_2-terminal or carboxy-terminal part of GR and with a second antimouse immunoglobulin G (IgG) coupled to fluorescein isothiocyanate (FITC). In confocal microscopy, fluorescence was observed mainly in the cytoplasm. Double labeling with anti-COXI antibodies or with mitotracker CMX revealed the localization of the GR in mitochondria.

The appearance of GR in mitochondria was also examined by means of immunoblotting (Scheller *et al.,* 2000) (Fig. 11). In Western blots of total cell extracts, the monoclonal antibodies G1 and G2 as well as the polyclonal antibodies E20 and M20 specifically recognized a polypeptide band with an apparent molecular mass of approximately 95 kDa (Fig. 11, lanes t). Bands with the same mobility were also detected in preparations of nuclear (n), mitochondrial (m), and cytosolic (c) fractions. Upon protease treatment of mitochondria and cytosol, the cytosolic receptor was completely digested (Fig. 11C, lane cp) whereas the enzyme did not affect the GR in the mitochondrial fraction (Fig. 11C and D, lanes mp). When the mitochondria were first disintegrated by ultrasonic treatment and successively

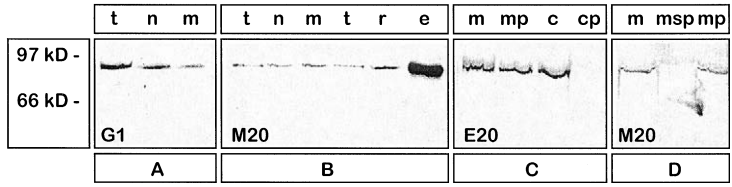

FIG. 11 Detection of GR by Western blots in HeLa (A, B) and Hep-2 cells (C, D) probed with the monoclonal GR antibodies G1 (A) and the polyclonal antibodies M20 (B, D) or E20 (C). Total cell extract (t), isolated nuclei (n), cytosol fraction (c), cytosol treated with protease (cp), isolated mitochondria (m), mitochondria treated with protease (mp), sonicated mitochondria treated with protease (msp), rat liver cytosol (r), translation product of GR-cDNA expressed in a baculovirus system (e). Note that the GR is absent in protease-treated cytosol (C, lane cp) and mitochondria which were sonicated and digested with protease (D, lane msp).

treated with protease, the GR band dissappeared from the gel (Fig. 11D, lane msp). When probed with rat liver proteins, the antibody also reacted with a band of approximately 95 kDa (Fig. 11B, lane r) corresponding to the molecular mass of rat GR protein (Evans, 1988; Picard and Yamamoto, 1987). Finally, a recombinant GR protein that was expressed in insect Sf9 cells after transfection with rat GR-cDNA exhibited a band migrating at the same position as GR (Fig. 11B, lane e).

2. Thyroid Hormone Receptors

High-affinity T_3 binding sites were first identified by Sterling and Milch (1975) in the mitochondrial inner membrane. These findings were confirmed by Goglia *et al.* (1981) and Hashizume and Ichikawa (1982) who demonstrated that the mitochondrion is a major compartment of T_3 accumulation. Similarly, an early mitochondrial uptake of [^{123}I]T_3 administered to mice has been shown. Sterling *et al.* (1984a,b) detected a mitochondrial receptor for T_3 of $M_r = 28$ kDa localized at the organelle's inner membrane. Sterling (1986, 1991) also reported high-affinity binding of T_3 by adenine nucleotide translocase purified from beef heart mitochondria and also by the carrier protein in intact beef mitochondria. Morel *et al.* (1996) also studied the kinetics of the internalization and specific subcellular binding of radiolabeled T_3 in mouse liver and confirmed binding of the hormone to mitochondria.

The alkylating T_3 derivative ^{125}I-labeled *N*-bromoacetyl-T_3 (BrAc[^{125}I]T_3) and its thyroxine analogue (BrAcT_4), which was used for the identification of TH-binding proteins of the nucleus, nuclear envelope, endoplasmatic retinculum, and plasma membrane, was applied to detect mitochondrial T_3-binding protein by Rasmussen *et al.* (1989). Incubation of rat liver mitochondria with BrAc[^{125}I]T_3 resulted in the highly selective labeling of a protein doublet with an apparent $M_r = 45$ kDa. Several arguments made it attractive to suggest that this protein doublet represented mitochondrial creatine kinase (mt-CK) and that interaction of thyroid hormones with mt-CK was of physiological importance. The mt T_3-binding proteins were localized in the mitochondrial inner membrane and coincided therefore in location with mt-CK, which is attached to the outer surface of the inner

membrane. As thyroid hormones influence and regulate the energy metabolism of the cell, mt-CK fulfills all preconditions to be an attractive site of regulation of cellular energy metabolism. In contrast to their cytosolic counterparts, mt-CK isoenzymes are thought to be displaced from thermodynamic equilibrium and thus are under kinetic control. Regulation of the enzyme activity of mt-CK by thyroid hormones could thus directly influence the flux of high-energy phosphates out of the mitochondria (Wyss *et al.,* 1993).

Evidence for the presence of α- and β-related T_3 receptors in rat liver mitochondria has been produced by Ardail *et al.* (1993). Five minutes after [^{125}I]T_3 injection in rats, specific binding sites could be detected in liver mitochondria, assessed by ultrastructural autoradiography. The presence of two T_3-binding proteins with $M_r = 53$ kDa and $M_r = 48$ kDa, respectively, were revealed in mitochondria, which the authors correlated to the rapid T_3 mitochondrial effects.

In addition to T_3 binding to mitochondria, Lanni *et al.* (1994), Leary *et al.* (1996), and Goglia *et al.* (1994b) demonstrated specific binding sites for 3,5-diiodo-L-thyronine (3,5-T_2) in this organelle, sites of which could be mediators of the metabolic effects of 3,5-T_2.

Definite proof for the mitochondrial localization of TR was provided by Wrutniak, Cabello, and co-workers (Wrutniak *et al.,* 1995). Using the photoaffinity labeling technique, they showed the presence of three T_3-binding proteins in rat liver mitochondrial extracts, one with $M_r = 43$ kDa, one with $M_r = 41$ kDa, and a third one with $M_r = 28$ kDa (Fig. 12). No 30-kDa binding protein could be detected, indicating that the ADP/ATP translocator does not bind T_3, as also concluded by the inability of the purified translocator to be labeled with [^{125}I]T_3-PAL. Subsequently, applying the immunoblotting technique using antibodies against the T_3 nuclear receptor cErbAα_1, the same group identified two proteins, with molecular weights the same (p43 and p28) as those observed with photoaffinity labeling (Fig. 12). Mitochondrial subfractionation revealed that the p43 was localized in the mitochondrial matrix, whereas p28 was mainly found in the membrane fraction. Further studies of the same group (Wrutniak *et al.,* 1995) showed that p43, in contrast to full length c-ErbA T_3 nuclear receptors, is imported into mitochondria in an in organello system. Two major steps in the import process is translocation through the outer and inner membranes driven by the mitochondrial membrane potential and unfolding of the protein to an active conformation involving ATP-dependent interactions with mitochondrial heat shock proteins. That these steps are not involved in the import of p43 is demonstrated by the lack of their inhibition both by FCCP, which decreases the mitochondrial membrane potential, and by apyrase, which depletes ATP and ADP stores. The protein is rapidly imported, maximal intramitochondrial levels being attained after 15 min (Fig. 13).

In transfection experiments using simian CV_1 cells, moderate expression of p43 displayed a predominantly mitochondrial localization, as demonstrated by cytoimmunofluorescence (Fig. 14), whereas overexpression led to the presence of the protein also in the nucleus, suggesting that mitochondrial p43 import is a saturable process (Casas *et al.,* 1999).

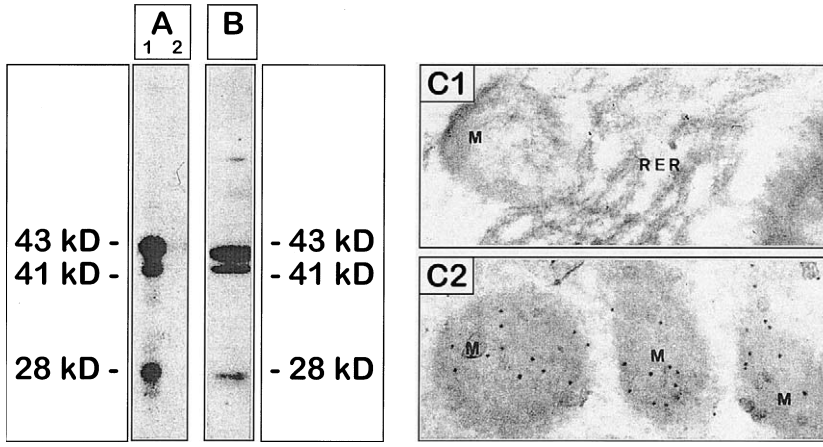

FIG. 12 A truncated c-ErbAα1 protein is located in rat liver mitochondria. (A) Photoaffinity labeling of mitochondrial T_3-binding protein using [^{123}I]T_3-PAL. T_3-PAL labeling was performed without (lane 1) and with (lane 2) a previous incubation of mitochondrial proteins with a 1000-fold molar excess of cold T_3-PAL in order to assess labeling specificity. (B) Western blot of highly purified mitochondrial proteins using an antibody raised against c-ErbAα. Tests of specificity indicate that only the 28- and 43-kDa proteins are related to c-ErbA. (C) Electron microscopic view (×42,000) of rat liver mitochondria showing a specific staining of the organelles by an antibody raised against c-ErbA. C1, preimmune serum; C2, specific antiserum. From Wrutniak *et al.* (1998). Physiological importance of the T3 mitochondrial pathway. *Ann. N.Y. Acad. Sci.* **839,** 93–100.

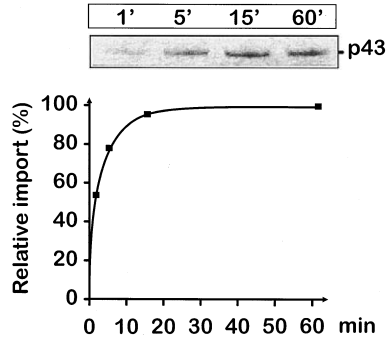

FIG. 13 Import of p43 in isolated rat liver mitochondria. Time-related changes in the amount of labeled p43 imported into mitochondria and relative p43 import expressed as the percentage of the value recorded after 60 min of incubation. Mitochondrial proteins were collected after increasing times of incubation at 30°C. From Casas *et al.* (1999). A variant form of the nuclear triiodothyronine receptor c-ErbA 1 plays a direct role in regulation of mitochondrial RNA synthesis. *Mol. Cell. Biol.* **9,** 7913–7924, with permission from American Society for Microbiology.

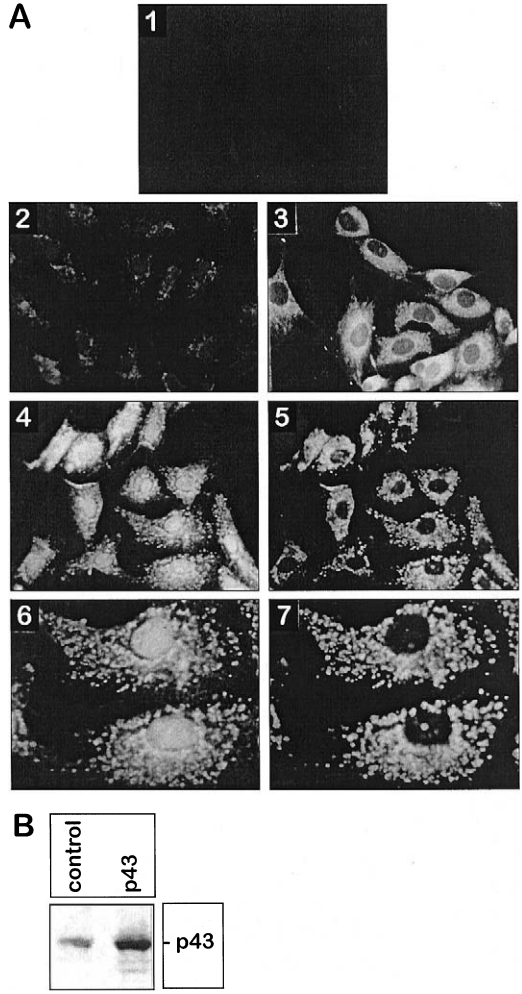

FIG. 14 Import of p43 in mitochondria of CV1 cells stably transfected with plasmid encoding p43 and assayed by cytoimmunofluorescence. p43 mitochondrial import occurs according to a saturable process. (A) 1, staining of CV1 cells with a c-ErbA-preabsorbed RHTH antiserum (antibody raised against c-ErbA; final dilution, 1/100); 2, staining of control CV1 cells transfected with empty vector; 3–7, staining of CV1 cells overexpressing p43 after stable transfection; 2–4, staining with rabbit RHTH antiserum (final dilution, 1/100); 3, mild p43 overexpression level leads to a major mitochondrial p43 location; 4, high p43 overexpression level leads to simultaneous mitochondrial and nuclear p43 locations; 5, staining with an antibody raised against a mitochondrial antigen (Anti-Mitok; final dilution, 1/30) (same microscopic field as in 4) (magnification, ×400); 6 and 7, magnification of microscopic fields 4 and 5 demonstrating colocalization of the two antigens. (B) High p43 overexpression in CV1 cells. Western blot experiments were performed with RHTH antiserum; 100 μg of proteins were loaded into each lane. From Casas *et al.* (1999). A variant form of the nuclear triiodothyronine receptor c-ErbA 1 plays a direct role in regulation of mitochondrial RNA synthesis. *Mol. Cell. Biol.* **9,** 7913–7924, with permission from American Society for Microbiology.

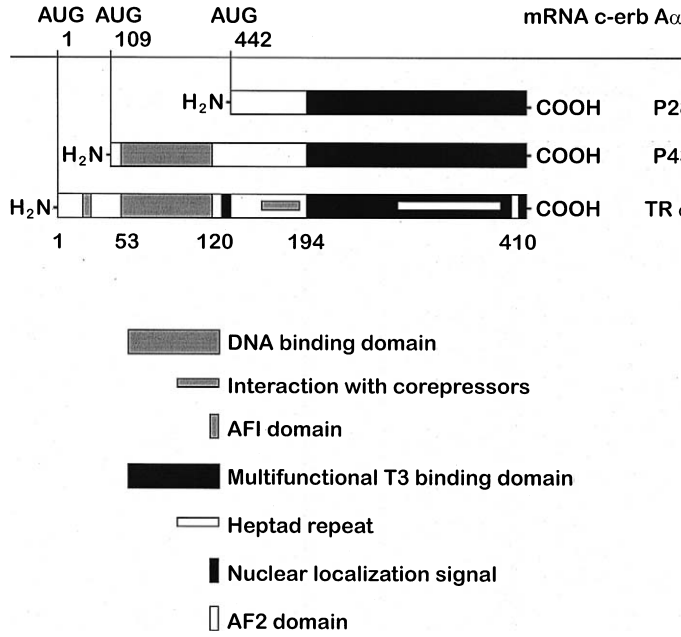

FIG. 15 The p28 and p43 isoforms of c-ErbAα_1. The two isoforms (p28 and p43) are synthesized by alternative translational initiation at internal AUG in the message encoding the full-length TR. Whereas p28 is detected in the mitochondrial inner membrane, p43 is localized in the matrix of the organelle where it stimulates mt genome transcription in the presence of T_3 (Wrutniak *et al.,* 1995; Casas *et al.,* 1999). TRα, T_3 nuclear receptor c-ErbAα_1 (47 kDa); 1, 109, and 442, number of nucleotides on the transcript (1 = A of the first AUG); 1, 53, 120, 194, and 410, number of amino acids on the c-eErbAα_1 receptor. From Wrutniak-Cabello *et al.* (2001), reproduced by permission of the Society for Endocrinology.

The p28 form has been identified as a species resulting from alternative translational initiation at the internal AUG of c-ErbAα_1 (Fig. 15). This protein, like p43 and in contrast to the intact c-ErbAα_1 receptor, is actively transported into the mitochondria. Peptide p43 represents a second truncated form of c-ErbAα_1, resulting from alternative translational initiation at another internal AUG. Neither p28 nor p43 could be found in nuclei. p43 has affinity to the ligand similar to the full length c-ErbAα_1 protein and, in contrast to p28, harbors the DNA-binding domain. As both p28 and p43 are actively transported into mitochondria the question of the process involved in the mitochondrial import of these receptors is raised. No putative mitochondrial localization signals have been detected in p43. However, deletion of the DNA-binding domain abrogates import, emphasizing the importance of this sequence. p28, which lacks the DNA-binding domain, is imported, but only in the presence of T_3. Perhaps the hormone acts by unmasking mitochondrial localization sequences.

Using the immunofluorescence technique applied for the detection of GR receptors in mitochondria of HeLa and Hep2-cells, Scheller *et al.* (1998) detected thyroid hormone receptor in mitochondria of the same cells. In these studies most of the receptor was found localized in the cytoplasm with only small amounts being present in the nucleus (Fig. 9B–B″).

D. Hormone Response Elements (HREs) in the Mitochondrial Genome

The identification of glucocorticoid and thyroid hormone receptors in mitochondria raised the question as to their possible interaction with mitochondrial DNA. Due to the fact that the mt genome of several mammals has been completely sequenced (Anderson *et al.,* 1981; Clayton, 1991), a search for sequences, similar to the nuclear HREs, in the mt genome was feasible and led to the detection of several such putative HREs (Ioannou *et al.,* 1998; Sekeris, 1990). As shown in Tables I and II, responsive elements, either full palindromes or half-sites for glucocorticoids and mineralocorticoids, androgens and progesterone, and for estrogens and thyroid hormones, were detected. The putative HREs were located at various sites of the mitochondrial genome, in the D-loop region, the regulatory part of the genome, some within the ribosomal subunit genes, between 16 S rRNA and the leu-tRNA genes, and some within structural genes.

TABLE I

Sequences Showing Partial Similarity to GRE (Class I HREs) Consensus Sequence Detected in the Human (H), Mouse (M), and Bovine (B) Mitochondrial Genome[a,b]

		GGTACA	NNN	TGTTCT		Consensus sequence
(H)	1195	AGAGGA	NNN	TGTTCT	1209	12 S rRNA
	3228	AACAAA	NNN	TGTTCT	3242	16 S rRNA/tRNA$^{Leu(UUR)}$
	4102	TAACCT	NNN	TGTTCT	4116	ND 1
	16489	CCGACA	NNN	GGTTCC	16503	D-Loop
(M)	614	AGAGGA	NNN	TGTTCT	628	12 S rRNA
	2742	CCCTAA	NNN	TGTTCT	2756	tRNA$^{Leu(UUR)}$
	6018	ACCAGC	NNN	TGTTCT	6032	COXI
	6455	TTTCCA	NNN	TGTTCT	6469	COXI
	6709	ACTAAC	NNN	TGTTCT	6723	COXI
	9177	GGTATC	NNN	GGTTCT	9191	COXIII
	15799	CAGACA	NNN	GGTTCT	15793	D-Loop
	16161	CAAACC	NNN	TGTCCT	16176	D-Loop
(B)	16257	CAGGCA	NNN	TGGTCT	16271	D-Loop

[a] From Ioannou *et al.* (1988) and Demonakos *et al.* (1996).

[b] The numbers refer to the position of the sequence in the genome. Denoted also are the genes in which the response elements have been detected.

TABLE II

Sequences Showing Partial Similarity to ERE and TRE (Class II HREs) Consensus Sequences Detected in the Mouse Mitochondrial Genome[a,b]

	AGGTCA	NNN	TGACCT		Consensus sequence
318	CGGTCA	TAC	GATTAA	334	12 S rRNA
748	AGGTCA	AGG	TGTAGC	764	12 S rRNA
2107	CAGTGA	AAT	TGACCT	2123	16 S rRNA
2246	CGGTTG	GGG	TGACCT	2262	16 S rRNA
3848	AGGTCA	GCT	AATTAA	3864	$tRNA^{Gln}$
5449	AGGTCA	ACC	AGGTGC	5465	COXI
5746	AGCATC	AGT	AGACCT	5762	COXI
8942	GGAGGC	TGC	TGACCT	8958	COXIII
10838	GGGTCA	ATA	ATTCTA	10854	ND 4
12018	TTGTCA	CAT	GATCAA	12034	ND 5
14185	CTCATT	CAT	TGACCT	14201	Cytochrome *b*
14652	AAGCCA	CCT	TGACCC	14668	Cytochrome *b*
15178	TATTAT	CAT	TGGCCA	15194	Cytochrome *b*
15514	AGGTCA	TAA	AATAAT	15530	D-Loop

[a] From Ioannou *et al.* (1988) and Sekeris (1990).

[b] The numbers refer to the position of the sequences in the genome. Denoted also are the genes in which the response elements have been detected.

The group of Cabello has confirmed the presence of TRE-like sequences in the 12 S RNA, 16 S RNA, and COXII genes and in the D-loop region (two TREs) (Wrutniak *et al.,* 1998; Casas *et al.,* 1999) (Table III). Similarly, Iglesias *et al.* (1995) confirmed the presence of a TR specific binding site in the mitochondrial ND3 gene, whose mRNA is decreased in cerebral cortex and liver of hypothyroid rats and is induced by T_3.

To further characterize these sequences as HREs, gel retardation and transfection experiments were performed. Tsiriyotis *et al.* (1993) carried out gel retardation assays in which two putative glucocorticoid responsive elements (GREs) (GREI and GREII, see Table IV) interacted with the COXI gene of the mitochondrial genome. The substrates were chemically synthesized and reacted with mitochondrial and nuclear extracts from MCF7 and $LATK^-$ cells untreated or subjected to dexamethasone treatment. As control GRE they used the human metallothionein II_A promoter. They could demonstrate that the cell extracts contained protein(s) that bind to the oligonucleotides used and that this binding was considerably enhanced when the extracts were derived from the dexamethasone-treated cells (Fig. 16). Demonakos *et al.* (1992, 1996a,b) tested six putative GREs (GRE I–IV and GRα and β) as specific binding sites for GR in gel shift assays and indeed demonstrated binding of the receptor to these sequences. Similar results

TABLE III

TRE-Like Nucleotide Sequences in the Rat Mitochondrial Genome[a,b]

Nucleotide sequence	TRE type	Gene	Position
AGGTCAAGGTGT	DRO[c]	12 S rRNA	748–760
CGACCTatttaagAGGTGA	Ipal7[c]	16 S rRNA	2374–2392
TGACCTaaaaccAGGTGA	Ipal6	COX II	7367–7384
TGCCTTcctcaacatagccgtcAAGCA	TRE RSV[c]	D-Loop	15909–15936
AGGCATgaAGGTCA	DR2[c]	D-Loop	15932–15945

[a] From Wrutniak *et al.* (1998).

[b] The number of the first and last nucleotide of each sequence is given according to Gadaleta *et al.* (1998).

[c] Sequences tested in gel-retardation experiments showing specific binding of p43 DR, direct repeat; Ipal, inverted palindrome; TRE RSV, TRE identified in the genome of Rous sarcoma virus.

were obtained in gel shift assays by the group of Cabello, using mitochondrial extracts partially purified on heparin-agarose and ^{32}P-labeled putative TREs mentioned above (in the D-loop and within the 12 S and 16 S rRNA genes) (Wrutniak *et al.,* 1998; Casas *et al.,* 1999). In gel shift experiments Braliou *et al.* (1995) also demonstrated binding of TR to a putative TRE present in the COXIII subunit.

TABLE IV

The Chemically Synthesized Nucleotide Sequences Containing the Putative Mitochondrial GREs Present in the COXI Gene, mtGREI and mtGREII[a]

mtGREI 6018–6032						
5′- AGCTT		ACCAGCATCTGTTCT	GGATCC	A	-3′	[F]
3′-	A	TGGTCGTAGACAAGA	CCTAGG	TTCGA	-5′	[R]
		GR	*Bam*HI			
mtGREII 6455–6469						
5′- AGCTT		TTTCCACTATGTTCT	GGATCC	A	-3′	[F]
3′-	A	AAAGGTGATACAAGA	CCTAGG	TCCGA	-5′	[R]
		GR	*Bam*HI			
hMT II_A GRE						
5′- AGCTT		GGTACACTGTGTCCT	GGATCC	A	-3′	[F]
3′-	A	CCATGTGACACAGGA	CCTAGG	TCCGA	-5′	[R]
		GR	*Bam*HI			

[a] From Tsiriyiotis *et al.* (1993, 1997).

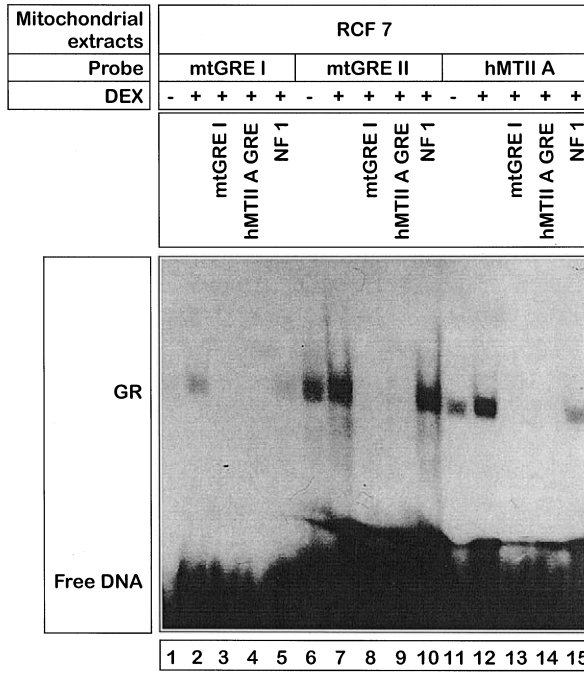

FIG. 16 Binding of mitochondrial proteins to mt-GREs. Electrophoretic mobility shift analysis using mt COXI GREI (mtGREI), mtCOXI GREII (mtGREII), and hMTII$_A$GRE oligonucleotides and mitochondrial extracts from MCF7 cells not treated and treated with 1 μM dexamethasone. Binding to mtGREI, lanes 1–5; to mtCOXGREII, lanes 6–10; and to hMTII$_A$GRE, lanes 11–15. Nontreated cells, lanes 1, 6, and 11; dexamethasone treated, lanes 2, 3, 4, 5, 7, 8, 9, 12, 13, 14, and 15. Competition with mtCOXIGREI, lanes 3 and 14; with mtCOXIGREII, lane 8; with hMTII$_A$GRE, lanes 4, 9, and 13; and with NF1, lanes 5, 10, and 15. mtCOXIGREI and mtCOXGREII, putative GREs in the cytochrome oxidase gene. hMTII$_A$GRE, GRE in the human metallothionine gene promoter. NF1, nuclear factor 1. For the sequence and positioning of the putative GREs in the mitochondrial genome, see Table IV. From Tsiriyotis *et al.* (1993) with permission.

To support the concept that the putative GREs have characteristics of ligand-activated enhancers, transfection experiments were performed with a reporter gene, chloramphenicol acetyltransferase (CAT) linked to putative mt GREs. Chemically synthesized oligonucleotides (27-mers) containing the GRE II sequence (Table IV) were inserted into the plasmid p^{ct}11 carrying the thymidine kinase promoter linked to the CAT gene. LATK$^-$ cells were stably transfected with the plasmids and exposed to dexamethasone. It was shown that dexamethasone induces CAT activation and that the induction is inhibited by the glucocorticoid antagonist RU38486, supporting the role of the GRE II as glucocorticoid receptor-activated enhancer (Tsiriyotis *et al.,* 1997; Demonakos *et al.,* 1996b) (Fig. 17). Although the expression of the CAT gene is taking place in a nuclear environment, these results strongly favor the HRE function of these mitochondrial sequences.

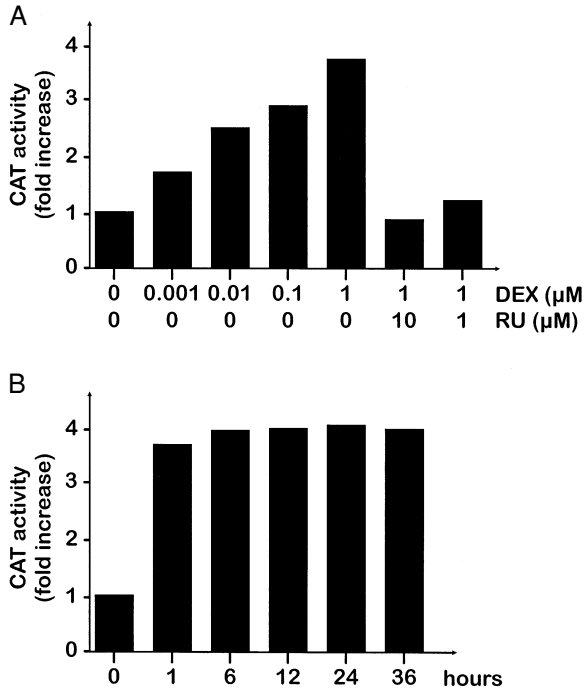

FIG. 17 GR induces a dexamethasone-dependent stimulation of the activity of a reporter gene driven by mitochondrial GREII. (A) CAT assay histogram representing the dose–response of the LACT11 cell line to dexamethasone and the effect of the antiglucocorticoid RU38486. The cells were harvested 24 hr after dexamethasone or RU38486 administration. The LACT11 cell line contains the GREII of the cytochrome oxidase subunit I mitochondrial gene upstream of the HSV-1 thymidine kinase gene promoter. (B) CAT assay histogram representing the time–response of the LACT11 cell line to 1 μM dexamethasone. From Tsiriyotis *et al.* (1997). The mitochondrion as a primary site of action of glucocorticoids: Mitochondrial nucleotide sequences, showing similarity to hormone response elements, confer dexamethasone inducibility to chimaeric genes transfected in LATK-cells. *Biochem. Biophys. Res. Commun.* **235,** 349–354, with permission from Academic Press.

Similar results with T_3 have been obtained by Casas *et al.* (1999) in transient transfection assays using QM7 cells with reporter genes driven by the mitochondrial D-loop or by two mt TREs located in the D-loop. p43 induced an increase in mitochondrial reporter gene transcription, but only in the presence of T_3.

The presence of the HRE-like sequences in the mitochondrial genome raises a question as to their origin. The possibility that they could be of nuclear origin being incorporated into the mt genome by translocation from the nucleus is highly improbable, as such translocations are rare exceptions (Attardi, 1985). Taking into account the endosymbiont hypothesis of origin of the mitochondria (Margulis, 1970; Yang *et al.,* 1985) and the high rate of mitochondrial gene mutations (Jacobs, 1988)—several times more frequent than these of the respective nuclear genes—the

putative mt HREs could have been generated during the evolutionary adaptation of mitochondrial physiology and regulation to that of the cell (Jacobs *et al.,* 1988). Another possibility could be that the bacterial symbionts already possessed such sequences in their genomes before being incorporated in their hosts. The detection of nucleotide sequences in both eubacteria and archaea (Table V) with a high similarity to nuclear consensus class I and class II HREs and to the mitochondrial putative HREs (Hatzoglou and Sekeris, 1997) favor this possibility. It is interesting to note that several of the bacterial genes that contain the HRE-like sequences encode proteins involved in energy production, in replication, and in transcriptional control.

E. Hormone Effects on Transcription in Isolated Mitochondria

The wealth of data amassed on the action of glucocorticoids and thyroid hormones on mitochondrial RNA and protein metabolism referred to in Section IV.A led to the conclusion that the hormones regulate transcription and perhaps also the stability of the transcripts. Furthermore, the hormones act indirectly, by way of induction of nuclear genes encoding mitochondrial transcription, and other regulatory factors.

The development of an in organello mitochondrial system capable of maintaining DNA transcription for several hours was instrumental in evaluating the possible direct action of steroid and thyroid hormones on this process (Enriquez *et al.,* 1996). The authors demonstrated that the processing of the RNA precursors and the stability of the mature rRNAs, but not transcription itself, are severely impaired after short periods of incubation of mitochondria, indicating that these processes are strongly dependent on the mitochondrial interaction with the nucleocytoplasmic compartment. However, the events that lead to the synthesis, processing, and turnover of the mt-mRNAs do not require the continuous supply of nucleocytoplasmic factors that are accumulated in excess by mitochondria. Provided that ADP plus oxidizable substrates are added to the system, allowing endogenous ATP synthesis, transcription is maintained in the in organello system in the absence of nuclear gene transcription, rendering the system autonomous for several hours. High ATP levels exert an inhibitory action on the mitochondrial RNA polymerase and these authors propose that mRNA synthesis can be regulated in response to changes in intramitochondrial ATP levels thus adjusting the optimal levels of mRNA to local energy demands. This in organello system has been exploited by Enriquez *et al.* (1999a,b), and the group of Cabello applied a different in organello system (Casas *et al.,* 1999; Wrutniak-Cabello *et al.,* 2001; Ostronoff *et al.,* 1996) to demonstrate direct effects of T_3 on mitochondrial transcription.

Enriquez *et al.* (1999a) isolated mitochondria from liver of euthyroid and hypothyroid rats and allowed them to perform *de novo* synthesis of RNA. The *in vitro* system reflected the *in vivo* situation as regards the thyroid hormone status. In

TABLE V

Sequences Found in Bacterial Genes with Strong Similarity to Class I and Class II HRE[a]

Bacterium	Gene	Accession number in EMBL data library	Sequence	No. of sequence
		Class I HREs		
Bacillus licheniformis	5A2 divlB	BLUO1958	GGTACAgaaTGTTCg	1823–1837
Bacillus subtilis	168 trrnD	BSTGTRDND	cGTACAagcTGTTCT	2141–2155
Prevotella disiens	ATCC 29426-16ss ribosome	PVORR16SS	GGTACAgaaTGTTgg	1243–1257
Klebsiella pneumoniae	rmpA	KPRMPA	GGTACAaaaTGTTaa	713–727
Bacillus thuringiensis	IS231D	BTIS23ID	GGTACAgaaTGTTta	636–650
Mycoplasma capricolum	CONTIG MC007	MC007	ctTACAaatTGTTCT	777–791
Vibrio cholerae	Tox T transcriptional activator	VCTCPFTOX	acTACAaaaTGTTCT	1371–1385
Lactococcus lactis	Ree A-like functions	LLRECA	GGTACAaaaTGTTtg	3259–3273
		Class II HREs		
Haloferax sp.	Gyrase subunit A and B	HSGYRB	AGGTCAtcaTGACCg	5610–5624
Pseudomonas putida	gyrB gene for gyrase	PPGYRB	AGGTCAtcaTGACCg	398–412
Pseudomonas siringae	Penicillin-binding protein	PSPONA	AGGTCAaaaTGACCg	2300–2314
Caulobacter crescentus	DNA gyrase subunit B	CC592	AGGTCAtcaTGACCc	405–419
Mycobacterium tuberculosis	Cosmid TBC2	MTO24	tGGTCAagcTGACCT	2728–2742
Bacillus subtilis	Penicillin-binding protein	BSPBPF	AGGTCAgaaTGACaT	396–410
Neisseria gonorrhoeae	pilB	NGI7PILB	ccGTCAaagTGACCT	989–1003
Mycobacterium leprae	Cosmid B229	ML020	AGGTCAaaaTGACtg	20420–20434
Azotobacter vinelardii	Hox and hyp operons	AVHOXHYP	cGGTCAacaTGACCa	1973–1987
Paracoccus denitrificans	Cytochrome c_{550}	PDCYT550	tGGTCAagaTGACCg	552–566
Echerichia coli	β-Hydroxydecanoylthio ester dehydrase	ECFABAA	tGGTCAaaaTGACCg	229–243

[a] From Hatzoglou and Sekeris (1997). The detection of nucleotide sequences with strong similarity to hormone responsive elements in the genome of eubacteria and archaebacteria and their possible relation to similar sequences present in the mitochondrial genome. *J. Theor. Biol.* **184,** 339–344, with permission from Academic Press LTD.

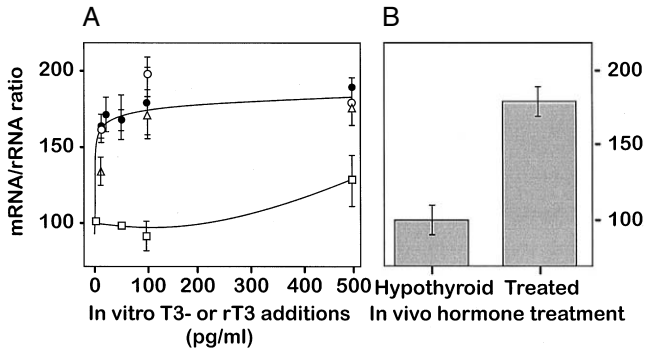

FIG. 18 Effect of thyroid hormone addition to the incubation medium on in organello mRNA synthesis. Comparison of the *in vivo* effect of T_3 and the *in vitro* addition effect of T_3 or rT_3 on the in organello mRNA/rRNA synthesis ratio. (A) Average change in the mRNA/rRNA ratio after addition of T_3 in the three independent experiments (Δ, ○, ●) or rT_3 (□) to the incubation medium. The differences in the mRNA/rRNA ratio at any concentration of T_3 added to the incubation medium compared with no T_3 were significant ($p < 0.001$) by Student's *t* test. The difference in mRNA/rRNA ratio at 500 pg of rT_3/ml compared with no addition was significant ($p < 0.01$) by Student's *t* test. (B) Effect of the *in vivo* treatment of hypothyroid animals with T_3 on the mRNA/rRNA ratio. The difference was significant ($p < 0.01$) by Student's *t* test. From Enriquez *et al.* (1999). Direct regulation of mitochondrial RNA synthesis by thyroid hormone. *Mol. Cell. Biol.* **19,** 657–670, with permission from American Society for Microbiology.

mitochondria from hypothyroid animals, as *in vivo,* a 1.85-fold reduction in the overall transcription rates, a 1.6-fold reduction in the mRNA/rRNA ratio, and a two-fold increase in mRNA stability are observed. Administration of T_3 to the animals restores the mRNA/rRNA ratio and this effect can be reproduced by the direct addition of T_3 to the isolated hypothyroid mitochondria (Fig. 18). The dimethyl sulfate footprinting patterns of mitochondria derived from euthyroid and hypothyroid rats show conspicuous differences at the transcription initiation sites but not at the termination factor (mTERF)-binding region. These differences can be abolished by the addition of T_3 to the mitochondria, which apparently acts on the initiation of the transcriptional process and this effect is very probably mediated by the thyroid receptor.

Casas *et al.* (1999) incubated rat liver mitochondria with the p43 truncated form of the c-ErbAα1 receptor synthesized in a reticulocyte system and subsequently measured mitochondrial transcription (Fig. 19). Using a COXIII probe, they could detect on Northern blots two precursor transcripts >14 kb, which were only faintly detectable in mitochondria incubated in the absence of p43. In addition, the precursor transcripts induced by p43 were also detected in Northern blots performed with other mt gene probes (ND1, 2, 4, 5, 6L, COXI, II, ATPase 6, cytochrome *b*). No precursor transcript reacting with 12 S and 16 S rRNA probes could be found. The addition of T_3 to the system induced a strong rise in the precursor transcript levels within 5 min. Similar effects could be observed in the absence of p43

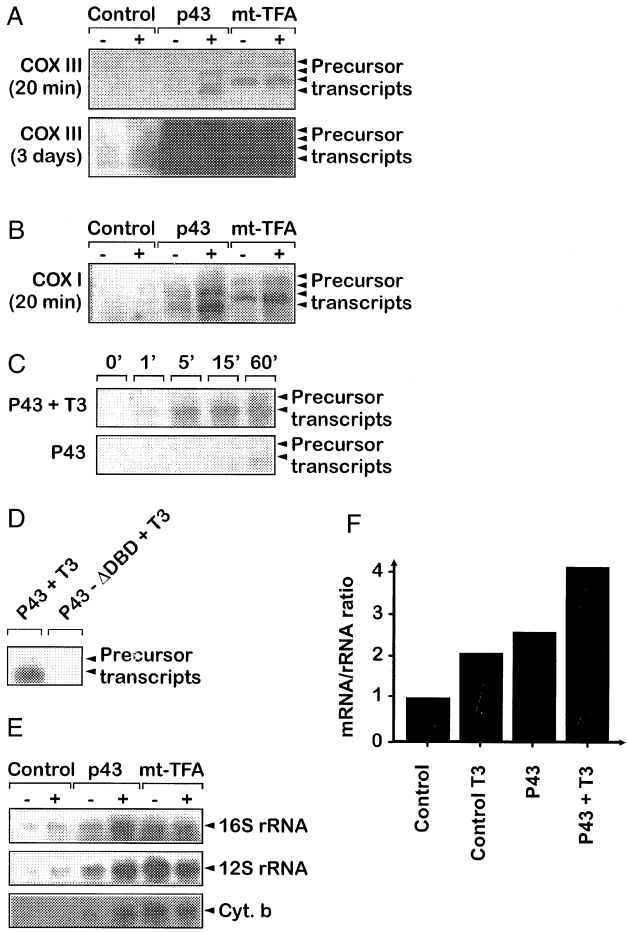

FIG. 19 p43 induces a T_3-dependent increase in mitochondrial transcript levels. (A–F) In organello transcription experiments were performed as described by Ostronoff *et al.* (1996) with purified rat liver in the presence of 2% rabbit reticulocyte lysate (unprogrammed lysate for control mitochondria, p43, or mt-TFA) and incubated at 37°C for 60 min. When indicated, 10^{-8} *M* T_3 was added to the incubation medium. Transcription experiments were stopped by cooling on ice. Mitochondria were collected by centrifugation and mitochondrial RNA was extracted twice at room temperature. (A–D) After in organello transcription experiments, precursor transcript were detected by Northern blotting with the indicated mitochondrial probes. Duration of film exposure is indicated under the probe. (A and B) Precursor transcript levels recorded after 60 min of in organello transcription. (C) Time-related changes in precursor transcript levels in the presence of p43 (0 or 10^{-8} *M* T_3). Transcription experiments were performed in the presence of cold p43 and incubated at 37°C for increasing times. (D) Transcription experiments were performed in the presence of cold p43 or p43-ΔDBD and then incubated at 37°C for 60 min with T_3 (10^{-8} *M*). (E) Northern blot experiments were performed with agarose stab gels containing deionized CH_3H_gOH after in organello transcription studies. Mature transcripts are detected by hybridization with the indicated mitochondrial probes after 60 min of in organello transcription. (F) Influence of T_3 and/or p43 on the value of the mitochondrial mRNA/rRNA ratio. The ratios were obtained after densitometric analysis of cytochrome *b* and 12 S plus 16 S rRNA. From Casas *et al.* (1999). A variant form of the nuclear triiodothyronine receptor c-ErbA 1 plays a direct role in regulation of mitochondrial RNA synthesis. *Mol. Cell. Biol.* **9,** 7913–7924, with permission from American Society of Microbiology.

provided that mt transcription factor A was present. Although no 12 S or 16 S rRNA precursor transcript could be detected, mature ribosomal RNAs were found in increased amounts after addition of p43 to the in organello system. The ratio of mRNA/rRNA was increased up to 4-fold under these conditions. The authors suggest that the extent and rapidity of the increase in precursor transcript levels make the possibility of effects on RNA stability unlikely. The failure of a mutated p43 lacking the DNA-binding domain (p43-ΔDBD) to stimulate precursor transcript accumulation indicates that p43-DNA binding is absolutely necessary to activate transcription.

As already mentioned, the precursor transcripts induced by p43 do not contain the genes for the 12 S and 16 S rRNAs. The presence of a putative TRE in the 16 S rRNA gene suggests that initiation of transcription in the presence of p43 could start at this site, accounting for the lack of the ribosomal transcripts. In the in organello system, the addition of mt-TFA also led to an increase in precursor transcript levels and, in the presence of T_3, to an increase in the levels of mature transcripts. Preliminary evidence has been presented that p43 and mt-TFA use different response elements of the D-loop.

The direct action of T_3 on transcription in the in organello mitochondrial system raises the question as to the molecular mechanism of the hormonal action. There are significant differences regarding the structure of the genome, the RNA synthesizing machinery, and the transcription process per se between the nucleus and the mitochondrion. Nuclear genes, each endowed with its own regulatory region, most having exons and introns, are organized in nucleosomal structures composed of DNA and the well-characterized histone octamer (Pruss *et al.,* 1996). Mitochondrial DNA is a tightly packed genome forming complexes with not well-characterized proteins (Fisher *et al.,* 1992) and possessing a single regulatory region (the D-loop). In contrast to the multiple nuclear RNA polymerases with specific functions, the mitochondrium harbors a single RNA polymerase. The nucleus is packed with several transcription factors, coactivators, histone-modifying enzymes, and other coregulators, whereas only a few transcription factors have to now been demonstrated in mitochondria. The nuclear genome is monocistronic, each individual gene transcribed and processed separately, whereas the mitochondrial genome is polycistronic, transcription starts at two promoters, each in one of the two DNA strands, the whole strands are read through, and the RNA transcripts are subsequently cleaved to produce the 38 RNA species. The mt genome and the RNA transcription process are obviously of a prokaryotic type and this should be taken into consideration when considering the possible role of the mitochondrial receptors on the mitochondrial transcription process. The hormone receptors could act as ligand-activated positive regulators, but also as repressors, dissociating from the D-loop initiation sites in the presence of the hormonal ligand or from other HREs interspersed in the mitochondrial genome, raising the possibility of control of transcript elongation and termination.

F. Coordination of Nuclear and Mitochondrial Gene Transcription

The energy-yielding capacity of the mitochondria is tuned to the organisms' needs, depending on growth and developmental states and on environmental influences. Various levels of adaptive regulation of OXPHOS genes are possible. Increase in gene dosage, i.e., mitochondrial biosynthesis, is a way to increase the oxidative capacity in conditions of considerable energy needs, as shown to be the case in chronic skeletal muscle stimulation in rat (Hood *et al.,* 1989) and rabbit (Williams, 1986), and in heart muscle of rats subject to aortic occlusion (Rajamanickam *et al.,* 1979; Wiesner *et al.,* 1994), although even under severe hyperthyroid conditions mitochondrial mass seldom increases much above the euthyroid level. An increase in transcription and OXPHOS-mRNA availability in the absence of mitochondrial biogenesis as well as effects on mRNA stability could lead to similar less intense and more rapidly adjustable effects and indeed mitochondrial genome expression can be specifically increased through mechanisms independent of mitochondrial genome abundance (Martin *et al.,* 1993). It should be mentioned, however, that in the mitochondrial compartment, in contrast to the cytosol, mRNAs are present in molar excess and to increase the synthesis of proteins encoded by mitochondrial DNA the availability of ribosomes is more important than of mRNAs, a situation that is observed in cardiac growth (Wiesner *et al.,* 1994). Allosteric activation of OXPHOS (Fig. 20) by metabolites, such as ADP, would even more subtly and rapidly adapt mitochondrial energy yield to cellular needs (see Enriquez *et al.,* 1999a,b).

One possibility for the elevated resting metabolic rates observed upon hormonal induction could be the increased expression of uncoupling proteins. UCP_3, expressed in skeletal muscle, is induced by TR (Barbe *et al.,* 2000; Gong *et al.,* 1997) and dexamethasone (Gong *et al.,* 1997). However, Weber *et al.* (2002) found no such effect of dexamethasone on UCP-3 in rat skeletal muscle or myotubules under conditions of increased RMR and stimulation of mitochondrial OXPHOS gene transcription.

The biosynthesis of mitochondria and the *de novo* formation of complexes of the respiratory chain necessitate coordination of nuclear and mitochondrial gene transcription and translation (Nagley, 1991). In steady-state conditions, COX activities and levels of representative mRNAs derived from the two genomes in several tissues are correlated and the ratio of nuclear and mitochondrial mRNA expression is constant and coordinated. However, in the case of hormonal regulation of oxidative metabolism and in the absence of mitochondrial replication and although both nuclear and mitochondrial gene products are needed for a correct assembly or activity of respiratory complexes [e.g., for Complex I, Hofhaus and Attardi (1993) and for Complex III, Attardi and Schatz (1988)], coordination of nuclear (Luciakova and Nelson, 1992) and mitochondrial transcription does not seem, and need not be, temporally tightly correlated, as the turnover and pool of

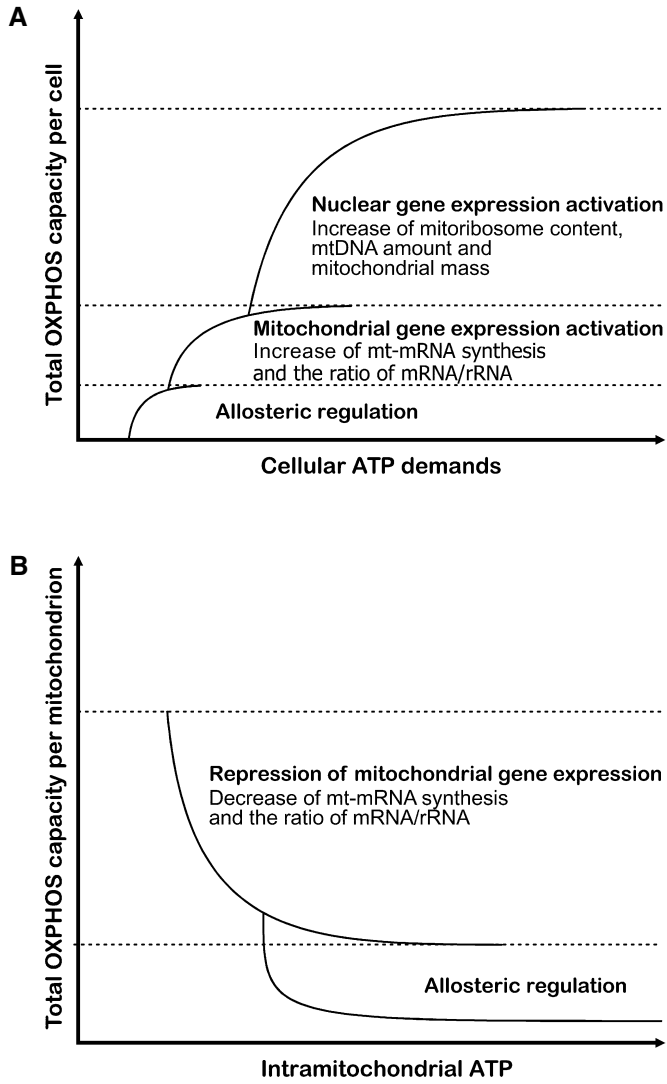

FIG. 20 Model for adaptive regulation of OXPHOS capacity. A model is proposed suggesting that different levels of regulation may allow adapting the OXPHOS capacity in response to changes in cellular ATP demands (A) or in the intramitochondrial ATP levels (B). These mechanisms could be sequentially activated: a first level of regulation would be allosteric activation of enzymes; when ATP demands were not covered by this activation, an increase in the expression of mt-mRNAs would further increase OXPHOS capacity; finally, a general activation of mitochondrial biogenesis involving nuclear-encoded genes would be triggered in those situtations requiring an increase in mitochondrial mass, like cell proliferation (A). In the mitochondrial compartment, programmed by the nucleus with a given transcription potential, repression mechanisms activated by a local increase in ATP concentrations would determine the level at which this potential is used in each individual organelle (B). From Enriquez *et al.* (1999). Autonomous regulation of OXPHOS capacity. *Biol. Chem.* **380,** 743–747.

the individual respiratory complex subunits differ. Thus, although T_3 induces both nuclear and mitochondrial genes encoding OXPHOS, the time sequence of their activation is different. T_3 induces transcripts for nuclear-encoded subunits IV, Va, and VI of cytochrome oxidase within 24 hr in rat liver, whereas the mitochondrial mRNAs were elevated with a considerable time lag. The reverse is true in the skeletal muscle (rapid increase of mitochondrial mRNAs and delay of accumulation of nuclear mRNAs). In every case, however, transcript levels are correlated with enzyme levels in both tissues, which conforms to a regulation of transcription as the main control mechanism by T_3 in liver and muscle (Wiesner *et al.,* 1992). The possibility of hormonal regulation of gene products controlling assembly or stability of mitochondria (Altamura *et al.,* 1996; Glerum *et al.,* 1995) would allow rapid adaptation to energy needs, a prerequisite being that the respiratory chain components are constitutively available.

In some cases, the hormone stimulates only the transcription of mitochondrial OXPHOS genes. As already mentioned, dexamethasone stimulates transcription of the genes encoding cytochrome *c* oxidase subunits II and III, but not of the nuclear-encoded subunit VI gene. As reported by Capaldi (1990) and Kadenbach *et al.* (1998), complex IV mitochondrial-encoded subunits alone are sufficient to catalyze the reduction of molecular oxygen and proton translocation.

Based on our knowledge of the molecular mechansims of steroid and thyroid hormone regulation of nuclear genes, it is possible to hypothesize that not only the nuclear but also the mitochondrially encoded OXPHOS are likely regulated. As regards nuclear genes, although more than 100 such genes encode subunits of OXPHOs, only nine are induced by T_3, and only in rare cases have sequences corresponding to TREs in their regulatory regions been detected (Pillar and Seitz, 1997). In only one case, that of the cytochrome c_1 gene, has the sequence proved by TR-DNA binding to be involved in T_3 regulation and to respond directly to thyroid receptor-mediated induction (Nelson *et al.,* 1995). These observations and the time period needed for manisfestation of the hormonal effect led Pillar and Seitz (1997) and Scarpulla (1997) to question a direct effect of T_3 on the majority of genes involved in oxidative phosphorylation.

The fact that many of the nuclear genes encoding subunits of Complex I, II, IV, and ATP synthase, whose products contribute to mitochondrial respiratory functions, possess binding sites (T/C GCGCA T/C CGC A/G consensus sequence) for one or both of the two transcription factors of OXPHOS genes, nuclear respiratory factor I (NRF-1) and nuclear respiratory factor 2 (NRF-2) (Watson *et al.,* 1995; Evans and Scarpulla, 1990; Virbasius and Scarpulla, 1991, 1994; Chau *et al.,* 1992; Virbasius *et al.,* 1993), moreover that NRF-1 expression in induced by T_3 (Pillar and Seitz, 1997), led to the hypothesis that T_3 regulates nuclear OXPHOS gene transcription by way of NRF-1 induction and that NRF-1 acts as the general transcription activator for these genes.

As mentioned in Section IV.B, mitochondrial transcription initiation is dependent on the presence of mitochondrial transcription factors A and B (TFA

and TFB), which are nuclearly encoded. Virbasius and Scarpulla (1994) made the important observation that the mt TFA gene possesses NRF-1 but also NRF-2-binding sites in its promoter, and that activity of mt TFA is dependent on binding of the nuclear factors on its promoter, providing a link between the expression of nuclear and mitochondrial genes. It is important to note that the mt TFA gene possesses a TRE in its regulatory region and is T_3 induced, indirectly linking T_3 action and mitochondrial gene transcription. However, as regards dexamethasone, no effect of the hormone on TFA gene transcription could be observed in rat muscle under conditions of increased mt gene transcription (Larsson *et al.,* 1993, 1997) and no glucocorticoid-responsive element was found in the rat and mouse mt TFA promoter using Mat Inspector Software (Weber *et al.,* 2002).

In addition to NRF, other factors linking nuclear and mitochondrial OXPHOS gene transcription have been discovered. Suzuki *et al.* (1990, 1991) showed the presence of similar regulatory sequences in the transcriptional regulatory region of human nuclear genes and in the D-loop of mitochondria. Two sequences, Mt_3 and Mt_4, located at the 5′-flanking region of human nuclear genes encoding subunits of the cytochrome bc_1 complex, are present also in the mitochondrial D-loop. Nuclear protein factors that recognize nuclear Mt_3 and Mt_4 *cis*-elements and the respective *trans*-acting protein regulatory factors are shared by the two genetic systems and these *trans*-acting factors are involved in the communication between the nuclear and mt genomes. Other relevant factors are the CREB element and the OXBOX–REBOX elements, *cis*-acting sequences mediating regulation by cellular redox status—found in several nuclear respiratory genes (Haraguchi *et al.,* 1994), including the promoter of the human muscle specific adenine nucleotide translocator 1 (ANT1) and the β-subunit of ATP synthase. Binding of proteins to these REBOX sequences in gel mobility assays is sensitive to the presence of T_3 or the reducing agent dithiothreitol (Chang *et al.,* 1992). In rats, ANT1, although possessing REBOX elements, is not induced by T_3, whereas ANT2, which does not have such elements, is induced by the hormone (Dümmler *et al.,* 1996). The scanning of the mt genome for putative binding sites for other regulatory molecules has revealed sequences homologous to ones present in the nuclear genome for several known transcription factors (Solakidi and Sekeris, submitted).

Coordination of nuclear-mitochondrial transcription also involves signals emanating from the mitochondria and directed to the nucleus, as demonstrated by the sensitivity of expression of nuclear genes to the functional state of the mitochondria. This interorganelle communication, called retrograde regulation, is beeing studied mainly in yeast. In this cell three genes (RTG1, RTG2, and RTG3) have been detected (Liao and Butow, 1993; Sekito *et al.,* 2000), two of which (RTG1, RTG3) encode basic helix–loop–helix leucine zipper transcription factors and one (RTG2) a protein of still unknown characteristics, which participate in the retrograde signaling pathway. Available data point to a role of the Rtg1 protein as both a positive and negative regulator of the retrograde response. A novel mechanism

of retrograde regulation has been demonstrated in the case of a mitochondrial ketogenic enzyme, 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoAS), a key enzyme in ketogenesis, whose biosynthesis is induced by the peroxisome proliferator-activated receptor (PPAR)α, a member of the nuclear receptor superfamily, acting at the level of gene transcription (Rodriguez *et al.,* 1994). Meertens *et al.* (1998) demonstrated that mHMG-CoAS can bind to (PPAR)α, by way of an LXXLL motif present in the enzyme, leading to potentiation of (PPAR)α-dependent transcriptional activation through the PPAR response element of the mHMG-CoAS gene.

The presence of glucocorticoid and thyroid hormore receptors in mitochondria of animal cells, described in Section IV.C considered in the context of the effects of these hormones on mt-RNA synthesis, was a strong indication that the respective hormones, by way of their receptors, could directly affect mitochondrial transcription. This hypothesis was further supported by the demonstration of the presence of putative GREs and TREs at various sites of the mitochondrial genome, hormone response elements that were shown to bind the respective receptors in gel shift assays and to confer hormone responsiveness in transfection studies, applying various constructs consisting of reporter genes linked to the HREs. These findings were in line with the molecular mechanisms of steroid/thyroid hormone action on responsive nuclear genes. The in organello mitochondrial system described in Section IV.E, which responds in a saturable way to the addition of very low amounts of thyroid hormone with increased transcription in the absence of exogenous nuclear/cytoplasmic factors and the indispensable need of the 43p mt-specific TR for this effect, provided direct proof of a primary action of T_3 on mitochondial transcription. Although initiation, on the basis of the dimethyl sulfate footprinting patterns, is the transcription stage affected by T_3, the placing of putative HREs for glucocorticoid hormone and TH within the structural mt genes, some in the boundary between the 16 S ribosomal gene and leu-tRNA (Demonakos *et al.,* 1996a,b) (Fig. 21), a transcription "arrest" site where the mtTERF interacts as a terminator of transcription (Kruse *et al.,* 1989), could suggest a role of these hormones on other stages of the transcription process. In liver (Gadaleta *et al.,* 1975) and other organs, the rates of synthesis and the steady-state levels of rRNAs are higher than the levels of each individual mRNA, due to premature termination of transcription after reading through the rRNA genes and this premature termination could be the target for the differential regulation of mitochondrial rRNAs and mRNAs.

The results of the studies mentioned above lead to the conclusion that mitochondrial gene transcription is subjected to hormonal regulation, both by signals emanating from the nucleus in response to a primary action of the hormones on nuclear genes encoding mitochondrial transcription factors and by the direct action of the hormones on mitochondrial genes by way of the mitochondrially localized hormone receptors (Fig. 22).

FIG. 21 Binding site of mitochondrial termination factor (mt-TERF). The region at the 16 S rRNA/Leu-tRNA interface of the mitochondrial genome protected by the mt-TERF (black region) (Kruse *et al.,* 1989) and the site of a putative GRE (gray region) (Demonakos *et al.,* 1996a,b).

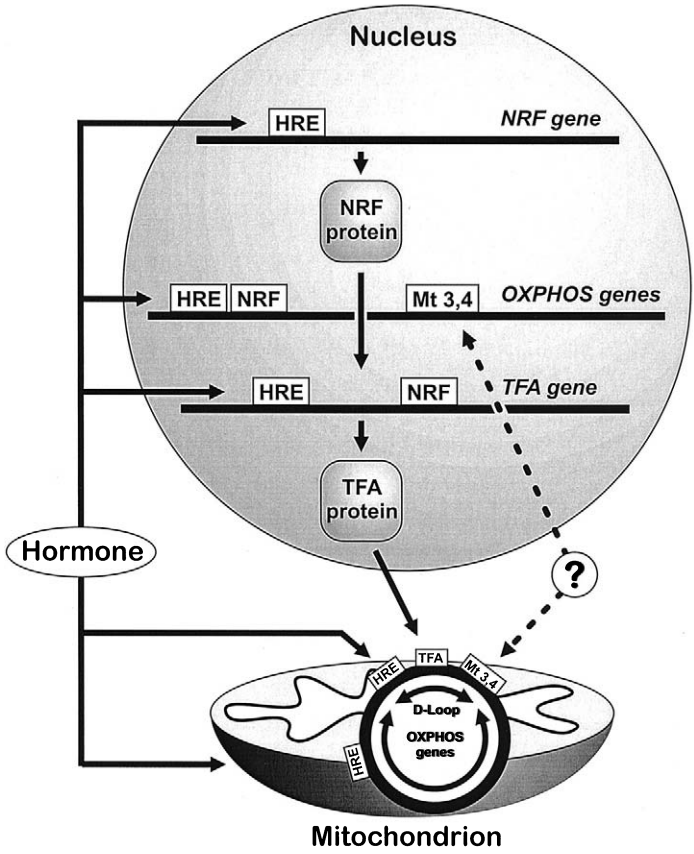

FIG. 22 Coordination of transcription of mitochondrial and nuclear genes encoding subunits of OXPHOS by steroid and thyroid hormones. Details are in the text (Section IV.F).

V. Concluding Remarks

The detection of glucocorticoid and thyroid receptors in mitochondria of animal cells and of nucleotide sequences in the mitochondrial genome, with similarity to hormone responsive elements, focused attention on the potential role of these receptors in mitochondrial gene transcription. Steroid and thyroid hormones regulate mitochondrial energy production in part through stimulation of OXPHOS gene transcription and the known molecular mode of action of the hormones on nuclear gene transcription involves binding of the hormone to cognate receptors and interaction with HREs.

This hypothesis has found experimental verification by the introduction by Enriquez *et al.* (1999a) and Casas *et al.* (1999) of in organello transcription systems, responding to exogenous TR with increased transcription rates by a receptor-mediated mechanism, under conditions that exclude nuclear participation. In contrast to the progress in understanding the fine mechanisms of nuclear gene transcription and in the identification of nuclear transcription factors, coactivators, and corepressors, the field of mitochondrial transcription has lagged behind; only a few transcription factors, such as mtTFA and mtTFB, and the mitochondrial transcription termination factor, mtTERF, have been identified as being important in sustaining the transcription and termination process, respectively. In analogy to the importance of physical interaction of the nuclear receptors with transcription factors and coregulators for transcription modulation, it is possible that similar interactions of the mitochondrial receptors with mitochondrial transcription factors could be significant for the modulation of transcription in mitochondria. The presence of HREs in the mt genome at sites other than the D-loop, e.g., at the 16 S rRNA-Leu-tRNA border, and within structural genes, raises the possibility of receptor involvement in other steps of the transcription process, in addition to transcription initiation.

In hypermetabolic states, precipitated by increased glucocorticoid or thyroid hormone levels, both nuclear and mitochondrially encoded OXPHOS are induced and the coordinate effect of these hormones on nuclear and mitochondrial OXPHOS gene transcription is observed. The presence of GRs and TRs in the regulatory regions of some, although not many, nuclear OXPHOS genes and in the D-loop can ensure a parallel increase of OXPHOS gene transcription in the two organelles by direct action of the respective receptors on the nuclear and mitochondrial genes through common hormone response elements. However, most of the nuclearly encoded OXPHOS genes lack HREs, therefore a direct action of thyroid hormones on these genes is not propable. The search for regulatory genes and factors (such as NRF-1, NRF-2, TFA, and TFB) and of *cis*-acting sequences (such as Mt3, and Mt4) that can control OXPHOS gene transcription and are hormonally regulated would shed light on how the steroid and thyroid hormones could coordinately regulate OXPHOS biosynthesis.

In addition to the long-range effects, glucocorticoids and thyroid hormones also show rapid action on the mitochondria and on oxidative phosphorylation, independent of transcription and translation events. It is still unknown whether these effects are mediated by the same receptors that act on the genes or by direct interaction of the hormones with other mitochondrial components. Mitochondria are the main actors in the events leading to apoptosis and both glucocorticoid and thyroid hormones are involved in this process. A role of the mitochondrial glucocorticoid and thyroid receptors in apoptosis (Kraemer *et al.,* 1995) is a possibility that should be considered in future research.

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Plastid Division: Its Origins and Evolution

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Photosynthetic eukaryotes have evolved plastid division mechanisms since acquisition of plastids through endosymbiosis. The emerging evolutionary origin of the plastid division mechanism is remarkably complex. The constituents of the division apparatus of plastids may have complex origins. The one constituent is the plastid FtsZ ring taken over from the cyanobacteria-like ancestral endosymbionts. The second is the doublet of concentric plastid dividing rings (or triplet in red algae), possibly acquired by ancestral host eukaryotes following the primary endosymbiotic event. Placement of the division apparatus at the correct division site may involve a system analogous to the bacterial Min system. Plastid nucleoid partitioning may be mediated by binding to envelope or thylakoid membranes. Multiple copies of plastid DNA and symmetrical distribution of the nucleoids in the plastids may permit faithful transmission to daughter plastids via equal binary plastid divisions. Cyanelles retain peptidoglycan wall and cyanelle division occurs through septum formation such as bacterial cell division. Cyanelle division involves the cyanelle ring analogous to the inner stromal plastid-dividing (PD) ring. According to the prevailing hypothesis that primary endosymbiosis occurred only once, cyanelle division may represent an intermediate stage between cyanobacterial division and the well-known plastid division among extant plants. With the secondary plastids, which are surrounded by three or four membranes, the PD ring also participates in division of the inner two "true" plastid envelope membranes, and the third and the outermost membranes divide by unknown mechanisms.

KEY WORDS: Chloroplast division, Cyanelle division, Endosymbiosis, Envelope membrane, FtsZ, Nucleoid, Plastid dividing ring, Plastid division.

I. Introduction

It is now widely accepted that two energy-converting organelles in eukaryotes, mitochondria and plastids, were acquired via endosymbiosis of previously free-living eubacteria but not by continuous evolution of the nuclear genome of the eukaryotes. The endosymbiosis hypothesis for origins of organelles was originally proposed by Schimper (1883) and Mereschkowsky (1905) for plastids and by Altmann (1890) for mitochondria, respectively. Since the discovery of organelle DNA, ample evidence collected by ultrastructural, biochemical, and molecular studies has reinforced this hypothesis (Gray, 1992). Recent sequence analyses indicate that ancestors of mitochondria and plastids are endosymbionts of α-proteobacteria-like and cyanobacteria-like eubacteria, respectively (Douglas, 1998; Gray, 1999). The plastids of glaucocystophytes, rhodophytes, and chlorophytes/metaphytes are believed to have originated from such an ancestral cyanobacterial endosymbiont, called the primary endosymbiont. The plastids are referred to as primary plastids. There is another history of the origins of plastids in other diverse algal taxa: heterokont and haptophyte algae, euglenoids, dinoflagellates, cryptophytes, chlorarachniophytes, and apicomplexan parasites harboring the vestigial nonphotosynthetic plastids (apicoplasts). It is accepted that in these algae the plastids are not directly derived from cyanobacteria but have been acquired via secondary endosymbiosis, in which a eukaryote already harboring plastids is captured by a nonphotosynthetic eukaryote and only its plastids are permanently retained (Gibbs, 1993; Williamson *et al.,* 1994; McFadden *et al.,* 1996; Cavalier-Smith, 1999; Delwiche, 1999). Furthermore tertiary endosymbiotic uptake of plastids is known in several dinoflagellates, and is seemingly a temporary or on-going phase of plastid acquisition (Schnepf, 1993). Sequence analysis of nuclear and plastid genes shows that secondary endosymbiotic events independently occurred in different lineages of algal taxa (Douglas, 1998; Delwiche, 1999). But all the plastids of secondary endosymbiotic origin ultimately derive from the ancestor of the primary plastids (Douglas, 1998; Martin *et al.,* 1998).

According to recent analyses of concatenated gene clusters, primary plastids have a common ancestor acquired through a single endosymbiotic event. Among primary plastids of the three lineages, plastids (cyanelles) of glaucocystophytes are closest to cyanobacteria (Bhattacharya and Medlin, 1998; Martin *et al.,* 1998; Moreira *et al.,* 2000). Thus, if monophyly of primary plastids is correct, the cyanelle may be the earliest diverged plastid from the common ancestor of all the plastids. Morphological and biochemical characteristics also suggest cyanelles may be closest to the ancestral cyanobacteria-like endosymbiont (Löffelhardt and Bohnert, 1994).

Plastids arise only by division of existing plastids but never arise *de novo* (Schimper, 1883). Since the primary endosymbiotic event, the descendants have been faithfully transmitted by the mechanisms of plastid division and partitioning.

Acquisition of the division mechanism of the plastid must have been a crucial step for the host eukaryotes to establish the endosymbionts as permanent cellular components. In other words the evolution of the mechanism of plastid division, i.e., plastokinesis and nucleoid partitioning, underlies the evolution of the plastids themselves.

No less than 90% of the genes of the endosymbionts were drastically lost and transferred into host nuclear genomes. Surprisingly loss of the plastid genes occurred independently in different taxa of plants and algae (Martin *et al.,* 1998). In most plastids the genome size is around 120–150 kb with about 120 or more genes or open reading frames (Sugiura, 1992; Gillham, 1994). However, the proteome of the plastids is estimated to consist of 2000–5000 proteins (Martin and Herrmann, 1999). Most of these proteins are encoded by the nuclear genome and translated in cytoplasm, thus they must be imported back to the plastid compartments. To equip the import machinery for nuclear-encoded plastid proteins is a vital requisite for establishing the endosymbiosis as much as the evolution of the plastid division mechanism. Virtually all bacterial (endosymbiont's) cell division genes are lost in the plastid genomes. In 1995 a plant homologue of *ftsZ* was discovered in the nuclear genome of *Arabidopsis thaliana* by Osteryoung and Vierling (1995). It is well known that FtsZ, a GTPase with a significant homology to eukaryotic tubulins, assembles to form a cytokinetic ring (FtsZ ring) at the division site of the bacterial cells (Lutkenhaus, 1993). The discovery of Osteryoung and Vierling (1995) provided momentum for molecular studies on plastid division. Subsequently it was shown that plant FtsZs play a key role in plastid division (Osteryoung *et al.,* 1998; Strepp *et al.,* 1998; Osteryoung and McAndrew, 2001).

Prior to the discovery of plastid FtsZs, a landmark finding that electron-dense annular structures (plastid-dividing ring, PD ring) are present at the isthmus of dividing plastids was achieved by ultrastructural studies using three-dimensional reconstruction of serial thin sections (Mita *et al.,*1986; Mita and Kuroiwa, 1988; Hashimoto, 1986). The annular structures are composed of two concentric PD rings, i.e., PD ring doublet; the outer cytosolic and the inner stromal PD rings, respectively (Hashimoto, 1986), and considered essential components of the plastid division apparatus. An acute question is whether FtsZ is a component of PD rings. Recently it has been evident that this is not the case in a primitive red alga *Cyanidioschyzon merolae* (Miyagishima *et al.,* 2001c). It is emerging that photosynthetic eukaryotes evolved the plastid division apparatus by complex pathways due to the background of acquisition of the plastids.

In this article I summarize our present knowledge about plastid division, focusing on the origin and the evolution of plastid division. To gain insight into the evolution of the plastid division mechanism, it is definitely important to understand the relationship between the division of the cyanelle and the other primary plastids. It is also fascinating to ask what mechanism has been acquired for division of the secondary plastids. Therefore cyanelle division, as an intermediate stage between

cyanobacterial and plastid division, and division of the secondary plastids are particularly highlighted.

II. Endosymbiosis and Origins of Plastid Envelope Membranes

A. Primary Endosymbiosis

As plastokinesis entails fusion and the subsequent fission of the surrounding membranes of the plastids, to understand the biochemical and evolutionary nature of these membranes is fundamentally important to elucidate the mechanism and its origin of plastid division. The primary plastids (the plastids acquired via primary endosymbiosis) lost peptidoglycan wall after primary endosymbiosis except for cyanelles in glaucocystophytes, and became enveloped by lipid-bilayered double membranes separated by a distance of 2–10 nm (Douce and Joyard, 1990). The cytosolic face of the outer envelope membrane is the interface to the nucleocytoplasmic system. Import of nuclear-encoded proteins involved in plastid division must be targeted inside plastids and probably also on the cytosolic surface of the outer envelope membrane (Section III.A).

The plastids of glaucocystophytes, rhodophytes, and chlorophytes/metaphytes are considered to be of primary endosymbiotic origin on the basis of the number of surrounding membranes and the phylogenetic trees of the plastid and nuclear genes. The inner membrane of the double plastid envelope may be homologous to the plasma membrane of the ancestral cyanobacterium-like organism. However, the origins of the outer membranes still remains in dispute. The first possible explanation is that the outer envelope membrane may have derived from the phagocytotic vacuole of the ancestral host eukaryote and either the outer membrane or the peptidoglycan layer of the endosymbiont must have been lost during evolution. The second possible explanation, on the contrary, is that the outer envelope membrane may have evolved from the outer membrane of the cyanobacterium-like endosymbiont and the peptidoglycan layer and the phagocytotic vacuolar membrane of the host must have been lost during evolution. Interestingly galactolipid and sulfolipid are present in the cytosolic leaflet of the outer envelope membrane (Billecocq *et al.,* 1972) as in the cell envelope of cyanobacteria (Murata *et al.,* 1981). However, in the outer envelope membrane phosphatidylcholine, which is undetectable in either the cell envelope or thylakoids of cyanobacteria, represents about 30–35% of glycerolipids (Douce and Joyard, 1990). A nonspecific porin-like protein was reported in the outer envelope membrane (Flügge and Benz, 1984), implying the cyanobacterial origin of this protein. Sequence analysis has revealed that *Synechocystis* sp. PCC6803 has homologues of the protein import components of the plastids. Among them Toc75, the component of the translocon of the outer

envelope membrane, has a homologue in *Synechocystis* sp. PCC6803. Intriguingly the homologue referred to as SynToc75 is analogously located in the outer envelope membrane of cyanobacteria (Reumann and Keegstra, 1999). However, Toc159 and Toc34, the components of the outer envelope, and Tic110, that of the inner envelope, may not be related to *Synechocystis* sp. PCC6803 proteins (Reumann and Keegstra, 1999). They seem to be of eukaryotic origin. Therefore the origin of the membrane proteins is not always coincident with the membrane origin.

B. Secondary Endosymbiosis

In heterokonts, haptophytes, cryptophytes, chlorarachniophytes, and protozoan parasites apicomplexa, the plastids are surrounded by four membranes. The plastids in euglenoids and the majority of photosynthetic dinoflagellates are surrounded by three membranes (Whatley, 1993). Secondary endosymbiotic origins were first hypothesized by Gibbs (1978, 1993) on the basis of membrane counting and topology. It was postulated that organelles other than plastids in algal endosymbionts have been eliminated during endosymbiosis. The inner two membranes are thought to correspond to the dual envelope of the primary plastids. The third and the outermost membranes may have derived from the plasma membrane of the eukaryotic endosymbiont and the phagocytotic endomembrane of the secondary eukaryotic host, respectively. The space between the inner and the outer pair of the surrounding membranes, the periplastidal space, is homologous to the cytosolic compartment of the algal endosymbiont. There is no clear explanation for the origin of the outermost third membrane in euglenoids and dinoflagelates. With euglenoid chloroplasts, Whatley (1993) considered that the third membrane might have been derived from the host endomembrane system because the third membrane is sometimes continuous with the endoplasmic reticulum (ER) and the nuclear envelope in *Euglena gracilis* (Ehara *et al.,* 1990).

In cryptophytes and chlorarachniophytes the secondary endosymbiotic origin of the plastids seems likely because a relic of the vestigial nucleus of a eukaryotic endosymbiont, namely the nucleomorph, is present in the periplastidal compartment (Greenwood *et al.,* 1977; Hibberd and Norris, 1984; Ludwig and Gibbs, 1985, 1989; Gilson and McFadden, 1996). The nucleomorph retains a highly reduced genome (480–660 kb in cryptophytes and 380–455 kb in chlorarachniophytes) coding 300–450 genes (Maier *et al.,* 2000). Nucleomorph-encoded genes are translated on the periplastidal ribosomes (McFadden, 1990). Sequence analyses of the genomes of the nucleomorph and of the plastid unequivocally show both genomes are placed in the same clade: rhodophytes for cryptomonads and chlorophytes for chlorarachnions (Bhattacharya and Medlin, 1998). By analyzing the protein-targeting mechanism it was shown that genes for the light-harvesting complex were probably transferred into the secondary nuclear genome from the nucleomorph

(the primary nucleus) in cryptophytes and chlorarachniophytes (Deane *et al.,* 2000).

With the algae that do not possess nucleomorphs, the secondary endosymbiosis hypothesis is also supported by comparative analyses of plastid and nuclear genes. All plastid genes in such algae belong to the lineage of the primary plastids of rhodophytes or chlorophytes/metaphytes and ultimately converge into the cyanobacterial clade whereas the nuclear genes show divergence coincident with their taxonomy (Bhattacharya and Medlin, 1998; Martin *et al.,* 1998). The secondary plastids (the plastids acquired via secondary endosymbiosis) that originate from cyanelles of glaucocystophytes are not known. Localization of the PD ring also provides further evidence for the secondary hypothesis (Hashimoto, 1997) as mentioned in detail in Section IV.A.

III. Division of Plastids Acquired via Primary Endosymbiosis

A. Plastokinesis: Division of Envelope Membranes

1. Binary Division in the Middle

Since the classic studies by Schimper (1883), many microscopic observations have been made of plastid division. Among them direct evidence of plastid division in living cells was compiled in angiosperms *Hydrilla verticillata* (Kiyohara, 1926), *Conandron remondioides* (Kusunoki and Kawasaki, 1936), and *Agapanthus umbellatus* (Fasse-Franzisket, 1955). For a charophyte *Nitella axillaries* (Green, 1964) and a moss *Mnium cuspidatum* (Ueda *et al.,* 1970), division cycles of the plastids in living cells were recorded in cinematographs.

It can be generally said on the basis of microscopic observations that plastids divide by binary fission at the midpoint, with the dumbbell-shaped configuration as an intermediate stage. Even in plant materials in which division of an individual plastid cannot be followed, it is reasonable to assume that the dumbbell-shaped configuration represents the division profile because the time course of the formation of dumbbell-shaped plastids and the increase in plastid number are in a precursor–product relationship (Chaly *et al.,* 1980; Hashimoto, 1986).

Equal binary division is important for transmitting nucleoids into two daughter plastids because nucleoid separation seems not to involve any specific apparatus such as a spindle and because the nucleoids are distributed symmetrically in each plastid. Equal binary division is established by setting the plastid division apparatus (PD rings and FtsZ rings, see Section III.A.4 and 5) at the midpoint of the plastids. In *Arabidopsis thaliana,* the nuclear mutation *arc11* (Marrison *et al.,* 1999) and reduced expression of *AtMinD1,* a homologue of a bacterial *minD* gene (Colletti *et al.,* 2000), result in an inability to define the division site of the plastids.

MinD is an inhibitor of FtsZ assemblage in bacterial and an essential component of the Min system that determines the division site in cooperation with MinC and MinE. Amino acid sequencing and *in vitro* chloroplast import experiments show that the *AtMinD1* product contains a transit peptide that targets it to the chloroplast (Colletti *et al.,* 2000). A homologue to MinE (AtMinE1) was also identified and shown to play a role in plastid division in *Arabidopsis* (Itoh *et al.,* 2001). In this case, it is not clear whether AtMinE1 participates in placing the FtsZ ring, whereas the overexpression causes impairment of the plastid division (Itoh *et al.,* 2001). In a green alga *Chlorella vulgaris* homologues of *minE* as well as *minD* are coded by the plastid DNA (Wakasugi *et al.,* 1997), but it is not known whether these homologues function in determining the division site of the plastids. These observations indicate that MinD and MinE were taken over for the mechanism of plastid division from the cyanobacterial ancestor together with the FtsZ ring. However, caution is needed in assuming that AtMinD1 and AtMinE1 mediate the determination of the division site because genes encoding MinC, another essential protein of the Min system in bacteria, are not found in either of the completely sequenced nuclear or plastid genome in *Arabidopsis* (Arabidopsis Genome Initiative, 2000). Although AtMinD1 may play a role in determining the division site, the precise function of AtMinD1 and whether the analogous Min system exists in plants have not yet been determined.

2. Plastid Division with Multiple Constrictions in Cultured Cells and in Embryonic cells

Multiple constriction sites have been observed in pleomorphic plastids of developing mesophyll cells in a fern *Ophioglossum reticulatum* (Duckett and Ligrone, 1993), in embryonic cells of *Pelargonium zonale* (Kuroiwa *et al.,* 2001), and in cultured tobacco cells (Miyazawa *et al.,* 2001). Although there is no direct evidence for multiple division of these plastids, such multiple constriction sites are supposed to be true division sites because of the presence of both PD rings and FtsZ rings (Duckett and Ligrone, 1993; Kuroiwa *et al.,* 2001; Miyazawa *et al.,* 2001). It has yet been shown whether multiple constriction division is accompanied by multiple partitioning of the plastid nucleoids.

3. Division of Double Envelope Membranes

Types of plastids can divide irrespective of the presence or absence of inner thylakoid membrane systems. Although partitioning of the thylakoid membrane system may be a regulated process, constriction division of the double envelope membranes is essential for the mechanism of plastokinesis. This is much different from divisions of single-membraned organelles and of plasma membranes in cytokinesis. Division of cell organelles entails membrane fusion at the constriction site followed by the separation of the pair of daughter organelles. Both

double-membraned plastids and mitochondria divide via concurrent constriction of the inner and outer membranes at the division site. Fusion and temporal disorder of the lipid bilayer of plastid envelope membranes must occur at the narrow isthmus of dividing plastids during the final stage of plastokinesis. However, these processes may not occur simultaneously but in order, first in the inner envelope membrane and then in the outer one. Following fusion, the inner envelope membrane may immediately retract from the fusing point to allow the outer envelope membrane subsequently to fuse and to separate into two halves. As suggested in mitochondrial division (Bleazard *et al.,* 1999; Sesaki and Jensen, 1999), completion of plastokinesis may occur by sophisticated balanced membrane fusion and fission.

In glaucocystophytes, the cyanelles are limited by the outer and inner envelope membranes with intervention by the peptidoglycan wall and their division quite resembles cyanobacterial cell division. In cyanobacteria and other Gram-negative bacteria cytokinesis occurs by invagination of the inner membrane and simultaneous ingrowth of the septum at the division site, then by lagging constriction of the outer membrane. Cyanelle division in *Cyanophora paradoxa* also takes place in a similar manner (Figs. 1 and 2) as described in detail in Section III.C.1.

4. Plastid Dividing Ring

When dividing plastids are carefully observed under an electron microscope, electron-dense structures are detected at the narrow isthmus of the constricting plastids. Suzuki and Ueda (1975) first reported such electron-dense deposits across the isthmus of the dumbbell-shaped proplastids in the root tip of pea, and described them as a septum. Chaly and Possingham (1981) and Leech *et al.* (1981) reported electron-dense annuli around the isthmus of extremely constricting plastids of spinach, pea, wheat, and other several higher plants. Chaly and Possingham (1981) noticed that the annuli are present between the inner and outer envelope membranes. Mita *et al.* (1986) detected such annuli not only in the late stage but also in the initial stage of the plastid division in a primitive red alga *Cyanidium caldarium,* and coined the term "plastid-dividing ring (PD ring)" for the annular structure (see also Mita and Kuroiwa, 1988; Kuroiwa *et al.,* 1998).

Hashimoto (1986) observed plastid division in a monocot *Avena sativa* and revealed that a pair of concentric rings girdled the isthmus of the dividing plastids; the outer ring attached on the cytosolic face of the outer envelope membrane and the inner one attached to the stromal face of the inner envelope membrane (Fig. 1). To date the doublet configuration of the PD rings is established in diverse taxa of plants and algae (summarized in Kuroiwa *et al.,* 1998). Although causal relationships between the PD ring and plastokinesis have not been verified by experimental or genetic studies, the PD ring is most likely the major component of the plastid division apparatus.

In higher plants the width of the inner and outer PD ring is about the same, ranging from 15 to 40 nm (Hashimoto, 1986; Oross and Possingham, 1989). The

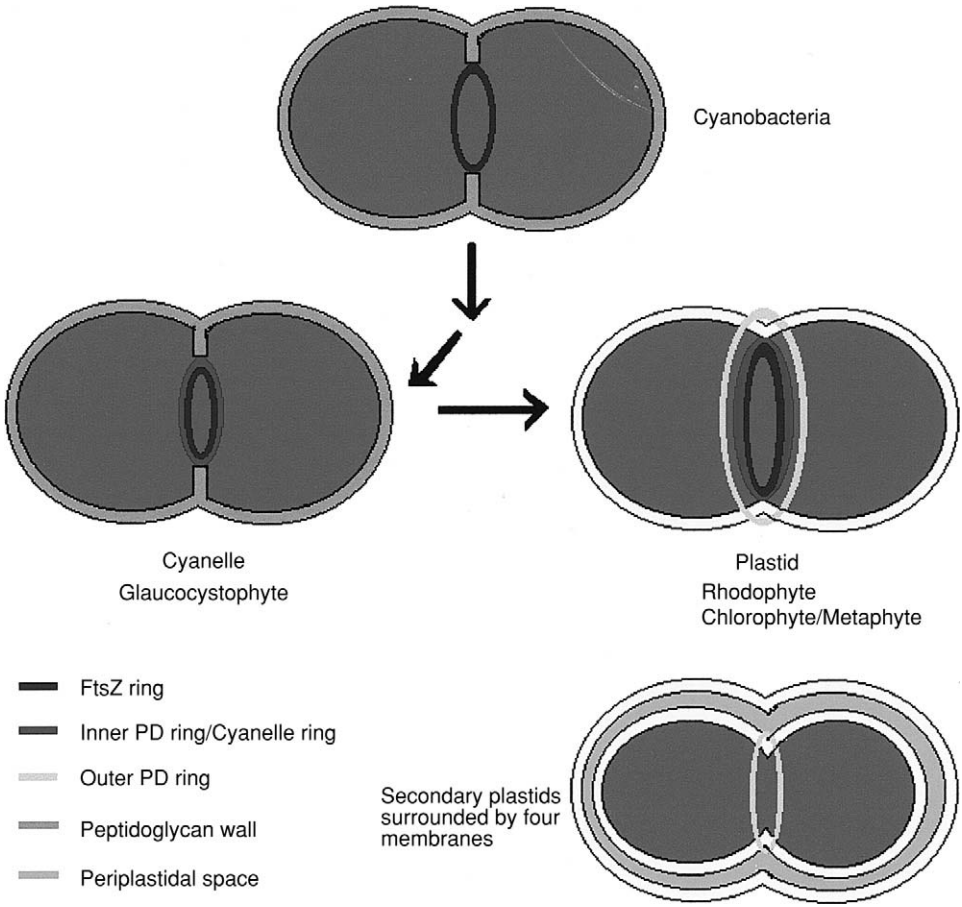

FIG. 1 A hypothetical model of the evolutionary pathway of plastid division according to the hypothesis of monophyly of the primary plastids. The model shows the transition from septum-based division to the contractile outer PD ring-based division. In the primitive plastids such as the cyanelles the peptidoglycan wall is retained and division progresses by ingrowth of the septum and formation of the cyanelle ring (inner stromal PD ring). Probably the FtsZ ring is also localized inside the cyanelle ring. In the primary plastids of rhodophytes and chlorophytes/metaphytes, the peptidoglycan wall is lost, and the outer cytosolic PD ring is acquired. The division apparatus is composed of the PD ring doublet and the FtsZ ring. In the secondary plastids the outer PD ring locates on the periplastidal face of the outer envelope membrane. (See also color insert.)

thickness of the inner and outer rings is not significantly different from each other and measured around 5 nm on electron micrographs. By contrast, in algae the PD rings are larger than those in higher plants. In the green alga *Trebouxia potteri* both inner and outer rings are approximately 100 nm wide and 20 nm thick (Chida and Ueda, 1991). In primitive red algae *Cyanidium caldarium* and *Cyanidioschyzon merolae,* the dimensions of the inner and outer rings are 100 nm × 20 nm and

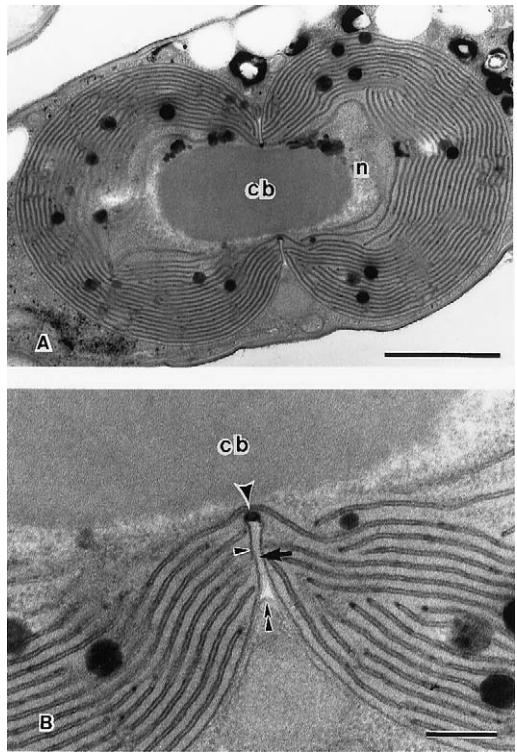

FIG. 2 Electron micrographs of a cyanelle of *Cyanophora paradoxa*. (A) A dividing cyanelle with an ingrowing septum. Nucleoids (n) are observed as translucent areas around the central body (cb). Scale bar = 1 μm. (B). Constriction site in the same section as in (A) shown with higher magnification. Arrow, septum; large arrowhead, cyanelle ring; small arrowhead, inner envelope membrane; small double arrowhead, outer envelope membrane. Scale bar = 0.2 μm.

120 nm × 5 nm (width and thickness), respectively (Miyagishima *et al.,* 1998b, 1999b, 2001a).

Miyagishima *et al.* (1999a) succeeded in isolatating dividing chloroplasts from highly synchronized cultured cells of *C. merolae*. The isolated dividing chloroplasts were extracted with a detergent Nonidet-P40 and the retained outer PD rings were analyzed by ultrastructural observations and biochemical characterizations (Miyagishima *et al.,* 2001c). Negatively stained outer PD rings were visualized with a higher resolution as a bundle of 5-nm filaments in which globular proteins are spaced 4.8 nm apart. The bundle of the filaments is very rigid and stable in 2 *M* urea. Electrophoretic analysis of the outer PD ring fraction revealed a 56-kDa protein as the main band, suggesting it is a candidate component of the cytosolic PD rings in *C. merolae*. The 56-kDa protein is immunologically distinct from the FtsZ homologue of *C. merolae* (CmFtsZ) (Miyagishima *et al.,* 200lb).

Miyagishima *et al.* (200lb) considered the novel 5-nm filaments are of eukaryotic origin. This protein is probably not related to actin, which had once been considered to be a component of the cytosolic PD ring. In a primitive red alga *Cyanidium caldarium,* and a green alga *Closterium ehrenberghii,* plastokinesis is arrested by cytochalasins, which are actin-depolymerizing reagents, and the constricting isthmus can be stained with rhodamine-conjugated phalloidin (Mita and Kuroiwa, 1988; Hashimoto, 1992). However, no consistent evidence has been obtained from similar experiments with the other species. Cytochalasins may affect plastid division indirectly through other cellular processes. However, the molecular nature and function of the inner stromal ring are totally unknown.

In *C. merolae,* another electron-dense ring, the middle ring has been reported to be localized in the lumen between the inner and outer envelope (Miyagishima *et al.,* 1998a). However, it is possible that the electron-dense image referred to as the middle ring is really the inner leaflet of the outer envelope membrane because the outer and inner leaflets of the envelope membranes are not clear in electron micrographs (Fig. 2 in Miyagishima *et al.,* 1999b). So far there have been no reports of such a middle ring for the other plants and algae.

In published reports the electron-dense PD ring doublet in higher plants appears only in extremely constricted plastids. However, in the primitive red algae *Cyanidium caldarium* and *Cyanidioschyzon merolae* the dense PD rings are readily observed from the onset of plastokinesis (Mita *et al.,* 1986; Mita and Kuroiwa, 1988; Kuroiwa *et al.,* 1998). In a number of species of green algae such as *Trebouxia potteri* (Chida and Ueda, 1991), *Nannochloris baccilaris* (Ogawa *et al.,* 1995) and *Klebsormidium flaccidum* (Hashimoto, unpublished observations) PD rings are detected in early or midphase plastokinesis. It is not clear whether the ring begins to form only during a late stage or if it is present before constriction starts but merely undetectable. One possibility is a difference in accumulation of the ring materials or stability of the ring structure during fixation and the following specimen preparation. Another possibility is the method of fixation. The PD rings in the above-mentioned microalgae were observed using rapid freezing followed by freeze substitution, which is regarded as superior to preserving the ultrastructure by chemical fixation, the method used in previous observations in higher plants. Provided that the PD ring generates mechanical force to divide plastids, ring formation should be a cause of the constriction of plastids.

PD rings in cyanelles of glaucocystophytes and in plastids of secondary endosymbiotic origins will be described in Sections III.C and IV.A, respectively.

5. Plastid FtsZ Ring

The bacterial cell division gene *ftsZ* was first identified by screening from a large number of *f*ilament-forming *t*emperature-*s*ensitive mutants of *Escherichia coli* (Hirota *et al.,* 1968; Lutkenhaus *et al.,* 1980). These *fts* mutants form filamentous

cells with regularly spaced nucleoids at a nonpermissive temperature. Genetic analyses show that FtsZ is a pivotal component in bacterial cell division machinery and is found in virtually all eubacteria and archebacteria as well as in higher plants and algae (Rothfield *et al.,* 1999; Beech and Gilson, 2000; Takahara *et al.,* 2000). The FtsZ protein is GTPase related to eukaryotic tubulins and can assemble to form filaments and loops *in vitro* (Erickson *et al.,*1996). In bacteria the FtsZ protein is recruited to assemble the division apparatus, i.e., the FtsZ ring, at the division site of the midcell in the earliest stage of cell division (Lutkenhaus and Addinall, 1997; Rothfield *et al.,* 1999). The FtsZ ring at the midcell was first identified by immunoelectron microscopy of serial thin sections of dividing *E. coli* cells (Bi and Lutkenhaus, 1991). FtsZ rings have also been clearly visualized by immunofluorescence (Addinall *et al.,* 1996) and by expression of FtsZ–green fluorescent protein (GFP) fusion (Ma *et al.,* 1996; Wang and Lutkenhaus, 1996; Levin and Losick, 1996).

The plant homologue of nuclear-encoded *ftsZ* (*AtFtsZ*) was first identified in *Arabidopsis* by Osteryoung and Vierling (1995). Subsequently homologues of *ftsZ* were reported in other several lower and higher plants (Gilson and Beech, 2001). In *Arabidopsis thaliana* two distinct families of *AtFtsZ1* and *AtFtsZ2* have been identified (Osteryoung *et al.,* 1998) in contrast with only a single *ftsZ* gene in bacteria. To date no *ftsZ* genes are known in plastid genomes. The discovery of plant homologues of *ftsZ* evoked the question of whether the plant FtsZ proteins are essential for plastokinesis and do form the electron-dense PD ring. The plant FtsZ proteins have unequivocally been shown to play a key role in plastid division in *A. thaliana* by the antisense technique (Osteryoung *et al.,* 1998) and by gene knockout using homologous recombination in a moss *Physcomitrella patens* (Strepp *et al.,* 1998). Vitha *et al.* (2001) demonstrated by immunofluorescence microscopy using anti-AtFTsZ antibodies and transfomants with a GFP-tagged *AtFtsZ* that AtFtsZ1 and AtFtsZ2 colocalize to form ring structures at the midpoint of dividing plastids in *A. thaliana, Nicotiana tabacum,* and *Pisum sativum*. It has not yet been clarified whether the FtsZ ring is composed of heterodimers of AtFtsZ1 and AtFTsZ2 or distinct AtFtsZ1 and AtFtsZ2 rings are colocalised at the division site. FtsZ rings have also been visualized in *Lilium longiflorum* (Mori *et al.,* 2001) and a red alga *Cyanidioschyzon merolae* (Miyagishima *et al.,* 2001c) by immunofluorescence microscopy. As mentioned above, multiple FtsZ rings have been visualized in plastids with multiple constriction sites in proplastids in cultured tobacco cells (Miyazawa *et al.,* 2001) and in embryonic cells in *Pelargonium zonale* (Kuroiwa *et al.,* 2001). In addition it has been demonstrated that FtsZ homologues of *Pisum sativum* can form multimers and suppress deficiency of an *Escherichia coli ftsZ* mutant (Gaikwad *et al.,* 2000).

Because both AtFtsZ1 and AtFtsZ2 are translated as preproteins with a transit peptide at the N-terminus and imported into the chloroplast stromal compartment (McAndrew *et al.,* 2001), it is unlikely either AtFtsZ1 or AtFtsZ2 is the constituent of the electron-dense outer cytosolic PD ring. Correspondingly in *C. merolae* FtsZ proteins are not detectable in the isolated outer PD ring fraction (Miyagishima

et al., 2001b). In view of stromal localization, the plant FtsZ ring was envisaged to be identical to the inner stromal PD ring. However, this was not supported by recent studies in *C. merolae* by Miyagishima *et al.* (2001c). They concluded that the FtsZ ring is a distinct structure from the inner PD ring based on the following evidence in *C. merolae:* (1) different stabilities between the two rings in isolated dividing chloroplasts under various biochemical conditions; (2) step-by-step formation of the FtsZ ring, the inner PD ring, and the outer PD ring in this order during the course of plastid constriction, and degradation in the reverse order during separation of the two daughter plastids; and (3) immunoelectron microscopy of the FtsZ that suggests the inner PD ring is located between the FtsZ ring and the inner plastid envelope membrane.

As mentioned above, the complex structure and characteristics of the plastid division apparatus have emerged as a multiannular contractile system. The dynamic features of the PD rings and the FtsZ ring have been documented in *C. merolae* (Miyagishima *et al.,* 2001a,b,c): the innermost FtsZ ring is assembled first, then the inner (and middle) ring, and last the outer PD ring is formed at the division site. Once all three (or four) rings are set up at the division site, constriction starts. As constriction progresses, the outer ring increases in thickness and maintains volume; in contrast the inner and middle rings maintain thickness but decrease in volume, indicating that components of the inner and middle rings are lost during the constriction and that those of the outer ring may be retained or remain in a stationary phase. The FtsZ ring first disappears at the late stage of constriction, then subsequently the inner and middle rings disassemble before completion of division. In contrast the outer ring is retained until separation of the fused outer envelope membrane. This suggests that the outer cytosolic ring generates the mechanical force needed for cleavage of the plastids. If the observations in *C. merolae* could be generalized over the other plants, the plastid division apparatus is composed of at least the electron-dense outer and inner PD rings (and the middle ring in *Cyanidioschyzon*) as well as the FtsZ ring (Fig. 1).

The *arc6* mutant of *Arabidopsis thaliana* resembles in phenotype the transformants with reduced expression of *AtFtsZ1* or *AtFtsZ2* by antisense transcription in which only a single or two huge chloroplasts reside in every mesophyll cell (Pyke *et al.,* 1994a,b; Osteryoung *et al.,* 1998; McAndrew *et al.,* 2001). *ARC6* maps to chromosome 5 at a position distinct from that of *AtFtsZ1,* and *AtFtsZ2* maps to chromosome 2 (Marrison *et al.,* 1999). Therefore *ARC6* is distinct from both *FtsZ1* and *FtsZ2*. *ARC6* has not yet been cloned and it is not known whether the product of *ARC6* functions as a component of the PD ring or some regulating factor in initiation of plastokinesis.

6. Mitochondrial Dividing Ring and FtsZ Ring

It is believed that mitochondria are also generated by endosymbiosis of ancestral eubacteria. The molecular phylogeny of mitochondrial genes suggests that α-proteobacteria are closest to the ancestor of mitochondria. By analogy with the

existence of the plastid FtsZ ring, the endosymbiosis hypothesis suggests involvement of the mitochondrial version of FtsZ in mitochondrial division. However, this is not the case in the yeast *Saccharomyces serevisiae* and the nematode *Caenorhabditis elegans,* in both of which no *ftsZ* homologues can be recognized in the complete genomic and mitochondrial sequences. But it is true in some algal groups (Rothfield *et al.,* 1999; Beech and Gilson, 2000). In a synurophyte alga *Mallomonas splendens* and a red alga *Cyanidioschyzon merolae,* not only plastid-destined FtsZ but also distinct mitochondrial versions of FtsZ are recognized in the nuclear genome (Beech *et al.,* 2000; Takahara *et al.,* 2000). The molecular phylogeny of mitochondrial *ftsZs* indicates a marked affinity with *ftsZ* of α-proteobacteria (Beech and Gilson, 2000), supporting the view of the mitochondrial origin of α-proteobacterial endosymbionts. In a recent immunoelectron microscopic examination in *C. merolae,* gold particle-conjugated antimitochondrial FtsZ antibodies were located at the cleavage site of the mitochondria, suggesting the mitochondrial FtsZ can form a ring and participate in mitochondrial division (Takahara *et al.,* 2001). *C. merolae* is the sole organism so far examined in which an electron-dense mitochondrial dividing-ring (MD ring) has been observed (Kuroiwa *et al.,* 1993; Miyagishima *et al.,* 1998b). The mitochondrial FtsZ ring in *C. merolae* seems distinct from the electron-dense MD ring, as is the case in plastids.

In *S. cerevisiae* and *C. elegans,* dynamin-related GTPases Dnm 1 and Mgm1p are suggested by genetic studies to control mitochondrial morphology and coordinated division of both the inner and outer membranes in yeast (Bleazard *et al.,* 1999; Shepard and Yaffe, 1999). In *C. elegans* another dynamin-like GTPase DRP1 may function in mitochondrial division (Labrousse *et al.,* 1999). Dynamins are 100-kDa GTPases and are known to assemble to form filaments that function in pinching off vesicles in cytoplasmic membrane trafficking in yeast and animals. By analogy of such a function of dynamin it is likely these dynamin-like proteins play a key role in fission of mitochondrial membranes. The Fzo1p protein is a dynamin-unrelated GTPase and functions in mitochondrial fusion. It became apparent that Fzo1p also participates in mitochondrial division and that the balance of membrane fusion and fission is an important factor in mitochondrial continuity (Bleazard *et al.,* 1999; Sesaki and Jensen, 1999). In yeast the other components, Mdv1 and Fis2, are also known to participate in fission of mitochondrial membranes (Yoon and McNiven, 2001).

Ancestors of eukaryotes may probably have recruited the bacterial FtsZ ring and the related components for mitochondrial division. But mitochondrial FtsZs may have been abandoned and replaced by the eukaryotic dynamin-related system in fungi, animals, and probably most plants (Beech and Gilson, 2000). There is no evidence that the components of the MD ring in *C. merolae* are related to dynamin-like GTPases. It is also unknown whether the outer PD ring consists of dynamin-related proteins. However, the 56-kDa protein that is detected in the outer PD ring fraction from isolated dividing plastids of *C. merolae* is unlikely to be a dynamin-related protein because of differences in the apparent molecular

weight among them (Miyagishima *et al.,* 2001b). MreB and the closely related Mbl proteins, which belong to the actin superfamily, are known to form bacterial actin-like filaments and to control cell shape in *Bacillus subtilis* (Jones *et al.,* 2001). However, there is so far no evidence for the involvement of MreB or its related proteins in either mitochondrial or plastid division.

7. Partitioning of Thylakoid Systems during Plastid Division

Dividing young chloroplasts contain a considerable amount of thylakoid membranes the content of which depends on the developmental stages. Whatley *et al.* (1982) proposed that a single continuum of the thylakoid membranes is severed into two portions in dividing young chloroplasts in primary leaves of *Phaseolus vulgaris*. There are many electron micrographs published showing strata of thylakoid membranes penetrate through the narrow isthmus of the dividing chloroplasts and are pressed together inside the isthmus. In this case, separation of the thylakoid system is completed just before the separation of the daughter plastids. Assuming that the thylakoid system is a continuum of complicated three-dimensional fretwork (Paolillo and Rubin, 1980; Whatley *et al.,* 1982), there may be a dynamic three-dimensional change in the entire configuration of the thylakoidal continuum in response to constriction of the envelope prior to partitioning of the thylakoid system.

B. Replication and Partitioning of Plastid Nucleoids

Plastid DNA molecules are organized as a discrete structure, a plastid nucleoid, together with specific DNA-binding and structural proteins and RNAs (Hansmann *et al.,* 1985; Nemoto *et al.,* 1988, 1990; Wu *et al.,* 1989; Sato *et al.,* 1993; Nakano *et al.,* 1997; Murakami *et al.,* 2000). The plastid nucleoid functions as an apparatus of replication and transcription of the plastome (the plastid genome) and as a unit of segregation and transmission of the plastome. Thus segregation and partitioning of plastid nucleoids underlie the genetic continuity of the plastid.

We can readily observe plastid nucleoids *in situ* in plant and algal cells after staining with a fluorochrome such as 4′,6-diamidino-2-phenylindole (DAPI). Microfluorometry using DAPI staining is a highly sensitive method to measure minute amounts of DNA and enables us to determine *in situ* DNA content in an individual plastid and even in a single nucleoid.

1. Plastid DNA Replication Uncoupled with Plastid Division Cycles

Usually plastids have a number of nucleoids, each containing multiple copies of plastid DNA. For example, mesophyll cells of mature oat leaves have approximately 160 chloroplasts containing about 100 to 130 copies of plastid DNA within

10 or more separate nucleoids (Hashimoto and Possingham, 1989a,b). In this case, compaction of plastid DNA molecules into the nucleoid is such that 10 or more copies of about 45-μm-long circular DNA are packed into a nucleoid approximately 0.1 μm in diameter.

Plastid DNA levels change during the course of plastid division, development, differentiation, and senescence (Lawrence and Possingham, 1986a,b; Miyamura *et al.,* 1986, 1990; Hashimoto and Possingham, 1989a,b; Sodmergen *et al.,* 1991, 1992; Itoh *et al.,* 1996; Inada *et al.,* 1998, 1999, 2000). In spinach it is established that there are three phases in changes in plastid DNA levels in developing leaves: (1) plastid DNA synthesis occurs in step with the plastid division in meristematic cells of emerging leaves, resulting in constant DNA levels per plastid; (2) in mesophyll cells in expanding leaves the rate of plastid division becomes lower than that of plastid DNA synthesis, resulting in a marked increase in the DNA level per plastid; and (3) thereafter the DNA level per chloroplast gradually decreases because of residual chloroplast divisions after cessation of plastid DNA synthesis (Scott and Possingham, 1980, 1983; Lawrence and Possingham, 1986b). A similar relationship between plastid DNA levels and plastid division has been observed for a monocot *Avena sativa* (Hashimoto and Possingham, 1989a). Such uncoupling between plastid DNA replication and the plastid division cycle implies that plastid DNA replication is not a prerequisite for plastid division. This may be relevant with "polyploidy" of plastid nucleoids or multiple numbers of plastid nucleoids in an individual plastid. Moreover, in spinach leaves there is no indication of a subpopulation of plastids that contains high or low levels of DNA, suggesting lack of a discrete S phase of plastids and even continuous DNA replication throughout the plastid division cycle (Lawrence and Possingham, 1986b).

Uncoupling of plastid division cycles to plastid DNA replication is also evident for several plants and algae other than angiosperms such as a chromophyte *Olisthodiscus luteus* (Cattolico, 1978), a red alga *Cyanidium caldarium* (Kuroiwa *et al.,* 1989), a characean alga *Chara corallina* (Sun *et al.,* 1988), and a fern *Pteris vittata* (Sugai and Kuroiwa, 1988). When nalidixic acid, a potent inhibitor of DNA gyrase, is administrated to cultured cells of *Euglena gracilis,* it causes loss of plastid DNA within a few generations, resulting from not only dilution out but also degradation of chloroplast DNA during chloroplast division cycles (Pienkos *et al.,* 1974; Lyman *et al.,* 1975; Hashimoto and Murakami, 1982). In *C. merolae,* nalidixic acid ceases both plastid and mitochondrial DNA synthesis but one round of plastokinesis occurs in the absence of DNA synthesis (Itoh *et al.,* 1997). Multiplicity of the plastid DNA copy number may permit uncoupling of the plastid division cycles to plastid DNA replication.

2. Partitioning of Plastid Nucleoids Mediated by Membrane Binding

Although plastids can divide without coupling to the plastid DNA replication as mentioned above, segregation and partitioning of plastid nucleoids underlie

genetic continuity of the plastid. Plastid DNA is transmitted in equal amounts to daughter plastid. This was first suggested by electron microscopic observations in a brown alga *Sphacelaria* sp. of which chloroplasts contain a single ring-shaped nucleoid around the periphery of the girdle thylakoids lining the envelope membrane (Bisalputra and Bisalputra, 1970). The nucleoid is entirely attached to the girdle thylakoids and orderly segregated via an 8-figure configuration accompanying membrane separation during plastokinesis. DAPI staining of dumbbell-shaped plastids also suggests equal partitioning of plastid nucleoids as observed in a range of plants and algae (Coleman, 1978, 1979; Boffey and Leech, 1982; Hashimoto, 1985; Hashimoto and Possingham, 1989a; Miyamura *et al.,* 1990; Itoh *et al.,* 1997). Quantitative evidence for transmission of equal amounts of plastid DNA has been provided by DAPI fluorescence microfluorometry done with individual plastids in leaves of *Spinacia oleracea* (Lawrence and Possingham, 1986b) and in a red alga *Cyanidium caldarium* M-8 (Kuroiwa *et al.,* 1989). As an exception, in vegetative cells of several species of *Acetabularia,* more than half of the chloroplasts lack their own DNA, suggesting that the lack of plastid DNA may result from one-sided partitioning of the plastid nucleoids during plastid division (Woodcock and Bogorad, 1970; Coleman, 1979; Lüttke, 1988).

Decatenation of newly replicated plastid DNA may be requisite to the division of nucleoids prior to partitioning. This notion is supported by evidence for the presence of topoisomerases in plastids. Gyrase activity was reported in pea chloroplasts (Lam and Chua, 1987). Eukaryotic topoisomerase type II is present in developing wheat chloroplasts (Pyke *et al.,* 1989) and colocalized with plastid DNA (Marrison and Leech, 1992). Nalidixic acid and novobiocin, both potent inhibitors of DNA gyrase, are known to inhibit plastid DNA synthesis in *Euglena gracilis* (Lyman *et al.,* 1975; Hashimoto and Murakami, 1982), *C. merolae* (Itoh *et al.,* 1997), and *Chlamydomonas* (Woelfle *et al.,* 1993), and in cultured tobacco cells (Heinhorst *et al.,* 1985) and in isolated tobacco plastids (Heinhorst and Cannon, 1993). In *C. merolae,* nalidixic acid affects equal partitioning of the plastid nucleoids (Itoh *et al.,* 1997).

By analogy with a model for the partitioning of bacterial chromosomes (Jacob *et al.,* 1964), Rose (1988) proposed on the basis of membrane binding of plastid DNA that segregation of plastid nucleoids is mediated by membrane growth during and after plastid DNA synthesis. For bacteria this model indicates that two daughter chromosomes are anchored to the cell envelope at the midcell division site and segregation is driven by insertion of new cell envelope materials between the anchoring sites of the two daughter chromosomes. To date, however, this model is not tenable, at least for bacteria, now that experimental results show random insertion of new materials over the entire region of the cell envelope (Nanninga *et al.,* 1990). Recent studies using GFP fusions and fluorescence *in situ* hybridization (FISH) suggest that newly replicated chromosomes are rapidly segregated in opposite directions by active movement rather than passive migration according to the growth of the cell envelope (Gordon *et al.,* 1997; Webb *et al.,* 1997, 1998; Niki and Hiraga, 1998). From these observations it has been proposed that a mitotic-like

apparatus is present in prokaryotes. This model involves centromere-like regions, condensation of nucleoids, and bidirectional extrusion of newly replicated DNA (Glaser *et al.,* 1997; Lin *et al.,* 1997; Mohl and Gober, 1997; Niki and Hiraga, 1998; Møller-Jensen *et al.,* 2000). Furthermore in this model membrane binding of chromosome DNA is not a prerequisite for the nucleoid partitioning in bacteria.

In the case of the plastid nucleoids, the notion of the passive migration of membrane-bound plastid DNA coupled with membrane growth seems still attractive. Indeed there is ample evidence suggesting membrane binding of plastid DNA. Correlation between the dynamic change in distribution of plastid nucleoids and the development of thylakoid systems suggests binding of plastid DNA to the thylakoid membranes (Hashimoto, 1985; Miyamura *et al.,* 1986; Lindbeck *et al.,* 1987). Biochemical evidence for membrane binding of plastid DNA was provided from studies in *Chlamydomonas reinhardtii* (Nie *et al.,* 1987; Wu *et al.,* 1989), *Spinacia oleracea* (Lindbeck and Rose, 1990), and *Pisum sativum* (Sato *et al.* 1993). In *Chlamydomonas* a protein bound to the cloned replication origin was identified from high-salt extract of the thylakoid membrane (Nie *et al.,* 1987). In spinach, Liu and Rose (1992) showed the rDNA region is a specific site that binds to the thylakoid membrane, and suggested a link between membrane binding and DNA replication, considering the fact that the replication origin in plastid DNA is mapped to rDNA regions in *Nicotiana tabacum* (Takeda *et al.,* 1992) and *Oenothera* (Chiu and Sears, 1992). Sato *et al.* (1993) identified by Southwestern blotting a 130-kDa plastid envelope DNA-binding (PEND) protein in the inner envelope membranes of developing and dividing chloroplasts in pea leaves. Several specific binding regions of the plastid DNA were identified, e.g., the downstream half of the *petA,* two regions within the *rpoC2,* and a region that includes the *psbM* (Sato *et al.,* 1993). The DNA-binding domain of the PEND protein is a novel type of basic plus leucine zipper (bZIP) consisting of sextuple repeats of a putative membrane-spanning region, referred to as cbZIP, which targets a sequence TAAGAAGT (Sato *et al.,* 1998; Sato, 2001). As the PEND protein is expressed only in young leaves where chloroplasts are actively developing and dividing, it may play a role in DNA replication and partitioning in such young chloroplasts (Sato *et al.,* 1999; Sato and Ohta, 2001). The homology search suggests that the PEND protein and most of the plastid DNA-binding proteins have no counterparts in cyanobacteria or other prokaryotes, and therefore they were probably acquired by the eukaryotic host after endosymbiosis (Sato, 2001).

In general, plastid nucleoids are distributed symmetrically or uniformly within the plastid in any stages of plastid division and development (Kuroiwa *et al.,* 1981; Sellden and Leech, 1981; Hashimoto, 1985; Hashimoto and Possingham, 1989b). Symmetrical or uniform distribution of multiple numbers of plastid nucleoids may permit automatic or passive separation resulting from equal binary division of the plastids. In spinach cultured leaf discs some nucleoids are still present in the extremely constricted isthmus of dividing plastids, suggesting lack of any mitotic-like apparatus for partitioning of the plastid nucleoids (Hashimoto and Possingham,

1989b). It is likely that partitioning of plastid nucleoids may not involve any mitotic-like apparatus but depend upon symmetrical or uniform distribution of the plastid nucleoids that needs membrane binding of the plastid DNA. However, the molecular mechanisms of DNA packing and segregation or division of the nucleoid itself and factors involved in the pattern of nucleoid distribution are totally unknown.

Plastid nucleoids in a number of chromophyte algae assemble to form a ring at the periphery of the plastid (Coleman and Nerozzi, 1999). In such cases the distribution of the nucleoids cannot be explained by isotropic membrane growth, but additional mechanisms might be needed. In *Cyanophora paradoxa* (glaucocystophyte) the cyanelle nucleoid locates around the central body, which resembles the cyanobacterial carboxysome in morphology (Fig. 2). The nucleoid is severed together with the central body by ingrowth of the septum during cyanelle division (see Section III.C.1). In this case adjoining or anchoring the nucleoid to the central body may be essential rather than membrane binding of the nucleoid.

Isolation of mutants coupled with various cytological methods could be a hopeful approach to dissect the molecular mechanism of segregation of plastid nucleoids. In a model photosynthetic eukaryote, *Chlamydomonas,* mutants defective in plastid nucleoid segregation (*moc* mutants: *mo*nokaryotic *c*hloroplast) have been isolated (Misumi *et al.,* 1999). The identification and characterization of the gene products are now awaited.

C. Cyanelle Division

Cyanelles of glaucocystophytes are probably the most primitive and the closest to cyanobacteria among known extant plastids because of the sequence phylogeny of cyanelle DNA and existence of the peptidoglycan wall surrounding the cyanelles. Because of the peptidoglycan wall, the cyanelle division of *Cyanophora paradoxa* presents unique features.

1. Septum Formation

Constriction of the dividing cyanelles entails centripetal formation of a septum at the cleavage site with the inner envelope membrane invaginating at the leading edge and the outer envelope membrane behind the septum (Fig. 2). This means the inner and outer envelope membranes do not constrict concurrently as they generally do in plastid divisions. The septum was intensely stained by silver grains after a silver methenamine staining procedure was applied to localize polysaccharides on thin sections (Iino and Hashimoto, submitted). Administration of β-lactams, a potent inhibitor of bacterial peptidoglycan synthesis, results in severe degradation or diminishing of the septum and arrest of the cyanelle division, and finally causes cell death due to rupture of the cyanelles (Kies, 1988; Iino and Hashimoto,

submitted). These observations suggest that cyanelle division of *C. paradoxa* resembles cyanobacterial cell divison rather than the known plastid divisions in the primary plastids. In the cyanelle genome of *C. paradoxa* a homologue to the *ftsW* gene is coded (Stirewalt *et al.,* 1995), the product of which is suggested to be a transmembrane protein from the predicted amino acid sequence and to be involved in septum formation in bacterial cells (Bramhill, 1997). It remains unknown whether the cyanelle homologue to *ftsW* is translated and plays a role in cyanelle division.

2. Cyanelle Ring

Only a single ring approximately 50 nm wide is detectable by electron microscopy on the stromal face of the inner envelope membrane at the cleavage site of dividing cyanelles (Fig. 2). The outer ring common in plastids is not detected on the cytosolic face of the outer envelope membrane. Such a feature is quite unique, so I propose to refer to the single inner ring structure as a *cyanelle ring*. Although localization of the cyanelle ring is the same as that of the bacterial FtsZ ring, the latter is not detectable by electron microscopy in thin sections (Bi and Lutkenhaus, 1991; Lutkenhaus, 1993). The cyanelle ring may be similar in morphology to the inner PD ring rather than the FtsZ ring. Further characterization of the cyanelle ring awaits immunolocalization of FtsZ in *C. paradoxa*. Lack of the outer PD ring is not surprising because septum-based cyanelle division may not require external mechanical force.

3. Cyanelle Nucleoid

The cyanelle nucleoid in *C. paradoxa* locates in a space between the innermost thylakoid membrane of the concentric thylakoid layers and the central body (Fig. 2), which is a carboxysome-like or pyrenoid-like inclusion occupying the central region of the cyanelle.

The cyanelle nucleoid covers the entire surface of the central body but it is not clear whether the nucleoids bind to thylakoid membranes. Rather, it is possible the nucleoid is adjoined to the periphery of the central body. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is localized by immunoelectron microscopy in the central body (Mangeney and Gibbs, 1987). Association of plastid nucleoids with pyrenoids is also known in different species of algae (Miyamura and Hori, 1991).

Considering the intensity of DAPI fluorescence of the nucleoid and the size of the cyanelle genome (ca. 136 kb), the nucleoid possesses multiple copies of cyanelle DNA. The nucleoid and the central body are severed into two halves by the ingrowing septum during cyanelle division (Fig. 2) Therefore nucleoid partitioning may depend upon symmetrical distribution of the cyanelle DNA molecules around the periphery of the central body and the ingrowth of the septum as a cleavage

force. In this case adjoining or anchoring of the nucleoid to the central body seems to be an important factor rather than binding of the nucleoid to the thylakoid membranes.

IV. Division of Plastids Acquired via Secondary Endosymbiosis

The plastids of secondary endosymbiotic origins are enveloped by three or four membranes. A pair of inner membranes originates from the double membranes of the true envelope of the primary plastid. The outer pair or third membranes outside the true plastid envelope form a completely closed sac that remains intact during the plastid division cycles. Therefore the sac must divide in a coordinate fashion with the division of the true plastid envelope. The mechanism for the division of the outermost one or two membranes of the three or four surrounding membranes may have been acquired by evolution of the nuclear genome of the secondary eukaryotic host.

A. Plastid Dividing Ring and Division of the "True" Plastid

A single PD ring is present at the periplastidal surface of the inner pair of four surrounding membranes of the plastids in a heterokont alga *Heterosigma akashiwo* (Raphidophyceae) (Fig. 3; Hashimoto, 1997). This finding suggests the PD ring is a conserved structure in plastid-harboring eukaryotes, irrespective of the different endosymbiotic origins of the plastids. In addition the localization of the PD ring is consistent with the idea that the inner pair of the surrounding membranes is derived from the true envelope of the primary plastids. In other words the localization provides novel evidence for the secondary endosymbiosis hypothesis. In this context, localization of the PD rings in three-membraned plastids of euglenoids and dinoflagellates would proffer a clue to elucidate the origin of each envelope membrane. The PD ring in the secondary plastids was reported in another heterokont alga *Mallomonas splendens* (Synurophyceae) (Beech and Gilson, 2000). There are no reports for the PD ring in the secondary plastids in other algae. As the eukaryotes harboring the secondary plastids are known in diverse taxa, extensive examination is awaited for insight into the mechanisms of division of the secondary plastids.

B. FtsZ in the Nucleomorph

A gene encoding a homologue of FtsZ is retained in the nucleomorph in a cryptomonad *Guillardia theta* and translated as a preprotein in the periplastidal space.

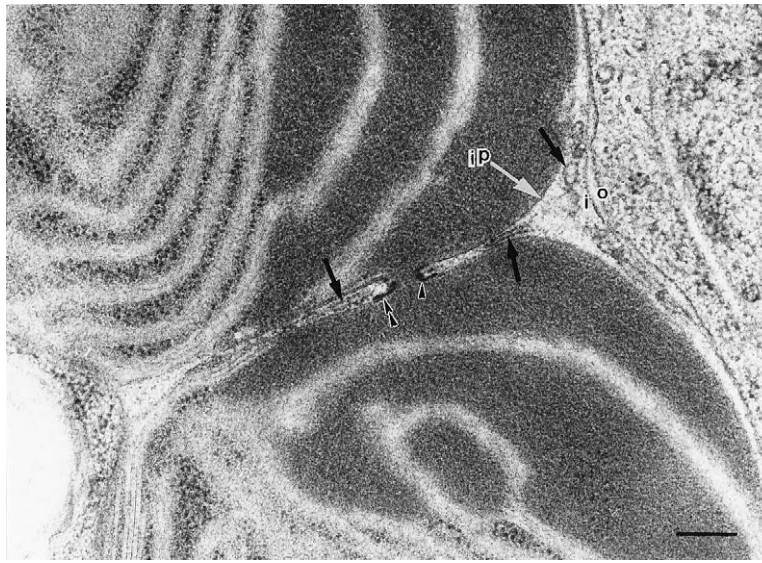

FIG. 3 Constriction region of the dividing chloroplast of *Heterosigma akashiwo*. An electron-dense outer PD ring (arrowhead) is present at the periplastidal face of the outer envelope membrane of the inner pair (ip) (true plastid envelope) at the constricting neck and the shoulder region (double arrowhead). The outer (o) and inner (i) membranes of the outer pair of the surrounding membranes are scarcely constricted. Many vesicles (arrow) are observed in the periplastidal space and some of them invade the narrow cleavage furrow. Seemingly they may fuse one another to form fretworks. Scale bar $= 0.2\ \mu$m. Reproduced with modifications from Hashimoto (1997). *Plant Morphology* **9,** 38–41, with permission of The Japanese Society of Plant Morphology.

Immunoelectron microscopy showed the FtsZ proteins are imported into stroma but failed to localize the FtsZ at the division site of the plastids (Fraunholtz *et al.,* 1998).

C. Division of the Outer Pair of Four Surrounding Membranes

The process and the mechanism of the outer pair of the surrounding membranes are scarcely known. The localization of the PD ring in the periplastidal space suggests the outer pair of the surrounding membranes divides in a distinct manner from that of the inner pair (true plastid envelope) that involves the PD ring. This view is consistent with the observation that the outer pair invaginates behind the inner pair of the plastid envelope during the course of plastid division (Fig. 3) (Magnussen and Gibbs, 1980; Hashimoto, 1997). It is known in *Cryptomonas* sp. (θ) (Mckerracher and Gibbs, 1982) that the inner membrane of the outer pair invaginates prior to the outermost membrane. In *Heterosigma akashiwo,* flat saccules of the periplastidal vesicles deeply invade the cleavage furrow of the inner pair (Fig. 3). A proposed

model is as follows. The periplastidal vesicles may be generated by pinching off from the inner membranes of the outer pair and may fuse to form a diaphragm. Possibly opening of the diaphragm comes to close centripetally into the deepest point of the cleavage furrow of the inner pair, and finally the leading edge may meet and fuse, resulting in division of the inner membrane of the outer pair. The outermost membrane of the outer pair may invaginate independently of the inner membrane of the outer pair by an unknown process.

It is well known that the outermost surrounding membrane in the secondary plastids is continuous to the ER and the outer envelope membrane of the nucleus. In many algae harboring the secondary plastids, the outermost membrane of the secondary plastids and the outer nuclear envelope is common in a broad area, constituting a continuum of the plastid and the nucleus. The process and the mechanism of the coordinated divisions of the plastid and nucleus remain to be investigated.

D. Division of Apicoplasts in Protozoan Parasites

Protozoan parasites of the apicomplexa such as the pathogens *Plasmodoium* and *Toxoplasma* possess nonphotosynthetic vestigial plastids called apicoplasts. The apicoplasts are surrounded by four membranes and contain 35-kb circular DNA, the sequence and gene organization of which unequivocally represent those of an algal plastid DNA. These unambiguously indicate that the apicoplasts were acquired via secondary endosymbiosis. The lineages of the eukaryotic endosymbionts are still unclear but are assumed to be rhodophytes or chlorophytes by sequence analyses of different genes. Because apicoplasts are essential for parasite survival, the division mechanism is envisaged as an important target in medical strategies against malaria and other diseases caused by those parasites.

In *Toxoplasma gondii* apicoplasts are severed by association with the centrosomes during nuclear division (Striepen *et al.,* 2000). Interestingly treatment with microtubule-disrupting reagents that cause aberration of the intranuclear spindle formation disturbs apicoplast division, giving rise to huge reticulated apicoplasts. To date, efforts have failed to identify *ftsZ* genes or ftsZ-related sequences in the apicomplexan genomes. Considering these observations, the parasites may have evolved an FtsZ-independent mechanism for apicoplast division. Recently electron-dense helical structures have been observed in constricting apicoplasts of *Toxoplasma gondii* by Matsuzaki *et al.* (2001) who assume that these structures are the PD rings. However, whether the helical structure corresponds to the PD ring cannot be determined unless the localization became clear. If the ring-like structures locate in the periplastidal surface of the inner pair, then it might be the PD ring. If, on the contrary, the helical structures are present on the outer pair of the surrounding membranes, it would be of a distinctive molecular nature and derived from a different evolutionary pathway.

V. Nuclear Control of Plastid Division

Nuclear control of plastid division was first suggested by investigations of a barley mutant albostrains, albino cells of which contain achlorophyllous plastids that lack their own ribosomes resulting in no significant protein synthesis (Börner *et al.,* 1976). The plastid number and the DNA level are, however, comparable to those in the normal green cells (Scott *et al.,* 1982; Hashimoto and Possingham, 1989c). This suggests that the plastids can be formed and divide only by nuclear-encoded proteins. The PD ring doublet is also detected in dividing ribosome-deficient plastids in this mutant (Hashimoto and Possingham, 1989c). These findings indicate that both replication of the plastid genome and plastokinesis are totally under nuclear control. This may also hold true with other cases of plastid ribosome deficiency in albino tissues of a mutant 'Mrs. Parker' of *Pelargonium zonale* (Börner *et al.,* 1976), *iojap* gene-affected albino tissues of *Zea mays* (Walbot and Coe, 1979), and high temperature-induced plastid ribosome deficiency in some cereals (Feirarbend, 1979).

No genes for plastid DNA synthesis are found in the plastid DNAs of which sequencing has already been completed. Plastid topoisomerase II is identified in pea (Lam and Chua, 1987) and wheat (Pyke *et al.,* 1989; Marrison and Leech, 1992). Although there is no genetic information, this enzyme is probably encoded in the nuclear genome.

Nuclear control of plastid division means that most of the nuclear-encoded proteins required for plastid division must be translocated into plastidal compartments and some of the nuclear-encoded proteins may operate for plastid division outside the plastids. The components of the outer PD ring may belong to the latter.

An array of *arc* (accumulation and replication of chloroplasts) mutants has been isolated from *Arabidopsis thaliana* by using as a criterion the fact that the chloroplast number in a mesophyll cell correlates with the cell plan area (Pyke and Leech, 1991, 1992; Pyke, 1999; Marrison *et al.,* 1999). Eleven independent nuclear-encoded *ARC* genes were identified and the interrelationship among the *ARC* genes was examined by analyzing the phenotypes of the double mutants (Marrison *et al.,* 1999). A mutant *arc6* has a striking phenotype: only two huge chloroplasts reside in every mesophyll cell, suggesting that onset of chloroplast division is arrested and the cellular content of the chloroplast mass is compensated for by increasing the chloroplast volume (Pyke *et al.,* 1994a,b). In *arc6* the plastid number is greatly reduced not only in mesophyll cells but also in meristematic cells (Robertson *et al.,* 1995). In a mutant *arc5* most of the chloroplasts have a dumbbell-shaped configuration, suggesting that ARC5 is responsible for progressing a late stage of plastokinesis and ARC3 ensures that young chloroplasts expand to a certain optimal size to coincide with the initiation of the division (Marrison *et al.,* 1999). Mesophyll cells in a mutant *arc11* have a heterogeneous population of chloroplasts in size suggesting that *ARC11* seems to be involved in the decision of

the equatorial division plane (Marrison *et al.,* 1999). So far, there is no evidence of relevance between *ARC* loci and plant *ftsZ, minD,* or *minE* genes. *ARC6* maps to chromosome 5 at a distinct position from that of *AtFtsZ1,* and *AtFtsZ2* maps to chromosome 2 (Marrison *et al.,* 1999). At present there is no evidence for the involvement of *ARC6* in PD ring formation.

Homologues to a number of bacterial cell division genes such as *ftsZ, minD,* and *minE* are present in the genomes of plants and algae as mentioned in the previous section. In higher plants the genes involved in plastid division that have been identified so far are all nuclear encoded. However, homologues to *minD* and *minE* are found in the plastid DNA in a unicellular green alga *Chlorella vulgaris* (Wakasugi *et al.,* 1997). Genes encoding FtsW, which is an intrinsic membrane protein probably involved in septum formation in bacteria (Ikeda *et al.,* 1989; Boyle *et al.,* 1998), were identified in the plastid genome of *Cyanophora paradoxa, Mesostigma viride* (Prasinophyceae), and *Nephroselmis olivacea* (Prasinophyceae) (Stirewalt *et al.,* 1995; Lemieux *et al.,* 2000; Turmel *et al.,* 1999). Furthermore in the plastid genome of *M. viride* and *N. olivacea* homologues of *minD* and *ftsI,* the latter of which encodes penicillin-binding protein 3 in bacteria, are encoded (Lemieux *et al.,* 2000; Turmel *et al.,* 1999). Also in *Cyanidioschyzon merolae, ipxA, ipxC,* and *glmS,* homologues of genes involved in peptidoglycan synthesis in bacterial cells, are coded in the plastome (Ohta *et al.,* 1998). There is as yet no information about the expression and function of these genes in these algal cells. Cyanelles of *C. paradoxa* are surrounded by a peptidoglycan layer and the division entails ingrowth of the septum as mentioned in Section III.C.1 (Fig. 2). If the cyanelle homologue of *ftsW* is expressed, it may play a role in septum formation in cyanelles. If it is true, cyanelle division is controlled by both nuclear and plastid genes.

VI. Concluding Remarks

It is established at least in higher plants that plastid division is entirely under nuclear control. The known plant homologues of FtsZ are all nuclear-encoded proteins. Although the function of plant FtsZs has not yet been determined, FtsZs undoubtedly participate in the mechanism of plastid division. The existence of FtsZ homologues in plant genomes and the phylogeny of their sequence definitely indicate the cyanobacterial ancestry of the plastids.

Both inner and outer PD rings are undoubtedly distinct from the FtsZ ring (Vitha *et al.,* 2001; Miyagishima *et al.,* 2001c). Consequently the present model for the division of primary plastids involves three (or four in red algae) distinct annular structures: the outer and inner PD ring (and possibly the middle ring as well in the red alga *Cyanidioschyzon merolae*) and the innermost FtsZ ring (Fig. 1). As bacterial cell division depends on septum formation but does not need force-generating rings outside the cell envelope, it is likely that the outer PD ring or the

PD ring doublet was acquired by the host eukaryote during the establishment of primary endosymbiosis in exchange for the loss of the "disused" peptidoglycan wall. Thus the apparatus of plastid division is of complex origin. However, it cannot be concluded whether the components of the outer PD ring are of eukaryotic origin. It is a possible consideration, as suggested by Osteryoung (2001), that although at present there is no evidence for genes encoding bacterial actin-like MreB or related proteins in a plant genome, they might be recruited to form the outer PD ring. Immunolocalization and homology search for the 56-kDa protein in the outer PD ring fraction in *C. merolae* (Miyagishima *et al.,* 2001b) will test the above possibility. A complex evolutionary origin also appears in the import apparatus for nuclear-encoded plastid proteins (Toc/Tic) (Reumann and Keegstra, 1999), in the composition of plastid nucleoid proteins (Sato, 2001), and in enzymes that catalyze the reactions of the Calvin cycle (Martin and Schnarrenberger, 1997), implying a common feature of eukaryotic cellular evolution.

The outer cytosolic PD ring may function to generate a mechanical force to sever plastids. At present the evidence is indirect. However, amino acid sequencing and biochemical characterization of the 56-kDa protein identified in the outer PD ring fraction of *C. merolae* will provide an opportunity to conduct experiments to determine the function of the outer PD ring in plants in which techniques of genetic manipulation are applicable.

Cyanelle division is an intermediate stage between plastid division and cyanobacterial division. If monophyly of the primary plastids is true, the cyanelle is the earliest diverged plastid from the ancestral endosymbiont. Actually the morphology and mechanism of division of the cyanelle quite resemble those of cyanobacteria. The mechanism of plastid division in rhodophytes and chlorophytes/metaphytes may have evolved from that of cyanelle division (Fig. 1). On the contrary, if acquisition of the cyanelle is a separate event from that of the other two lineages of primary plastids, the mechanism of plastid division may have evolved independently in glaucocystophytes and the other two lineages. Nevertheless, in this case cyanelle division may represent an intermediate evolutionary stage of the mechanism of plastid division as a living fossil.

In cyanelles of *Cyanophora paradoxa,* only the inner stromal PD ring (cyanelle ring) is present at the leading edge of the ingrowing septum (Figs. 1 and 2). Although lack of the outer PD ring is accounted for by the presence of the peptidoglycan wall, the function of the inner PD ring is not known. If the cyanelle ring is homologous to the inner PD ring not only in morphology but also in molecular nature, the acquisition of the inner PD ring may have been prior to that of the outer PD ring during the evolution of plastid division.

The rhodophytes and chlorophytes/metaphytes may have evolved the outer PD ring in exchange for loss of the peptidoglycan wall. In such a scenario a question arises as to the function of the cyanelle ring or the inner PD ring. Although cyanelle FtsZ rings have not yet been shown, the cyanelle ring is probably unrelated to the FtsZ ring because the cyanelle rings are visualized by electron microscopy unlike the FtsZ rings. A possible function of the cyanelle ring or the inner PD ring may

be placing the division apparatus at the correct division site. However, this notion conflicts with the observation that the FtsZ ring is formed prior to the inner PD ring in *C. merolae* (Miyagishima *et al.,* 2001c). Another possibility is that the cyanelle ring or the inner PD ring may mediate fusion of the inner envelope membrane at the isthmus just prior to separation of the two halves of the plastid. The fusion of the inner envelope membrane and the following retraction of the separated daughter inner envelope membranes are prerequisites for the subsequent fusion of the outer envelope membrane. Once fusion of the inner envelope membrane is complete, the cyanelle ring or the inner PD ring may disassemble. However, this function is also required for bacterial cell division and thus it cannot account for why the electron-dense inner ring is absent from the division site of bacterial cells. Otherwise, the putative fusion proteins in bacteria may have been replaced with the electron-dense cyanelle ring by the eukaryotic host.

The PD ring is conserved not only in the primary plastids but also in the secondary plastids. The location of the PD ring suggests the two membranes of the true plastids envelope divide by an apparatus that involves the PD ring, supporting the secondary endosymbiosis hypothesis (Figs. 1 and 3). The division mechanism for the outermost and third membranes is distinct from that of the inner pair and is almost unknown. As the outermost and third membranes are considered to derive from eukaryotic plasma membrane or phagocytotic membrane, it is possible that vesicular trafficking and fusion may be involved in the mechanism of division. Based on this, dynamin-related proteins would deserve attention. Mitochondrial division in yeast and animals is known to involve dynamin-related proteins (Bleazard *et al.,* 1999; Shepard and Yaffe, 1999; Labrousse *et al.,* 1999), although mitochondria are not of secondary endosymbiotic origin.

Apicoplast division in apicomplexan parasites has quite unique features that do not involve FtsZ but depend upon the function of centrosomes or microtubules. *Plasmodium* and *Toxoplasma* are the organisms that can be genetically manipulated among those harboring the secondary plastids. Although the apicoplasts show rather exceptional morphology, this will be a great advantage for molecular studies of the mechanism of division of the secondary plastids.

The secondary plastids have received less attention, but extensive investigations of the mechanism of division would be needed to gain insight into how and how many times eukaryotes could tame their endosymbionts.

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Cell-Cycle Responses to DNA Damage in G_2

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Cellular reproduction, at its basic level, is simply the passing of genetic information from a single parent cell into two daughter cells. As the cellular genome encodes all the information that defines a cell, it is crucial that the genome be accurately replicated. Furthermore, the duplicated genome must be properly segregated so that each daughter cell contains the exact same information as the parent cell. The processes by which this occurs is known as the cell cycle. The failure of either duplication or segregation of the genome can have disastrous consequences for an organism, including cancer and death. This article discusses what is known about checkpoints, the surveillance mechanisms that monitor both the fidelity and accuracy of DNA replication and segregation. Specifically, we will focus on the G_2 checkpoint that is responsible for ensuring proper segregation of the duplicated genome into the daughter cells and how this checkpoint functions to arrest entry into mitosis in response to DNA damage.

KEY WORDS: Cell cycle, Checkpoints, DNA damage, Genomic stability.

I. Introduction

Life on earth centers on the capacity of organisms to reproduce. For bacteria and single-celled eukaryotes, reproduction is the ability of one cell to make two identical daughter cells. Although higher eukaryotes have additional complexities to contend with, reproduction still requires the intrinsic ability of one cell to produce two genetically identical copies.

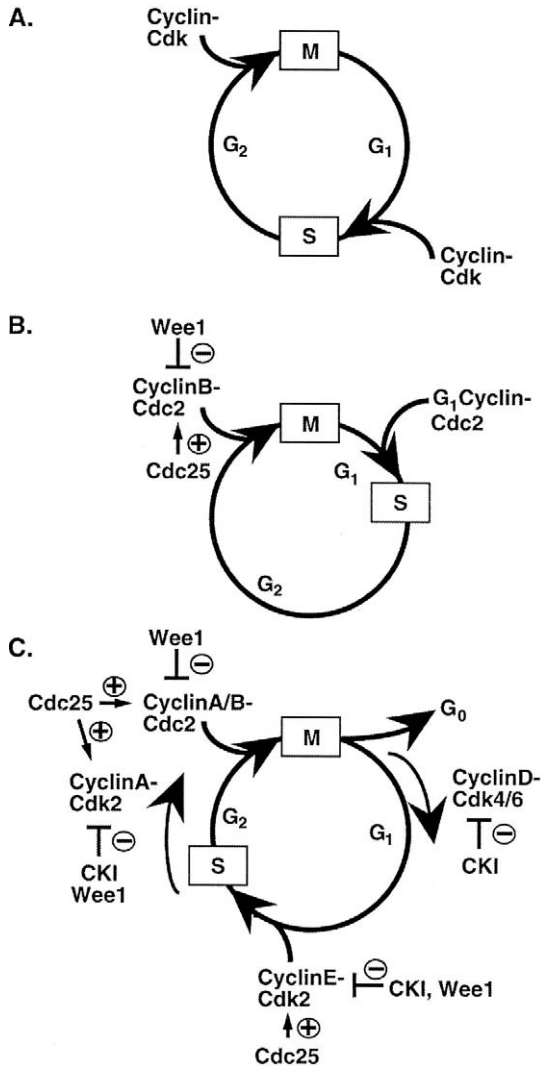

FIG. 1 The eukaryotic cell cycle. (A) General model of the cell cycle. The two key events that take place in the eukaryotic cell cycle are S phase (S), in which DNA replication occurs, and mitosis (M), in which chromosome segregation takes place. In between S phase and mitosis are two gap phases, G_1 and G_2, that provide the cell with time to prepare for DNA replication and mitosis, respectively. During these preparatory stages, cyclin-dependent kinases (Cdk) and their cyclin partners assemble into the active kinase complexes that positively regulate cell-cycle transitions. These Cdk–cyclin complexes are targets for both positive and negative regulation. (B) The fission yeast cell cycle. *S. pombe* has a very short G_1 phase, under the control of G_1 cyclins–Cdk complexes. The majority of its time is spent in G_2. Thus the G_2/M transition, under the control of the cyclinB–Cdc2 complex, is the major transition point, particularly in response to DNA damage. The inhibitory "stop" signal is provided by the Wee1 kinase, and the activating "go" signal by the Cdc25 phosphatase. (C) The mammalian cell

The processes by which cells reproduce themselves form a series of events collectively known as the cell cycle. Although details for the molecular mechanisms of cell division differ between prokaryotes and eukaryotes, there exists a basic principle in which the genome must be accurately replicated and then segregated into daughter cells. Here we will concentrate on the eukaryotic cell cycle, and specifically how this program is modified in response to DNA damage.

A. Basics of Cell-Cycle Control

The cell cycles of all eukaryotic cells obey a basic set of rules. First, cells must make an exact copy of themselves. Second, eukaryotic cells replicate their DNA during a discrete temporal period known as the synthesis or S phase and duplicated chromosomes are segregated into daughter cells at mitosis. These are the two key events in the life of a cell. Finally, S phase must be completed before the initiation of mitosis, and similarly, the segregation of chromosomes at mitosis must be completed prior to the onset of DNA replication during S phase.

This article deals with checkpoints—those functions that ensure the correct order of cell-cycle events and the accuracy with which they occur. Checkpoints function in the "gap" phases of the cell cycle—in Gap1 (G_1) to prevent the initiation of DNA replication until mitosis is successfully completed, and in Gap2 (G_2) to prevent mitosis until the genome has successfully and accurately been replicated (Fig. 1). There are also checkpoints that function during S phase that monitor DNA replication (Boddy and Russell, 1999), and during mitosis to ensure correct function of the mitotic spindle (Gardner and Burke, 2000; Wassmann and Benezra, 2001).

B. Keeping All in Check: Discovery of Cell-Cycle Checkpoints

Negative regulation of cell-cycle progression became evident in the mid-1970s with the identification of mutations in the simple fission yeast, *Schizosaccharomyces*

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cycle. Progression through the cell cycle requires coordination between several cyclin–Cdk complexes. Mammalian cells spend the majority of the cell-cycle in G_1. The cyclinD–Cdk4/6 complexes control commitment to cell-cycle progression and function early in G_1. CyclinE–Cdk2 complexes control the transition from G_1 into S phase. CyclinA–Cdk2 complexes act during S phase and in G_2. DNA damage-inducible inhibitory proteins known as CKIs, and, to a lesser extent, Wee1, negatively regulate all of these complexes. As with the *S. pombe* cell cycle, the progression through G_2 and into mitosis in higher eukaryotes is under the control of Cdc2, with both cyclinA–Cdc2 and cyclinB–Cdc2 complexes also playing a role in this stage. The relative roles of Wee1 and Cdc25 in the regulation of mitotic cyclin–Cdc2 activity are also the same as in *S. pombe*. Following exit from mitosis, if conditions permit, the cell may reenter another round of the cell cycle. Otherwise, if conditions are not favorable or a cell is programmed to do so, the cell will withdraw from the cell cycle into a quiescent stage, G_0. From this point the cell can wait for the appropriate conditions for cell-cycle reentry or withdraw from the cell cycle and undergo terminal differentiation.

pombe (Fantes, 1977; Nurse, 1975). These mutants entered mitosis early at a reduced cell size. This finding and the pursuant experiments by Nurse and colleagues raised the possibility that the cell cycle contained mechanisms that monitored the successful progression through S phase and mitosis. Moreover, restraint over further cell-cycle progression could be achieved. These observations, initially termed cell-cycle dependencies, were later refined by a seminal study by Weinert and Hartwell (1988) in which a checkpoint responding to genomic damage was first described. The authors reasoned that mechanisms existed as "feedback" (or indeed feedforward) signals to prevent cell-cycle progression in the presence of defects in the cellular division program. Among a collection of radiation-sensitive mutants, Weinert and Hartwell defined a subset of mutants that appeared to be hypersensitive to irradiation due primarily to an inability to halt cell-cycle progression in the presence of DNA damage to allow time for DNA repair. The mechanisms by which these checkpoints affect cell-cycle delay have been elucidated from experiments of many laboratories discussed herein. Checkpoints respond to a wide range of challenges—such as DNA damage, S phase/mitosis coordination, achievement of minimal cell mass, and chromosome organization. However, all checkpoints share the common components of detectors of the problem, signal transducers, and effectors to regulate the activity of cyclin-dependent protein kinases (CDKs), which ultimately drive the cell cycle. CDKs and how they are regulated were discovered by the groundbreaking work of Hunt, Nurse, and many others (Evans *et al.,* 1983; Nasmyth, 2001; Nurse, 1990; Swenson *et al.,* 1986). Together, the importance of discovery of the principles of regulation of CDKs, and the role of checkpoints was exemplified by awarding the Nobel prize in Physiology and Medicine to Hartwell, Nurse, and Hunt in 2001 (Nasmyth, 2001).

C. DNA Damage: The Achilles Heel of Cell-Cycle Progression

The genome of all cells is exposed to a damaging environment. By-products of metabolism and environmental mutagens such as ultraviolet light and the carcinogens created by metabolism attack the genome. To offset these attacks on the genome, cells have evolved repair mechanisms to cope with these stresses. However, cells require time to execute this repair, and this time is provided by cell-cycle checkpoints. Checkpoints that respond to genomic damage have been described in G_1, S, G_2, and perhaps mitosis, and the reader is directed to several excellent reviews (Bartek and Lukas, 2001b; Boddy and Russell, 1999; Chang and Nurse, 1993; Evan and Vousden, 2001; Hwang and Muschel, 1998; Khanna and Jackson, 2001; Nurse, 2000; Ohi and Gould, 1999; Smits and Medema, 2001). Here, we focus on checkpoint signaling events that prevent the entry into mitosis in the presence of DNA damage—the G_2 DNA damage checkpoint.

It is important to consider the consequences of losing such checkpoints—genome integrity relies on both cell-cycle progression and the ability to delay

progression when DNA damage occurs. In the G_1 phase of the cell cycle, failure to delay entry into S phase and repair damaged DNA results in replication of damaged templates, whereas failure at the G_2/M transition can result in the catastrophic segregation of damaged, partially repaired chromosomes. Failure of these checkpoints, particularly at G_1/S, is common in genomically unstable tumors, with the increased mutation rate perhaps contributing to the molecular evolution of cancer cells (Hartwell, 1992; Lengauer *et al.,* 1998; Paulovich *et al.,* 1997). At the G_2/M transition, the topic of this article, checkpoint failure can lead to cell death. Here, we will focus on current knowledge of these signaling events and how they may contribute to development and tumorigenesis.

II. Cdc2: The Mitotic Switch

The engines that drive a cell through the cell cycle are a family of serine/threonine protein kinases known as cyclin-dependent kinases (Cdks). As their name implies, activation of Cdks depends on their binding to the appropriate cyclin partner. Cyclins themselves were first identified in oocytes of marine invertebrates as proteins whose expression oscillated in a cell-cycle-specific manner (Evans *et al.,* 1983; Swenson *et al.,* 1986). Higher eukaryotes contain several Cdks, and, similarly, several cyclins, to guide the cell through S phase and mitosis (Fig. 1). The transition from G_2 into mitosis is regulated by the archetypal Cdk, Cdc2, together with its A- and B-type cyclin partners. As we will be considering the regulation of the G_2 to M cell-cycle transition, and how this is affected by the detection of DNA damage, we must first review the regulation of this complex within the context of normal cell-cycle progression.

A. Activation of Cdc2

Cdc2 was first identified in *S. pombe,* and is essential for mitotic entry in all eukaryotes (Beach *et al.,* 1982; Nasmyth, 1993; Nurse, 1990; Nurse and Bissett, 1981). Much of what we know about the regulation of Cdc2 has come not only from genetic analyses in yeasts and other models, but also from the study of oocyte maturation, which is also under the critical control of Cdc2 (Ferrell, 1999). As with all Cdks, Cdc2 activation requires binding to a cognate cyclin partner, both the B-type cyclins, which are found in all eukaryotes, as well as the related A-type cyclins, which are found only in higher eukaryotes (Evans *et al.,* 1983; Meijer *et al.,* 1991; Roy *et al.,* 1991; Swenson *et al.,* 1986).

Monomeric Cdks are not functional as protein kinases, largely due to the incorrect position of the C-terminal lobe of the protein (Jeffrey *et al.,* 1995). Whereas Cdc2 protein levels are invariant throughout the cell cycle, the protein levels of

A- and B-type cyclins begin to accumulate as the cell nears mitotic entry (Creanor and Mitchison, 1996; Moreno and Nurse, 1990; Pines and Hunter, 1991). Structural studies based on CyclinA–Cdk2 complexes have shown that the association of cyclins with Cdks causes a conformational change in the Cdk subunit, converting it to a more conventional structure similar to other S/T protein kinases (Jeffrey *et al.,* 1995). A region within the Cdc2 activation domain, the T-loop, moves away from the catalytic cleft of the enzyme. This conformational change is stabilized by phosphorylation of a conserved threonine residue within the T-loop by Cdk-activating kinases (CAKs) (Ducommun *et al.,* 1991; Gould *et al.,* 1991). The exposed catalytic cleft of the activated cyclin–Cdk complex can thus catalyze the phosphorylation of a range of target substrates that drive the cell through a particular cell-cycle transition. Thus the accumulation of cyclins creates a larger pool of cyclin-bound, and thus "primed," Cdc2 kinase and is a means of preparing the cell for entry into mitosis.

As the cell progresses through G_2, cyclinA–Cdc2 complexes are found exclusively within the nucleus (Girard *et al.,* 1991; Pines and Hunter, 1991), whereas cyclinB–Cdc2 complexes are largely cytoplasmic (Bailly *et al.,* 1992; Gallant and Nigg, 1992; Pines and Hunter, 1991). However, the cyclinB–Cdc2 complexes shuttle between the nucleus and cytoplasm under the control of sequences that regulate nuclear export and nuclear import (Hagting *et al.,* 1998; Toyoshima *et al.,* 1998). As a cell progresses through G_2, the nuclear export of cyclinB–Cdc2 complexes is predominant over nuclear import (Hagting *et al.,* 1998; Toyoshima *et al.,* 1998). When a cell enters mitosis, nuclear export is suppressed, and cyclinB–Cdc2 complexes accumulate in the nucleus where they phosphorylate substrates that promote progression through mitosis (Clute and Pines, 1999; Hagting *et al.,* 1998; Takizawa and Morgan, 2000).

B. Inhibition of Cdc2

Entry into mitosis represents a rapid switch resulting in massive reorganization to the cellular architecture. The accumulation of cyclins, and thus the potential to activate Cdc2, does not provide an efficient mechanism for a rapid switch promoting mitotic entry. To achieve this, the accumulating cyclin–Cdc2 complexes are inactivated as they form during G_2, and remain inactive until conditions are appropriate for mitotic entry. This control over Cdc2 activity is via reversible phosphorylation and ultimately the regulation of these events represents the critical point to regulate the G_2/M transition, and occurs with or without the imposition of a checkpoint. Alternative mechanisms exist to inhibit G_1/S cyclin–Cdk complexes through their interaction with a group of proteins known as Cdk inhibitory proteins (CKIs). In mammalian cells CKIs include $p21^{cip1}$, $p27^{kip1}$, and $p16^{INK4a}$ (El-Deiry *et al.,* 1993; Harper *et al.,* 1993; Hebert *et al.,* 1994; Polyak *et al.,* 1994) and these respond to checkpoints, but are not the subject of this review. The reader is directed to several

excellent reviews on the regulation of G_1/S Cdk complexes (Sherr and Roberts, 1995, 1999).

The key residue for inhibitory phosphorylation of Cdc2 is tyrosine-15 (Y15) (Fletcher *et al.,* 2002; Gould and Nurse, 1989; Ye *et al.,* 1996). This phosphorylation is catalyzed by the nuclear Wee1 kinases (Lundgren *et al.,* 1991; McGowan and Russell, 1993; Nurse, 1975; Nurse and Thuriaux, 1980; Parker *et al.,* 1991, 1992; Parker and Piwnica-Worms, 1992). In higher eukaryotes, but not the yeasts, the adjacent threonine-14 (T14) (Krek and Nigg, 1991; Norbury *et al.,* 1991) is also phosphorylated by a cytoplasmic kinase, Myt1 (Liu *et al.,* 1997), though only phosphorylation of Y15 is required to inhibit Cdc2 (Fletcher *et al.,* 2002). Once conditions are satisfied for entry into mitosis, Cdc25 phosphatases dephosphorylate Y15, thus activating cyclin–Cdc2 complexes, and cells rapidly enter mitosis (Dunphy and Kumagai, 1991; Gautier *et al.,* 1991; Gould *et al.,* 1990; Kumagai and Dunphy, 1991; Millar *et al.,* 1991; Russell and Nurse, 1986; Strausfeld *et al.,* 1991). The decision to enter mitosis is therefore under the control of opposing forces: a "stop signal" imposed by the Wee1 kinase and a "go signal" imposed by the Cdc25 phosphatase (Fig. 2). Altering this balance is an important mechanism for pathways acting upstream to alter the timing of mitotic entry.

C. Y15 Phosphorylation and the DNA Damage Response

Studies in a number of systems have shown that the maintenance of Y15 phosphorylation is the crucial molecular event that prevents mitotic entry when the G_2 DNA damage checkpoint is evoked. Expression of nonphosphorylatable Y15F mutants of Cdc2 in *S. pombe* (Rhind *et al.,* 1997), *Aspergillus nidulans* (Ye *et al.,* 1996), and human cells (Jin *et al.,* 1996) abrogates the checkpoint response. As the regulation of Cdc2 Y15 phosphorylation is a consequence of the opposing actions of Wee1 and Cdc25, logic would dictate that these two proteins are the key targets for the G_2/M DNA damage response. *S. pombe* cells lacking Wee1, the major Y15 kinase, divide at a smaller size than wild-type cells (hence the "wee" acronym), due to a reduced ability to inactivate Cdc2, which is then reliant on a minor Y15 kinase activity provided by Mik1 (Lundgren *et al.,* 1991). Mik1 is expressed only during S phase, and this window of Cdc2 Y15 phosphorylation accounts for the viability of cells lacking Wee1 (Christensen *et al.,* 2000). Although *wee1*$^-$ cells are radiation hypersensitive, they retain their ability to undergo arrest at G_2 in response to DNA damage. Therefore the inactivation of Wee1 alone is not sufficient to abolish the G_2 checkpoint (Barbet and Carr, 1993), and so upregulation of Wee1 cannot be the only mechanism of checkpoint-mediated arrest. However, cells lacking both Wee1 and Mik1 do not contain Y15-phosphorylated Cdc2 and enter catastrophically premature mitoses once sufficient levels of cyclin–Cdc2 complexes accumulate (Lundgren *et al.,* 1991).

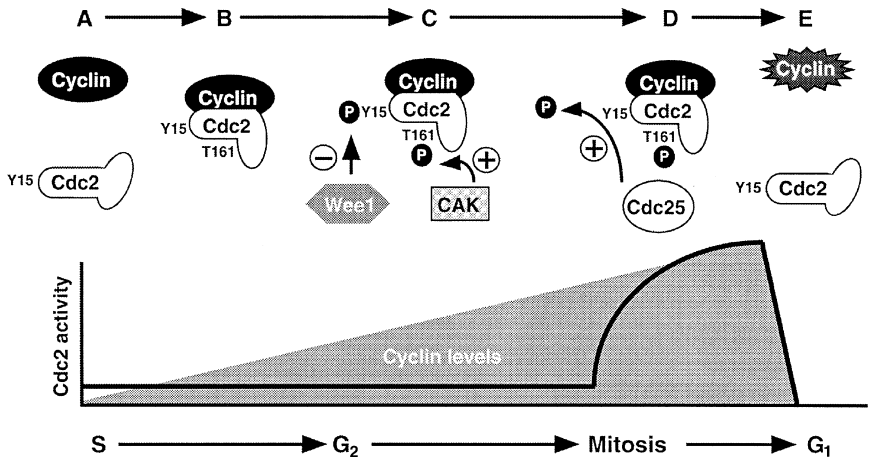

FIG. 2 Regulation of Cdc2 activity. (A) During S phase the levels of mitotic A- and B-type cyclins are low and Cdc2 exists as an inactive monomer. (B) As the cell progresses through S phase into G_2, the levels of mitotic cyclins rise and form complexes with Cdc2. Interaction with cyclin causes a conformational change in Cdc2; the T-loop moves away from the catalytic cleft and exposes a conserved threonine residue (T161 in human Cdc2). (C) The Cdc2–cyclin complexes are phosphorylated by the Cdk-activating kinase (CAK) on T161, but are also inhibited by the phosphorylation of tyrosine-15 (Y15) on Cdc2 by Wee1 kinases. These inactive complexes continue to accumulate throughout G_2 as the cyclins are synthesized. (D) Once the levels of cyclin–Cdc2 complexes have reached a threshold and the cell is ready to enter mitosis, Cdc25 phosphatases promote entry into mitosis by dephosphorylating Y15, thus leading to a rapid increase in Cdc2 kinase activity. (E) At the transition from metaphase to anaphase, mitotic cyclins must be degraded for progression through mitosis leading to chromosome segregation. Cdc2 is thus returned to its inactive, monomeric form, ready for the next round of the cell cycle.

S. pombe cells lacking Cdc25 are unable to enter mitosis and terminally arrest in G_2 due to an inability to activate Cdc2. Such cells can grow only in the presence of compensating (wee) mutations in *cdc2* (Russell and Nurse, 1986) or with the ectopic expression of other tyrosine phosphatases that bypass the requirement for Y15 dephosphorylation by Cdc25 (Gould *et al.,* 1990). However, as with *wee1* mutants, these cells are also checkpoint proficient (Al-Khodairy and Carr, 1992; Sheldrick and Carr, 1993). Only when both Wee1 and Cdc25 are inactivated is the G_2 DNA damage checkpoint abolished and subsequently the cells die due to catastrophic mitoses (Raleigh and O'Connell, 2000).

Therefore, it would appear that to arrest cells in G_2 in response to DNA damage, the signaling pathway that is activated ultimately targets both Wee1 and Cdc25, maintaining the activity of the former and inactivating the latter. In doing so, this ensures that cyclin–Cdc2 complexes are kept inactive by Y15 phosphorylation. A great deal is now known about the signaling pathway leading to DNA damage-activated G_2 checkpoint arrest, and subsequent sections will discuss these components in more detail.

III. The DNA Damage-Mediated G_2 Arrest Signaling Pathway

Having established that maintenance of Y15 phosphorylation of Cdc2 is the mechanism by which the G_2 DNA damage checkpoint prevents entry into mitosis, how is this phosphorylation regulated within the context of the G_2 checkpoint? There are several potentially separate responses to DNA damage that could cumulatively bring about what is currently understood as G_2 arrest in response to DNA damage. First, in terms of initiating the arrest, the recognition of damaged DNA or, alternatively, the presence of repair complexes on chromatin could result in an initiation signal being sent as an emergency stop signal. Then, mechanisms to maintain this arrest and coordinate cell-cycle events with DNA repair machinery could take over. Finally, reversal of the arrest and reentry into the cell cycle may either be a passive event, where the stop signal is no longer maintained, or facilitated by an active restart mechanism that overrides the stop signal. This linear order, or iterations of these events until damage/repair is no longer detected, may serve to prevent the catastrophe of attempting mitosis prior to completion of DNA repair. Most of the data gained thus far have suggested a linear pathway in which one component is affected by a protein upstream and signals to one or more proteins downstream ultimately leading to G_2 arrest in response to DNA damage. However, multiple events are likely to occur simultaneously, in parallel or in different subnuclear locations. Therefore, temporal and spatial arrangements of the components of the DNA damage-activated G_2 checkpoint pathway need to be resolved to gain a complete understanding of the biochemistry and molecular biology of this checkpoint. As our understanding of the molecular events surrounding the G_2 checkpoint improves, the model will likely evolve away from a linear to a more complex model involving several cross-talking pathways working in concert, a theme becoming typical of many signal transduction cascades.

A. The PI3-K-Like Kinases

To date, the earliest measurable step in the signaling pathway leading to the G_2 checkpoint is the activation of a group of phosphatidylinositol 3-kinase (PI3-K)-related proteins. These are large protein kinases, with a C-terminal catalytic domain that has sequence similarity to the phosphatidylinositol lipid kinases (Hunter, 1995). These proteins are divided into two groups based on structure and function. The Rad3 gene in *S. pombe,* and subsequently the ATR gene (for ATM and Rad3-related) in vertebrates, defined the first group. The second group was defined by the human *ATM* gene, which is mutated in the complex disorder ataxia telangiectasia (A-T) (Abraham, 2001) (Table I). Although there is a high degree of structural and functional similarity between the two groups of proteins, as will be discussed, they are likely to have distinct roles in the G_2 checkpoint.

TABLE I
The PI-3K-Like Kinases

S. pombe	Mammals	*Xenopus*	*S. Cerevisiae*	Checkpoints	Treatment
Rad3p	ATR	XAtr	Mec1p	S and G_2	IR, UVR, HU[a]
Tel1p	ATM[b]	XAtm	Tel1p	G_1, S (and G_2?)	IR, HU

[a] IR, UVR, and HU denote ionizing radiation, ultraviolet radiation, and hydroxyurea, respectively. IR induces double-stranded DNA breaks. UVR induces various lesions including cyclobutane dimers and 4-6-photoproducts. HU inhibits DNA replication by depleting nucleotide pools, creating stalled replication forks.

[b] ATM is the only PI-3K like kinase among ATM, Tel1p (from both *S. pombe* and *S. cerevisiae*), and XAtm that has a checkpoint function. The other proteins within this group have no checkpoint function and are involved in telomere length maintenance, although ATM itself is also involved in telomere maintenance. See text for details.

1. ATR/Rad3/Mec1

Rad3 is not essential for cellular viability in *S. pombe,* but is essential for mediating G_2 checkpoint arrest in response to a variety of DNA-damaging agents (Al-Khodairy *et al.,* 1994; Martinho *et al.,* 1998; Walworth and Bernards, 1996). From the analysis of conditional mutants of *rad3,* it has been shown that Rad3 is required for initiation of the G_2 checkpoint in response to DNA damage, but not for G_2 checkpoint maintenance (Martinho *et al.,* 1998). This clearly and importantly delineates that initiation and maintenance of the G_2 damage checkpoint are two separable phenomena.

ATR, the vertebrate homologue of Rad3, is similarly required for the G_2 checkpoint. Expression of dominant negative alleles of ATR renders cells susceptible to all forms of DNA damage (Cliby *et al.,* 1998; Wright *et al.,* 1998). Somatic $ATR^{-/-}$ cells generated by sequentially deleting both alleles are incapable of long-term viability in culture. However, these cells do lack a G_2 checkpoint response (Cortez *et al.,* 2001). Furthermore, the G_2 checkpoint is also abolished when ATR is immunodepleted from *Xenopus* oocyte extracts (Guo *et al.,* 2000). These data clearly show ATR, like Rad3, is an essential component of G_2 checkpoint signaling in vertebrates.

2. ATM/Tel1

Cells from A-T patients display defects in multiple DNA damage-activated checkpoints in response to ionizing radiation (IR) (Pandita *et al.,* 2000). Curiously, A-T cells are not hypersensitive to other types of DNA damage, such as that caused by ultraviolet radiation (UVR). Mice lacking ATM display the phenotypes seen in A-T, including checkpoint defects (Abraham, 2001; Hawley and Friend, 1996; Xu and Baltimore, 1996). This suggests that ATM plays a key role in cellular responses to specific types of DNA damage, including invoking the G_2 checkpoint.

In the yeasts, the closest ATM homolog, Tel1, is involved in the maintenance of telomere length (Greenwell *et al.,* 1995; Matsuura *et al.,* 1999). A-T cells also undergo premature senescence suggesting that ATM is also involved in telomere length maintenance in humans. Although XAtm, the *Xenopus* homolog of ATM, has been shown to inhibit replication in response to DNA damage, no evidence as yet has established a role for XAtm in the G_2 checkpoint. This raises the question as to the precise role of ATM homologs in the G_2 checkpoint (see below).

3. ATM versus ATR in the G_2 Checkpoint

Although ATM and ATR have been implicated in the G_2 checkpoint, the relative contributions of the two kinases remain to be determined. The current data suggest that ATM is involved in an IR-induced G_2 checkpoint, whereas ATR is involved in the G_2 checkpoint in response to many forms of DNA damage, including IR. There are considerable data to link ATM to G_1 and S phase checkpoint responses, through the phosphorylation of proteins such as p53 (Banin *et al.,* 1998; Canman *et al.,* 1998), BRCA1 (Gatei *et al.,* 2000) and Nbs1 (Lim *et al.,* 2000). With the lack of an essential G_2 checkpoint function for the yeast Tel1 proteins, it is possible that the ATR group is a more specific regulator of G_2 events, with the ATM group playing more diverse roles without being absolutely essential for DNA damage-mediated G_2 arrest. Further work is required to clarify the relative roles of these two kinases before the delineation of responsibility between ATM and ATR in the G_2 checkpoint can be more fully understood.

B. The Rad Checkpoint Proteins

A number of approaches have been used to identify genes required for G_2 checkpoint arrest. Genetic screens in the yeasts have proven particularly fruitful in this regard. From the cloning of vertebrate homologs and subsequent biochemistry and cell biology, a detailed picture of signaling events in this checkpoint has emerged in recent years. These additional G_2 checkpoint genes and what is known about their function are outlined in the following sections.

1. Partners of the PI3-K-Like Kinases

The earliest measurable biochemical event following DNA damage in G_2 is the phosphorylation of a Rad3-binding protein, Rad26, catalyzed by Rad3 itself. This occurs independently of all other proteins known to be involved in the G_2 checkpoint pathway (Edwards *et al.,* 1999). Cells lacking Rad26 are also G_2 checkpoint defective, and are defective for any other known biochemical events in the G_2 checkpoint pathway (see below). Recent biochemical evidence suggests a role for Rad26 as a positive regulatory subunit of Rad3 (Wolkow and Enoch, 2002),

although it remains unclear as to the significance of the phosphorylation of Rad26 by Rad3 in mediating a G_2 checkpoint response.

A homolog of *S. pombe* Rad26 has been identified in *S. cerevisiae,* Ddc2 (also known as Pie1 and Lcd1) (Paciotti *et al.,* 1998; Rouse and Jackson, 2000; Wakayama *et al.,* 2001). Like Rad26, Ddc2 interacts with, and is phosphorylated by, Mec1 (the Rad3 homolog), independently of other checkpoint proteins. Similarly, the *A. nidulans* Rad26 homolog, UVSD, interacts with the Rad3 homolog UVSB (De Souza *et al.,* 1999).

A putative human homolog of Rad26 has been identified, with weak homology to Rad26 and UVSD. Named ATRIP for ATR Interacting Protein (Cortez *et al.,* 2001), it was identified on the basis of its ability to interact with, and be phosphorylated by, ATR *in vitro*. *In vivo,* ATRIP and ATR colocalize in response to DNA damage, presumably at the sites of damage. Inhibition of either ATRIP and ATR expression caused defects in the DNA damage-mediated G_2 checkpoint. Given the similar effects observed with Rad3 and Rad26, it was suggested that ATRIP is a functional homolog of Rad26 (Cortez *et al.,* 2001). Although some functional details remain to be confirmed, given the similarities observed thus far, it is very likely that ATRIP is indeed a Rad26 homolog.

2. Rad17, Rad1, Rad9, and Hus1

Rad3 and Rad26 are two members of a family of six known checkpoint "Rad" proteins in *S. pombe.* The other members include Rad1, Rad9, Hus1, and Rad17 in *S. pombe* and mammals, and are known as Rad17, Ddc1, Mec3, and Rad24, respectively, in *S. cerevisiae* (see Table II). When any of these genes are deleted, cells lack a G_2 DNA damage checkpoint response. In addition to genetic data linking the function of these six proteins, there are physical and functional interactions between them that are important for DNA damage checkpoint responses.

Rad1, Rad9, and Hus1 bear limited primary amino acid similarity to the proliferating cell nuclear antigen (PCNA). During DNA replication, PCNA forms a homotrimeric donut-shaped structure that forms a "sliding clamp" that encircles DNA and helps to tether DNA polymerase δ to its template, thus providing processivity to the enzyme (Stillman, 1994). The PCNA clamp structure is loaded onto DNA initially by replication factor C (RFC), a complex consisting of one large subunit (RFC1) and four smaller subunits (RFC2, RFC3, RFC4, and RFC5). This complex recognizes primer–template junctions and serves to open the PCNA "donut" and loads it onto the DNA in an ATP-dependent manner (Cullmann *et al.,* 1995; Mossi and Hubscher, 1998; Waga and Stillman, 1998). Molecular modeling has postulated more extensive structural similarities between PCNA and Rad1, Rad9, and Hus1, raising the possibility that these proteins might form a heterotrimeric checkpoint sliding clamp (CSC) complex (Thelen *et al.,* 1999; Venclovas and Thelen, 2000). In fact, biochemical characterization has revealed a more complex pattern of higher order interactions between CSC members, with a heterotrimer being one

TABLE II
The "Rad" Checkpoint Proteins

S. pombe	Mammals	*S. Cerevisiae*	Function
Rad26p	ATRIP	Ddc2p	Regulatory subunit for Rad3/ATR/MEC1[a]
Rad17p	Rad17	Rad24p	RFC1-like protein Checkpoint loading complex[b]
Rad1p	Rad1	Rad17p	PCNA-like protein Checkpoint sliding clamp[c]
Rad9p	Rad9	Ddc1p	PCNA-like protein Checkpoint sliding clamp[c]
Hus1p	Hus1	Mec3p	PCNA-like protein Checkpoint sliding clamp[c]

[a] Rad26p, ATRIP, and Ddc2 interact with Rad3p, ATR, and Mec1p, respectively. This interaction is required for activation of the PI-3K-like kinases. In response to DNA damage, the complex then localizes to the site of DNA damage independently of other checkpoint Rad proteins.

[b] These replication factor C-1 (RFC1)-like proteins are part of multimeric complexes that include RFC2, RFC3, RFC4, and RFC5. They function to load the checkpoint-sliding clamp (CSC) onto DNA at the site of damage. Localization to the site of DNA damage depends on the CSC proteins and proximity to the ATR/ATRIP complex. Phosphorylation then occurs in a Rad3p/ATR/Mec1p-dependent manner.

[c] These three proteins are hypothesized to form a trimeric donut-shaped structure similar to that seen with the proliferating cell nuclear antigen (PCNA) (refer to Fig. 2) that encircles DNA proximal to the site of damage. They are also phosphorylated in a Rad3p/ATR/Mec1p-dependent manner. See text for details.

possible complex among others (Caspari *et al.,* 2000; Kaur *et al.,* 2001; Kostrub *et al.,* 1998). Recently, similar complexes among human Rad1, Rad9, and Hus1 were described using both *in vivo* and *in vitro* approaches (Burtelow *et al.,* 2001; Rauen *et al.,* 2000; St. Onge *et al.,* 1999). Furthermore, phosphorylation of both Hus1 and Rad9, most likely by Rad3/ATR, has been described. The functional significance of the phosphorylation of these proteins is not known, but two possibilities have been suggested: it may stabilize the trimeric complex, or, alternatively, stabilize the individual proteins themselves (M. J. Chen *et al.,* 2001), but direct evidence supporting these models is yet to be reported.

Rad17 bears limited similarity to RFC1 and physically interacts with the smaller RFC subunits that make up the RFC complex (Lindsey-Boltz *et al.,* 2001). Indeed, genetic studies in *S. pombe* (Shimada *et al.,* 1999), *S. cerevisiae* (Kim and Brill, 2001; Naiki *et al.,* 2000), and *Drosophila melanogaster* (Krause *et al.,* 2001) have demonstrated checkpoint functions for the smaller RFC subunits. Based on the similarities with RFC1, it has been suggested that Rad17 may be part of a checkpoint-loading complex (CLC) that loads the CSC onto DNA in response to DNA damage. Human Rad17 is able to associate with the CSC complex *in vivo* via

a direct physical interaction with Rad1 (Rauen *et al.,* 2000). This interaction can be induced in response to DNA damage and correlates with the ATM/R-dependent phosphorylation of Rad17 (Bao *et al.,* 2001). Nonphosphorylatable mutants of Rad17 fail to undergo G_2 arrest in response to DNA damage in human cells, and are not capable of interacting with Rad1. It is possible that phosphorylation of Rad17 by ATM/R perhaps promotes an interaction between Rad17 and the CSC complex, and may facilitate loading of the Rad1–Rad9–Hus1 PCNA-like structure onto DNA (Bao *et al.,* 2001).

In the absence of DNA damage, the complex of Rad17 and the small RFC subunits is associated with the CSC consisting of Rad1–Rad9–Hus1 in human cells (Rauen *et al.,* 2000). All of the proteins are readily extractable in nonionic detergent and low salt, suggesting that the entire nuclear CLC–CSC complex is soluble and only loosely associated with the chromatin (Rauen *et al.,* 2000). However, in response to DNA damage, the CSC complex becomes extraction resistant, suggesting it is tightly associated with DNA, whereas Rad17 remains in the soluble fraction. These data suggest that in both humans and in *S. pombe,* Rad17 recognizes structural alterations to DNA and clamps the CSC complex onto DNA, thus converting it to an extraction-resistant complex (Bao *et al.,* 2001; Burtelow *et al.,* 2000; Kai *et al.,* 2001).

The phosphorylation of Rad17 and members of the CSC by the PI3-K-like kinases suggests a linear pathway in which the CLC and the CSC function downstream of Rad3–Rad26. A number of recent studies have shown that a more complex level of coordination among the checkpoint Rad proteins. An elegant experimental system was utilized in *S. cerevisiae* whereby a single double-stranded break was generated. In this system, Ddc1 (Rad9 in humans and *S. pombe*) formed a single focus and this focus formation was dependent on the presence of both the CLC protein Rad24 (Rad17 in humans and *S. pombe*) and the CSC proteins. However, this localization was not dependent on the presence of Mec1 (Rad3 in *S. pombe* and ATR in humans) (Kondo *et al.,* 2001; Melo *et al.,* 2001). Ddc2 (Rad26 in *S. pombe* and ATRIP in humans) formed similar foci in response to DNA damage, which is dependent on Mec1 but not on any of the other Rad proteins. Thus two complexes, one consisting of Ddc2 and Mec1 and the other likely consisting of the CSC and CLC complexes, are recruited to the sites of DNA damage independently of each other (Kondo *et al.,* 2001; Melo *et al.,* 2001).

The apparent independence of the recruitment of the Rad3–Rad26 complex and the other checkpoint Rad proteins to chromatin in response to DNA damage was also demonstrated in mammalian cells (Zou *et al.,* 2002). Although the lack of Rad17 affected the localization of Rad9 to the chromatin in response to DNA damage, ATR was still capable of localizing to chromatin. Conversely, localization of Rad17 or Rad9 to DNA was not ATR dependent. However, it appears that ATR-dependent phosphorylation of chromatin-associated Rad17 following DNA damage requires that the CSC be localized to DNA lesions, suggesting that the CSC helps to target substrates for phosphorylation by ATR (Zou *et al.,* 2002).

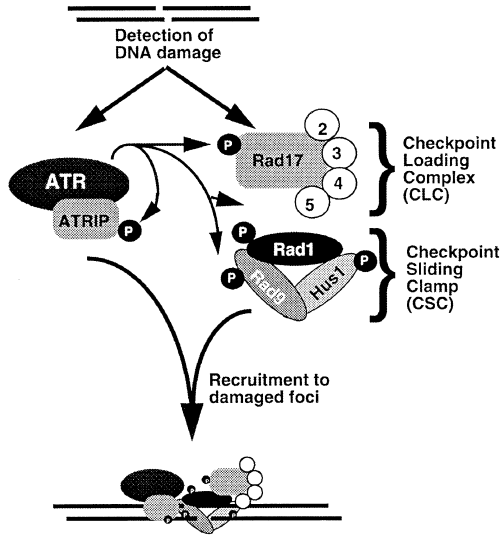

FIG. 3 Targeting of Rad checkpoint proteins to sites of DNA damage. A generalized model of events following checkpoint activation using data from a number of systems is depicted (see text for details). Although the upstream events involving detection of DNA damage and initiation of the checkpoint pathway have yet to be defined, DNA damage in G_2 causes the activation of the PI-3-like kinase ATR (Rad3 in *S. pombe*). Activated ATR phosphorylates its binding partner ATRIP (Rad26 in *S. pombe*), and the complex localizes to the site of DNA damage. Localizations of ATR and ATRIP are codependent, but this occurs independently of the other checkpoint Rad proteins. In parallel, both the checkpoint-loading complex and the checkpoint-sliding clamp are recruited to the site of DNA damage. The colocalization of all the checkpoint Rad genes to the site of DNA damage allows the phosphorylation of Rad17, Rad1, Rad9, and Hus1 in an ATR-dependent manner.

These data suggest that two major complexes, one consisting of the CSC and CLC and the other of Rad3–Rad26, may be modified simultaneously in some way in response to DNA damage. These modifications allow the two complexes to localize to the sites of DNA damage independently of each other. This would allow the CLC, the CSC, and Rad3–Rad26 to come into proximity with each other, and thereby the CLC and the CSC can become phosphorylated by the Rad3–Rad26 complex (Fig. 3). However, the fundamental question still remains as to how these two complexes localize to the sites of DNA damage. It remains to be seen whether these two complexes are bona fide DNA damage sensors or if one or more pathways play a role in targeting the complexes to DNA.

C. The Chk1 Effector Protein Kinase

Chk1 was identified in *S. pombe* as a protein that functioned downstream of the checkpoint Rad genes (Walworth *et al.,* 1993). Chk1 is not essential for viability in the absence of heightened levels of DNA damage, but is essential for G_2

checkpoint function in response to a number of DNA-damaging agents (Walworth *et al.,* 1993). Chk1 overexpression imposes a G_2 arrest in both wild-type cells and in all checkpoint Rad mutants. Moderate overexpression of Chk1 does not cause a G_2 arrest, although it can rescue the radiation sensitivity of the Rad mutants, placing *chk1* genetically downstream of the Rad genes (Al-Khodairy *et al.,* 1994; Martinho *et al.,* 1998; Walworth *et al.,* 1993; Walworth and Bernards, 1996). In *S. pombe,* the DNA-damage hypersensitivity of *chk1*Δ cells, and the damage-mediated phosphorylation of Chk1 occurs only after DNA has been replicated, placing Chk1 function in a temporal window between S phase and mitosis (Al-Khodairy *et al.,* 1994; Martinho *et al.,* 1998; Walworth *et al.,* 1993). Moreover, *chk1*Δ mutants are capable of arrest in the presence of the replication inhibitor hydroxyurea (HU), which arrests cells very early in S phase. Thus, at least in *S. pombe,* Chk1 is not involved in the replication checkpoint under these conditions, whereas all genes described above are required to prevent mitosis in the presence of HU (Martinho *et al.,* 1998).

Serine-345 (S345) of Chk1 is phosphorylated in response to DNA damage in *S. pombe,* human cells, and *Xenopus* (Guo *et al.,* 2000; Liu *et al.,* 2000; Lopez-Girona *et al.,* 2001). In *Xenopus* and human cells, phosphorylation of S345 of Chk1 in response to DNA damage is dependent on ATR activity, whereas in *S. pombe* this phosphorylation occurs in a Rad3-dependent manner. In humans, an additional site, serine-317, is also phosphorylated in an ATR-dependent manner (Weiss *et al.,* 2002; Zhao and Piwinica-Worms, 2001). Depletion of XAtr, the *Xenopus* ATR homolog, from oocyte extracts both prevents phosphorylation of XChk1 and leads to cell-cycle defects (Guo *et al.,* 2000). Based on these data, it is highly likely that Chk1 is activated directly by ATR-mediated phosphorylation.

The phosphorylation of Chk1 in *S. pombe* is dependent on the integrity of all the upstream checkpoint Rad genes (Walworth *et al.,* 1993) (Fig. 4). It was recently demonstrated in murine fibroblasts that the ability of ATR to phosphorylate Chk1 in response to DNA damage was dependent on Hus1 (Weiss *et al.,* 2002). Although Chk1 is a nuclear protein, there are no published data to place Chk1 at damaged foci coincident with the localization of Hus1, and so it is not clear whether Chk1 phosphorylation occurs at DNA damage foci or elsewhere within the nucleus.

In *S. pombe,* DNA damage and the subsequent phosphorylation of Chk1 stimulate an interaction between Chk1 and the homologs of 14-3-3 proteins, Rad24 and Rad25 (L. Chen *et al.,* 1999). 14-3-3 proteins are small acidic proteins that bind serine/threonine-phosphorylated proteins and regulate key aspects of signal transduction and intracellular signaling (Tzivion and Avruch, 2002; Tzivion *et al.,* 2001). Although this association requires the DNA damage-mediated phosphorylation of Chk1, it is not dependent on Chk1 kinase function. The significance of this interaction is not known. As 14-3-3 proteins are largely thought to function as scaffolding or adaptor proteins, perhaps the interaction between these two proteins may help to target Chk1 to specific substrates (L. Chen *et al.,* 1999). However, it is noteworthy that Chk1 does not bear a consensus 14-3-3 binding site; this interaction has so far been shown to occur only in *S. pombe*.

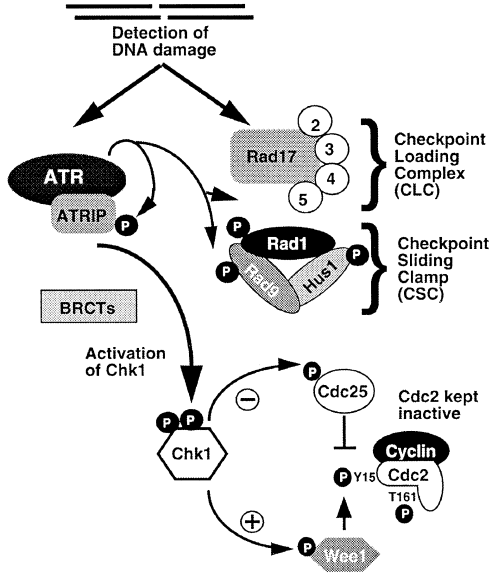

FIG. 4 Activation of Chk1 and the "double lock" model. DNA damage-induced Chk1 activation occurs via phosphorylation on serine-345 (in *S. pombe,* humans, and *Xenopus*) as well as serine-317 (in humans and *Xenopus*) by ATR/Rad3. Activation of Chk1 depends on the presence of all the checkpoint Rad proteins. In *S. pombe,* activation of Chk1 also depends on the presence of the BRCT-domain-containing proteins Crb2 and Cut5. Activated Chk1 phosphorylates Wee1, increasing protein levels, which in turn maintains inhibitory Cdc2 Y15 phosphorylation. Chk1 also phosphorylates Cdc25 and prevents it from dephosphorylating Y15 on Cdc2 by inhibiting its phosphatase activity. By acting on both Wee1 and Cdc25, Chk1 indirectly maintains Cdc2 in an inactive state and prevents mitotic entry after DNA damage has occurred.

Another Chk1-interacting protein, claspin, was identified by its copurification with Chk1 in *Xenopus* oocyte extracts (Kumagai and Dunphy, 2000). Phosphorylation of claspin, possibly by Chk1, is required for this interaction. Depletion of claspin blocks Chk1 activation and checkpoint responses, and so claspin seems to play a role in the phosphorylation and activation of XChk1 (Kumagai and Dunphy, 2000). Claspin may function to promote the activation of Chk1 by acting as a scaffolding or adaptor protein that presents Chk1 to an appropriate kinase, such as ATR, in a mechanism similar to the proposed role for the interaction between Chk1 and Rad24. Although a human homolog of claspin has been identified, homology searches have not identified any possible candidates for claspin in *S. pombe* or other lower eukaryotes, although functional homologs may exist.

D. The "Double Lock" Model of G_2 Arrest

As discussed above, two different proteins function in opposition to each other to regulate the activity of cyclin–Cdc2 complexes: the Wee1 kinases that catalyzes

the inhibitory Y15 phosphorylation on Cdc2 and the Cdc25 phosphatases that remove the inhibitory phosphorylation. Mutation of either *wee1* or *cdc25* alone does not lead to a loss of checkpoint proficiency in response to DNA damage in *S. pombe,* but when both genes are mutated checkpoint defects become apparent (Al-Khodairy and Carr, 1992; Barbet and Carr, 1993; Raleigh and O'Connell, 2000; Sheldrick and Carr, 1993). Deletion of *chk1,* or any one of the checkpoint Rad genes, does lead to G_2 checkpoint defects similar to a *wee1cdc25* double mutant. However, the G_2 checkpoint induced by overexpression of Chk1 depends on the presence of both Wee1 and Cdc25. This suggests that Chk1 functions upstream of both Wee1 and Cdc25 and targets both proteins when the G_2 checkpoint is invoked (Raleigh and O'Connell, 2000).

Under *in vitro* assay conditions, Chk1 phosphorylates both Wee1 (Lee *et al.,* 2001; O'Connell *et al.,* 1997; Price *et al.,* 2000; Raleigh and O'Connell, 2000) and Cdc25 (Kumagai *et al.,* 1998; Peng *et al.,* 1997; Sanchez *et al.,* 1997) proteins from a number of species. Although there are considerable data to indicate that Chk1 directly phosphorylates Wee1 in *S. pombe* cells, this phosphorylation did not appear to alter kinase activity of Wee1, at least when reconstituted *in vitro* (O'Connell *et al.,* 1997). However, in response to DNA damage the endogenous Wee1 becomes hyperphosphorylated by Chk1. This leads to a transient increase in Wee1 stability (Raleigh and O'Connell, 2000). This would significantly raise the cellular pools of Wee1 activity, which may be required to ensure inactivation of accumulating cyclinB–Cdc2 complexes. A similar stabilization of Wee1 has been demonstrated in *Xenopus* oocyte extracts with replication blocks (Michael and Newport, 1998), and in *S. cerevisiae* during meiotic DNA damage checkpoints when completion of recombination is blocked by a number of mutations (Leu and Roeder, 1999). Another alternative mechanism operating in *Xenopus* oocyte extracts has been proposed to modulate a Chk1-dependent Wee1 upregulation. Wee1 binds a 14-3-3 family member and coexpression of human Wee1 and 14-3-3 in baculovirus led to an increase in active Wee1 (Honda *et al.,* 1997; Wang *et al.,* 2000b). Dunphy and colleagues have demonstrated a link among Wee1, Chk1, and 14-3-3 proteins in the *Xenopus* oocyte system (Lee *et al.,* 2001). In this study, the association of XWee1 and 14-3-3 proteins could be detected in interphase but not during M phase, and this interaction was related to the phosphorylation state of XWee1. Furthermore, XChk1 phosphorylation of XWee1 on serine-549 (S549) promoted association with 14-3-3 proteins and mutation of this site to an alanine residue abolished 14-3-3 binding and with it XWee1 protein stability and kinase activity. Finally, recent studies with a tyrosine kinase inhibitor, PD0166285, implicate Wee1 as a checkpoint target in human cells (Li *et al.,* 2002; Wang *et al.,* 2001). Collectively, these accumulating data support a conserved role for Wee1 proteins in signaling a G_2 checkpoint arrest.

Genetic analyses in *S. pombe* clearly demonstrate the importance of maintaining Cdc25 in an inactive state throughout a G_2 checkpoint arrest (Furnari *et al.,* 1997; Lopez-Girona *et al.,* 1999; Zeng and Piwnica-Worms, 1999). However,

mechanisms of action are not yet clear, as some potentially conflicting studies have been published over the past 5 years. In *S. pombe,* it was initially proposed that phosphorylation of Cdc25 by Chk1 facilitated the interaction between Cdc25 and 14-3-3 proteins (Peng *et al.,* 1997). This interaction was further hypothesized to block the nuclear localization of Cdc25 in a number of experimental systems, thus sequestering it into the cytoplasm (Dalal *et al.,* 1999; Kumagai and Dunphy, 1999; Yang *et al.,* 1999; Zeng and Piwnica-Worms, 1999). In *S. pombe,* this would separate Cdc25 from the nuclear pools of cyclinB–Cdc2 (Alfa *et al.,* 1989, 1990), and was thus an attractive model. However, as discussed above, cyclinB–Cdc2 complexes are cytoplasmic during G_2 in higher organisms, which makes a model involving Cdc25 sequestration less attractive. For human cells, the localization of Cdc25 isoforms during interphase has been reported to be either nuclear, cytoplasmic, or both (Davezac *et al.,* 2000; Graves *et al.,* 2001; Karlsson *et al.,* 1999), and so it appears these assays may differ with reagents and/or cell type; live cell analyses would be extremely useful to clarify this. However, the phosphorylation and 14-3-3 association of Cdc25 occurs in normal G_2 cells, and in *S. pombe* these events are unaffected by deletion of *chk1* (L. Chen *et al.,* 1999). More recent evidence suggests that the localization of Cdc25 is not critical for checkpoint arrest; rather it is the catalytic inactivation of Cdc25 that appears to be more important (Lopez-Girona *et al.,* 2001). Chk1 phosphorylates Cdc25 on several sites and not all of these would form a consensus 14-3-3 binding site (Walworth, 2001). Thus the phosphorylation of Cdc25 by Chk1 may serve to inactivate the phosphatase activity in a more direct fashion rather than promoting a change in cellular localization.

Although there is only one predominant form of Cdc25 in the yeasts (Ducommun *et al.,* 1990; Moreno *et al.,* 1990; Russell and Nurse, 1986), mammals have three different isoforms of Cdc25, A, B, and C (Galaktionov and Beach, 1991; Nagata *et al.,* 1991). Of the three, only Cdc25B and C are thought to be important in the promotion of mitosis and appear to have nonredundant functions (Gabrielli *et al.,* 1996; Lammer *et al.,* 1998; Millar *et al.,* 1991b). These phosphatases primarily function in different phases of the cell cycle, with Cdc25B from S phase to mitosis, and Cdc25C only in late G_2 (Gabrielli *et al.,* 1996; Lammer *et al.,* 1998; Nishijima *et al.,* 1997). Overexpression of Cdc25B causes cells to inappropriately enter mitosis from either the S or G_2 phase, whereas overexpression of Cdc25C has no effect on the timing of mitosis unless cyclin B1 is also overexpressed (Heald *et al.,* 1993; Karlsson *et al.,* 1999).

Pharmacological studies utilizing caffeine, or the kinase inhibitor UCN-01, have shown that Chk1 is inhibited *in vitro* (Busby *et al.,* 2000; Graves *et al.,* 2000), and *in vivo* this leads to an abrogation of Cdc25C phosphorylation (Graves *et al.,* 2000). Although UCN-01 is not specific for Chk1 (Kawakami *et al.,* 1996), these data suggest that Cdc25C is a target for inactivation by Chk1 that may contribute to the G_2 delay (Graves *et al.,* 2000). However, Cdc25C nullizygous mice exhibit no discernible phenotype, at both the whole animal level and in cells derived from

these mice (M. S. Chen *et al.,* 2001). It is possible that Cdc25B and/or Cdc25A are able to compensate for the loss of Cdc25C, although disruption of these genes in mice is yet to be reported.

From the genetic and biochemical data discussed herein, a picture of a "double-lock" model emerges for initiating G_2/M arrest. In this model, DNA damage engages the Rad checkpoint proteins to signal to Chk1, leading to its activation in a Rad3/ATR-dependent fashion. Activated Chk1, in turn, phosphorylates Wee1, either helping to maintain Wee1 in an active state or enhancing the stability of Wee1, thus allowing Wee1 to more effectively maintain Cdc2-Y15 phosphorylation. Simultaneously, Chk1 also phosphorylates Cdc25 and downregulates Cdc25 activity, either through regulating the subcellular localization of Cdc25, its activity, or both (Fig. 4). Thus, preexisting cyclin–Cdc2 complexes are kept in an inactive, Y15 phosphorylated state, and Wee1 ensures the new cyclin–Cdc2 complexes are inactivated as they form. Given the importance of the G_2 checkpoint to cellular integrity, it is perhaps not surprising that such a mechanism has evolved, ensuring arrest even if one arm of the pathway is nonfunctional.

Although this model of checkpoint control is in agreement with the current data in *S. pombe, Xenopus,* and human cells, it is possible that variations on this theme or even alternative mechanisms may exist. One example of this is *S. cerevisiae,* in which inhibitory phosphorylation of Cdc28 (the Cdc2 homolog) appears to be less important in the mitotic cycle. Chk1, and another effector kinase, Rad53, signal cell cycle arrest at the metaphase–anaphase transition, by regulating the activity of the anaphase-promoting complex (Sanchez *et al.,* 1999). It is possible that species or context-specific variations on the double-lock model occur in other systems, although to date there have been no reports of experiments that support this hypothesis.

IV. The G_2 Checkpoint and Development

In *S. pombe,* deletion of the checkpoint Rad proteins does not have an effect on cellular proliferation. Therefore, they are dispensable for normal cell growth. It is only in response to DNA damage or when DNA synthesis is blocked that these proteins are activated and loss of any of these proteins leads to checkpoint defects resulting in cell death. In contrast, studies using both murine and *Drosophila* models have illustrated that several of the checkpoint Rad proteins have essential roles in embryonic development and, in some cases, the proliferation of mammalian somatic cells *in vitro*.

The first 13 cycles of a *Drosophila* embryo are controlled by maternally provided mRNAs. They consist of very rapid nuclear division cycles of alternating S phases and mitoses that occur without gap phases or cytokinesis. At cycle 14, zygotic transcription is initiated and the G_2 phase lengthens, cellularization starts to

occur, although G_1 phases still do not exist (O'Farrell *et al.,* 1989). Interestingly, *Drosophila* embryos that lack either Mei-41 (ATR), grapes (Chk1), and dWee1 (Wee1) exhibit the same phenotype. The first 13 mitoses in *Drosophila* embryos lacking these maternally supplied proteins are unusually short and fail to terminate at a stage when cellularization occurs. Instead the embryonic blastoderm degenerates without forming cells (Fogarty *et al.,* 1994, 1997; Price *et al.,* 2000; Sibon *et al.,* 1999).

The fact that lack of functional ATM leads to a definite clinical phenotype (ataxia telangiectasia) suggests that ATM is likely to be dispensable for development (Xu *et al.,* 1996; Xu and Baltimore, 1996). However, deletion of ATR in mice results in periimplantation embryonic lethality caused by extensive chromosomal fragmentation (Brown and Baltimore, 2000). Mice lacking Chk1 also die at the periimplantation stage (Liu *et al.,* 2000; Takai *et al.,* 2000). These data highlight the essential nature of an intact G_2 checkpoint for early murine embryonic development, particularly in light of the fact that early embryos lack a G_1 checkpoint (Schmidt-Kastner *et al.,* 1998).

Using conditionally deleted Chk1 ES cells it was shown that Chk1, like its yeast counterpart, plays an essential role in G_2/M arrest in response to DNA damage. In these Chk1-deficient ES cells, there were more cells in mitosis in response to DNA damage compared to unirradiated controls (Liu *et al.,* 2000). However, given that the conditional mutants die within 2 days of deletion of Chk1, it is not clear whether this accumulation of mitotic cells is the result of a specific checkpoint defect due to the absence of Chk1 or a general mitotic catastrophe. Disruption of Chk1 function in somatic cells grown in culture, however, leads to viable cells that are checkpoint defective (Koniaras *et al.,* 2001), suggesting that the essential nature of Chk1 in mammalian development may be restricted to embryogenesis.

Conditional deletion of ATR in mammalian cells causes a high level of apoptosis. Therefore, ATR is likely to have some essential function in normal cell proliferation, perhaps in ensuring successful DNA replication (Cortez *et al.,* 2001). It is notable that in *S. pombe,* the deletion of *rad3* confers the additional phenotypes of telomere shortening (Dahlen *et al.,* 1998) and reduced DNA damage tolerance (Bentley *et al.,* 1996; Martinho *et al.,* 1998), which are not evident in *chk1*Δ cells.

Deletion of Hus1, however, displays a later embryonic phenotype than that observed for $ATR^{-/-}$ and $Chk1^{-/-}$ mice. Hus1 null embryos gastrulate normally but display gross developmental and morphological defects. Lack of Hus1 leads to a slight but detectable increase in a number of DNA-damage-inducible genes such as $p21^{WAF1/Cip1}$, which is likely to be due to the increase in chromosomal abnormalities observed in primary cultures of cells lacking Hus1. As such, $Hus1^{-/-}$ MEFs failed to proliferate in culture, whereas deletion of both p21 and Hus1 allowed a small number of MEF cultures derived from the embryos to proliferate despite widespread chromosome damage (Weiss *et al.,* 2000).

Wiess *et al.* (2002) suggested that ATR has additional Hus1-independent functions required for embryonic development as a means of explaining the phenotype

differences between $ATR^{-/-}$ and $Hus1^{-/-}$ mice. As DNA damage-induced Chk1 activation is dependent on the presence of Hus1 (Weiss *et al.,* 2002), Hus1 must therefore act upstream of Chk1. There are three possible explanations that may account for these phenotypic observations. One is that Chk1, like ATR, also has Hus1-independent functions in early embryonic development. The other explanation stems from the fact that mice of different genetic backgrounds were used. The ATR and Chk1 knockouts were generated using mice of the same genetic background (C57BL), whereas the Hus1 knockout mouse was generated using a mouse of a different background (Swiss Black). It has already been established that knocking out the same gene in mice of different genetic backgrounds can have an effect on the observed phenotype (Pearson, 2002). A third possibility is that in $Hus1^{-/-}$ cells, the remaining members of the CSC complex, Rad1 and Rad9, are still able to form a trimeric complex (Caspari *et al.,* 2000), which can only partially compensate for the loss of Hus1.

Clearly the lethal phenotypes exhibited by deletion of most known G_2 checkpoint proteins demonstrate that an intact G_2 checkpoint plays a role in development, at least during embryogenesis. The chromosome fragmentation effect seen in these knockouts shows that chromosomal integrity is required in the embryonic cell cycle. It also means that human diseases resulting from highly penetrant germ-line mutation of these checkpoint genes are unlikely to exist.

V. Additional Components in Checkpoint Signaling

We have described the best understood components and the pathway leading to G_2 arrest via Y15 phosphorylation of Cdc2 in response to DNA damage. At the periphery of this pathway exist several other proteins for which a role in the G_2 checkpoint has been assigned (Table III). The precise function of many of these proteins in the G_2 checkpoint remains to be determined. This next section describes some of these additional components and their putative roles in G_2 checkpoint control.

A. The BRCT Domain Proteins

There exists an increasingly large group of proteins for which a role in the DNA damage checkpoint appears to be unambiguous, but their precise biochemical function is poorly understood. Several of these proteins are known as the BRCT domain proteins. Their name derives from domains that share homology to the C-terminal domain of the breast and ovarian cancer susceptibility gene product BRCA1 (BRCT = BRCA1 C-Terminus). The BRCT domain is found in many proteins, although all appear to be involved in DNA metabolism. The

TABLE III
Other G_2 Checkpoint Proteins

S. pombe	Mammals	*S. Cerevisiae*	Features and functions
Cut5p	TopBP1	Dpb11p	Essential BRCT[a] domain protein Regulator of Chk1 activation?[b]
Crb2p	N/A[c]	Rad9p	BRCT domain protein Regulator of Chk1 activation?[b]
N/A[c]	BRCA1[d]	N/A[c]	Essential BRCT domain protein Genome surveillance DNA repair Recruitment of other proteins to site of damage?
Rad18p	SMC6	Rhc18p	Essential SMC-like protein[e] G_2 checkpoint maintenance DNA repair

[a] BRCT = BRCA1 C-Terminus. These proteins contain domains with homology to the C-terminus of the breast cancer susceptibility gene BRCA1.

[b] Only Cut5 and Crb2 in *S. pombe* are required for DNA damage-induced activation of Chk1. How these proteins function in Chk1 activation is unclear. It is also not clear whether the mammalian or *S. cerevisiae* homologs possess similar functions.

[c] Indicates that no clear functional homolog has been identified.

[d] BRCA1 interacts with numerous proteins. Some of these BRCA1 interacting proteins are involved in checkpoint responses and DNA repair. The main role of BRCA1 seems to be one of recruitment of other proteins to the sites of DNA damage. Whether BRCA1 itself possesses an intrinsic function is not clear. No clear homolog of BRCA1 has been identified in either *S. cerevisiae* or in *S. pombe*.

[e] Indicates homology to the structural maintenance of chromosomes (SMC) family of proteins. So far similar roles for mammalian SMC6 in G_2 checkpoint maintenance and DNA repair have yet to be demonstrated.

BRCT domain itself is thought to facilitate both protein–protein and protein–DNA interactions (Bork *et al.,* 1997; Callebaut and Mornon, 1997).

The *S. pombe* BRCT-domain protein Cut5 (also known as Rad4), and its *S. cerevisiae* and mammalian homologs Dpb11 (Araki *et al.,* 1995) and TopBP1 (Yamane *et al.,* 1997), are required for the DNA damage and replication checkpoints (McFarlane *et al.,* 1997; Saka and Yanagida, 1993; Yamane and Tsuruo, 1999). They are essential genes (Araki *et al.,* 1995; Saka and Yanagida, 1993; Yamane *et al.,* 2002), and from the analysis of Dpb11, their essential function appears to be in the initiation of DNA replication, most likely through facilitating polymerase loading (Araki *et al.,* 1995; McFarlane *et al.,* 1997; Saka and Yanagida, 1993). However, in *S. pombe,* the inactivation of Cut5 also results in mitotic entry without DNA replication, a phenotype in common with some other mutants that fail to initiate DNA replication (D'Urso *et al.,* 1995; Hofmann and Beach, 1994; Muzi Falconi *et al.,* 1996; Saka and Yanagida, 1993). This suggests that the act of replication establishes the replication checkpoint and is an important issue in

defining the G_2 state (McFarlane *et al.,* 1997; Saka and Yanagida, 1993; Verkade and O'Connell, 1998). By using temperature-sensitive alleles of *cut5,* a postreplication role for Cut5 in G_2 checkpoints has also been demonstrated (McFarlane *et al.,* 1997; Verkade and O'Connell, 1998). TopBP1 not only associates with topoisomerase IIβ, but also undergoes DNA-damage-induced ATM phosphorylation (Yamane *et al.,* 1997; Yamane and Tsuruo, 1999). These data place this group of BRCT domain proteins at replicative sites on chromatin. This may suggest that these proteins may be involved in targeting or recruiting proteins to the sites of DNA damage, although a formal biochemical role has yet to be elucidated.

Cut5 has been shown to interact in two-hybrid assays with Crb2 [also known as Rhp9 (Willson *et al.,* 1997)], another BRCT domain protein. Crb2 has a homolog in *S. cerevisiae* (RAD9), although a clear mammalian homolog has not been identified (Saka *et al.,* 1997). Crb2 and Rad9 are required for the G_2 DNA damage checkpoint, and like Chk1, are dispensable for the DNA replication checkpoint. In *S. pombe,* Crb2 is required for the Rad3-dependent phosphorylation of Chk1 (Saka *et al.,* 1997), and Crb2 itself is modified (probably phosphorylated) in a manner dependent on the checkpoint Rad proteins (Saka *et al.,* 1997). Chk1 and Crb2 can interact via a yeast two-hybrid assay, although a stable interaction has not been detected *in vivo*. This suggests that such an interaction may be transient or may require certain unique DNA damage-induced modifications. To support the latter hypothesis, in *S. cerevisiae,* phosphorylated Rad9 not only undergoes dimerization, but is able to bind the FHA domain of Rad53, an alternative effector kinase (see below) (Soulier and Lowndes, 1999; Sun *et al.,* 1998; Vialard *et al.,* 1998). Mec1-dependent hyperphosphorylation of Rad9 has been shown to recruit Rad53 to a large protein complex. The recruitment of Rad53 to this complex allows the activation of Rad53 by autophosphorylation *in trans,* whereupon Rad53 dissociates from the complex (Gilbert *et al.,* 2001). The functional relationships between Rad9–Rad53 interactions in *S. cerevisiae* and Crb2–Chk1 interactions in *S. pombe* are presently unclear.

The archetypal BRCT domain protein, BRCA1, is involved in both S phase and G_2 phase arrest following DNA damage (Larson *et al.,* 1997; X. Xu *et al.,* 1999; B. Xu *et al.,* 2001) where it is phosphorylated in an ATM- and ATR-dependent manner (Tibbetts *et al.,* 2000; Xu *et al.,* 2001). However, it is not clear to what extent BRCA1 is involved in the G_2 checkpoint. It is possible that the checkpoint defects observed with BRCA1 mutants merely reflect an inability of the cells to repair DNA damage effectively. This is consistent with several reports implicating BRCA1 in repair responses (Y. Chen *et al.,* 1999; Cressman *et al.,* 1999; Frankish, 2001; Khanna and Jackson, 2001; Moynahan *et al.,* 1999; Scully *et al.,* 1997). Furthermore, BRCA1 is found in a large (>2 mDa) complex known as BASC (BRCA1 associated surveillance complex). This complex contains proteins known to be involved in checkpoint responses (ATM/ATR) but also a large number of proteins that have been implicated in various forms of DNA repair ranging from homologous recombination to transcription-coupled repair (Wang *et al.,* 2000a).

A large body of work has accumulated with regard to BRCT domain proteins and the G_2 checkpoint. We do not yet know whether these proteins are bona fide checkpoint-signaling proteins, or alternatively whether their apparent checkpoint function is a causal effect arising from defects in DNA repair processes. Nevertheless, they remain an interesting set of proteins for study in the thorough analysis of G_2 checkpoint signaling.

B. Cds1/Rad53/Chk2: Checkpoint Effector Kinases

The mammalian and *S. cerevisiae* homologs of the *S.pombe* Cds1 protein kinase (Lindsay *et al.,* 1998; Murakami and Okayama, 1995) are known as Chk2 (Blasina *et al.,* 1999; Brown *et al.,* 1999) and Rad53 (Allen *et al.,* 1994), respectively. Homology among these proteins was determined on the basis of a structurally similar kinase domain and a forkhead-associated (FHA) domain (Blasina *et al.,* 1999). FHA domains were originally identified in the forkhead transcription factor and have more recently been implicated in mediating protein–protein interactions (Rhind and Russell, 2000).

In *S. pombe,* Cds1 is activated in response to DNA damage; this activation depends on the checkpoint Rad proteins, but occurs only during S phase. Induction of a replication block by treating cells with hydroxyurea leads to activation of Cds1. Cells lacking Cds1 still undergo G_2 arrest in response to DNA damage, suggesting that in yeast *S. pombe,* Cds1 activation is limited to a very specific temporal window (Brondello *et al.,* 1999; Lindsay *et al.,* 1998). One of the targets for Cds1 phosphorylation, possibly leading to or stabilizing a replication arrest, is Hsk1, a homolog of the *S. cerevisiae* Cdc7 protein kinase that is involved in the initiation of DNA replication (Snaith *et al.,* 2000).

In mammalian cells, Chk2 activation in response to DNA damage depends on its ATM-mediated phosphorylation of threonine-68 (Ahn *et al.,* 2000; Melchionna *et al.,* 2000). Cells expressing a mutant form of Chk2 that cannot be phosphorylated (T68A) have a lower percentage of cells in G_1 even in the absence of DNA damage compared to wild-type controls, indicating a potential role for Chk2 in G_1/S arrest. Although Chk2 nullizygous embryonic stem (ES) cells, which like all ES cells lack a G_1 checkpoint (Schmidt-Kastner *et al.,* 1998), are able to arrest in G_2 in response to DNA damage, they are unable to maintain this arrest (Hirao *et al.,* 2000). It is thus possible that a role for Chk2 in the G_2 checkpoint may be in the regulation of some aspect of DNA repair or in the maintenance of an arrested state. Germ-line mutations of the Chk2 gene itself have been identified in a number of families with a Li-Fraumeni-like syndrome (Vahteristo *et al.,* 2001), which is generally caused by mutations in the p53 gene. This further suggests that Chk2 plays a more dominant role in G_1/S arrest than at the G_2 checkpoint.

In summary, although Cds1 and Chk2 seem to play an unequivocal role in the regulation of G_1/S arrest, their role in G_2/M arrest is not as straightforward. It

appears that these kinases do not play a significant role in the initiation of G_2/M arrest but may to some degree aid in the maintenance of the arrested state.

As mentioned earlier, *S. cerevisiae* lacks a G_2 checkpoint arrest per se. Instead, the cells arrest either in G_1/S or at the metaphase–anaphase transition in mitosis. Rad53 regulates both of these checkpoints. Although the point of arrest differs, the biological outcome of preventing chromosome segregation prior to completion of DNA repair is still achieved. With regard to the latter checkpoint, Rad53 is activated in response to DNA damage in a Mec1-dependent manner. Activated Rad53 then works to indirectly inhibit the metaphase–anaphase transition by downregulating the activity of proteins that are responsible for the degradation of mitotic cyclins (Sanchez *et al.,* 1999). In the case of the G_1/S checkpoint, Rad53 signals to inactivate transcription factors that regulate the expression of the G_1 cyclins that are ultimately required for progression through S phase (Rhind and Russell, 2000; Sidorova and Breeden, 1997).

These data are in contrast with the roles of Cds1 and, in particular, Chk2, which indirectly work to activate transcription of genes that are involved in inducing cell cycle arrest (Boddy *et al.,* 1998; Brondello *et al.,* 1999; Chehab *et al.,* 2000; Hirao *et al.,* 2000; Lindsay *et al.,* 1998). Furthermore, Rad53 contains two FHA domains whereas Cds1 and Chk2 both contain a single domain. It was recently established that only one of the two FHA domains was responsible for the ability of Rad53 to induce cell cycle arrest. Replacing that domain with the FHA domains from Chk2 did not restore a Rad53-dependent cell cycle arrest (Pike *et al.,* 2001). Thus, it is formally possible that Rad53 is not a functional homolog of either Cds1 or Chk2, but has perhaps acquired functions more akin to Chk1 in this organism.

C. Maintenance of the Checkpoint

The analysis of conditional *rad3* alleles in *S. pombe* clearly demonstrated that the signaling events leading to the initiation and maintenance of a G_2 checkpoint are functionally separable (Martinho *et al.,* 1998). Therefore there must be additional components that function either downstream of, or parallel to, Rad3 that are responsible for the maintenance of the G_2 checkpoint once it has been initiated. The analysis of several *S. pombe* mutants that fail to initiate DNA replication, and yet enter mitosis, displays a distinct response from those that stall during replication, and activate the replication checkpoint to prevent mitotic entry. This suggests that the act of ongoing replication establishes the dependency relationship between S phase completion and mitosis. Maintenance of the G_2 checkpoint may similarly involve a link with ongoing DNA repair, which, once completed, allows a cell to enter mitosis.

The *rad18* gene in *S. pombe* is one such component thought to be involved in maintenance of the G_2 checkpoint. The Rad18 protein is a member of the structural maintenance of chromosome (SMC) family of proteins involved in higher

order chromosome architecture (Ball and Yokomori, 2001; Harvey *et al.,* 2002; Strunnikov, 1998), and is essential for cellular proliferation. Rad18 was identified by a mutant allele, *rad18-X,* which is defective in DNA repair but largely retains its checkpoint proficiency (Lehmann *et al.,* 1995). Another allele of *rad18, rad18-74,* was identified during a screen for new genes involved in checkpoint arrest (Verkade *et al.,* 1999). In contrast to *rad18-X, rad18-74* is defective in both DNA repair and checkpoint arrest. However, *rad18-74* mutants are able to initiate checkpoint arrest, in that Chk1 becomes phosphorylated, but are unable to remain arrested and enter mitosis without completing DNA repair. Moreover, these cells fail to repair DNA even when given time to do so by an imposed cell-cycle delay through the inactivation of Cdc25 (Verkade *et al.,* 1999). This suggests that the cell needs to detect ongoing repair to signal to maintain the checkpoint arrest. Clearly, the molecular mechanisms behind the maintenance of the G_2 checkpoint are important aspects of this field that deserve further study.

D. p53: A Role in G_2/M Arrest?

It is clear that p53 plays a crucial role in arresting cells at the G_1/S checkpoint in response to DNA damage (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997; Oren, 1999; Prives and Hall, 1999). This is of particular significance given that over 50% of all human cancers lack functional p53 and therefore also lack a DNA damage-induced G_1/S checkpoint. However, whether p53 plays a role in the G_2 checkpoint has been the subject of controversy. It has been shown that cells lacking functional p53 are still capable of undergoing G_2/M arrest in response to DNA damage. In some cases, a combination of the absence of p53 and the addition of caffeine, which overrides the G_2 checkpoint, greatly sensitizes cells to DNA damage (Taylor and Stark, 2001). However, several lines of evidence have suggested that p53 does play a role in the G_2 checkpoint by functioning both as a transcriptional activator and as a transcriptional repressor. However, the lack of isogenic controls in these experiments made a clear determination for a role of p53 in the G_2 checkpoint somewhat difficult.

A derivate of the HCT116 human colon carcinoma cell line, in which both p53 alleles were sequentially deleted by homologous recombination, has provided such a control. It was revealed that both HCT116 wild-type and p53 null cells arrested in G_2 in response to DNA damage, but cells lacking p53 were unable to maintain this arrest and the cells eventually entered catastrophic mitoses (Bunz *et al.,* 1999). Similar phenotypes were observed in HCT116 cell lines lacking the p53-inducible genes $p21^{Cip1}$ (Bunz *et al.,* 1999) or 14-3-3σ (Chan *et al.,* 2000; Hermeking *et al.,* 1997). However, in these studies, the cells were generally subject to high doses of radiation from which they could never recover. Moreover, in all cases asynchronously growing cells were used; perhaps cyclinA–Cdk2 complexes acting in late S to early G_2 phase are being inhibited and thus halting cell-cycle progression.

In another study, the HCT116 wild-type and p53 nullizygous cell lines were synchronized into the G_2 phase and then exposed to levels of IR from which cells could recover. Both wild-type and p53 null cell lines underwent a transient cell-cycle delay for the same period of time and then reentered the cell cycle with normal kinetics. In both cell lines an increase in Y15 phosphorylation was observed, correlating with an increase in cyclin B1 levels (Koniaras *et al.,* 2001). Together, these studies show that p53 seems to be dispensable for both initiation and maintenance of the DNA-damage-mediated G_2 checkpoint arrest, although this does not rule out that in wild-type cells, p53 may contribute to DNA damage responses during the G_2 phase of the cell cycle.

VI. The G_2 Checkpoint as a Therapeutic Target

Unlike the G_1/S transition, where inactivation of key proteins that leads to abrogation of the checkpoint is a common event (Bartek and Lukas, 2001a; Bartkova *et al.,* 1997; McDonald and El-Deiry, 2001; O'Connor, 1997; Sherr, 1995), the upstream pathways that regulate the phosphorylation state of Y15 on Cdc2 are rarely if ever mutated or inactivated. This has important consequences for the progression and treatment of cancer. The lack of a DNA damage-activated G_1/S checkpoint means that cells experience a higher degree of genomic instability and a heightened mutation rate. However, the continued presence of an intact DNA damage-activated G_2 checkpoint means that a cancer cell is able to undergo relatively normal mitosis without experiencing a massive asymmetry in chromosome segregation that would lead to cell death. So although the fact that the G_2 checkpoint is relatively difficult to abrogate is advantageous for normal cell progression, it also provides for the continued division of tumor cells. The continued presence of the G_2 checkpoint even in the absence of a G_1/S checkpoint also has a deleterious effect on the efficacy of cancer treatments, many of which rely upon inflicting genomic damage.

It seems logical, therefore, that abrogation of the G_2 checkpoint within the context of p53-deficient cells (which already lack a G_1/S checkpoint) would enable cancer treatment to become much more effective. Expression of dominant negative alleles of Chk1 abrogates the G_2 checkpoint and sensitizes cells to DNA damage. This suggests that Chk1 would be a good drug target (Koniaras *et al.,* 2001). Several compounds have been found that are able to force cells that lack functional p53 to bypass G_2/M arrest (Curman *et al.,* 2001; Graves *et al.,* 2000; Jackson *et al.,* 2000; Roberge *et al.,* 1998; Wang *et al.,* 1996, 2001). When combined with radiation or chemotherapeutic drugs, killing of these cells is much more effective and also requires a much lower dose of the anticancer treatment. In contrast normal cells, which retain wild-type p53, will arrest largely in G_1/S and in this case abrogation of the G_2 checkpoint has little effect. In many cases, the drug under study is known to be a kinase inhibitor based on its chemical structure. However,

the problem is that the *in vivo* target of the drug is not known, the doses of the drug required to cause an effect are so high so as to be impractical for use in therapy, or the drug is relatively nonspecific. UCN-01 is one such drug undergoing clinical trials for cancer treatment (Graves *et al.,* 2000; Wang *et al.,* 1996; Yu *et al.,* 1998). It sensitizes cancer cells to cytotoxic agents by abrogating the G_2 checkpoint, thus promoting the efficacy of anticancer agents. Although a recent report suggests that Chk1 is the target for this particular drug (Graves *et al.,* 2000), it does not rule out the fact that other kinases may be affected. A more selective Chk1 inhibitor, SB218078, has been reported to abrogate G_2 checkpoint arrest and synergize with p53 mutations to sensitize cells to genotoxins (Jackson *et al.,* 2000). As stated above, the Wee1 inhibitor PD0166285 (Li *et al.,* 2002; Wang *et al.,* 2001) has also been shown to be an effective agent in checkpoint abrogation and radiation sensitivity. We will doubtlessly see the development of a number of compounds in years to come, showing how basic molecular and cell biology is proving to be important in the design of targeted therapies.

VII. Concluding Remarks and Future Directions

In recent years, our understanding of the G_2 DNA damage checkpoint has gone from little more than a list of genes described in the yeasts to the relatively well-understood signaling pathway described in this article. A large hole in our understanding remains, however, as to how the signal is initiated, and this is a vital question that must be answered. Furthermore, how is DNA damage sensed, and how does the cell know DNA repair is completed, so that progression toward mitosis can resume?

It is possible that the sensing mechanism is a component inherent to maintaining the architecture of the genome. Mutagenesis screens to detect DNA damage-signaling components of this nature would be difficult as it is likely that they would be lethal if such proteins were important for the maintenance of genomic integrity. However, such an approach has been successfully employed to uncover specific checkpoint defects in essential cellular components such as RFC subunits (Kim and Brill, 2001; Krause *et al.,* 2001; Naiki *et al.,* 2000; Shimada *et al.,* 1999), Cut5 (McFarlane *et al.,* 1997; Verkade and O'Connell, 1998), and Rad18 (Verkade *et al.,* 1999). Despite the depth of understanding we have achieved, it is unlikely that all the components involved in the G_2 checkpoint have been identified. Additional genetic screens, perhaps with a novel design that builds on available data rather than the conventional radiation sensitivity phenotype, will be likely to uncover either novel G_2 checkpoint genes or assign a role in the G_2 checkpoint for previously identified genes. Although such screens will be laborious and doubtlessly will identify many alleles of the known checkpoint genes, they have the promise of making significant contributions and we are likely to see results of these in the near future.

Although we have developed a good basic understanding of G_2 checkpoint mechanisms, the precise molecular details controlling the events described here are largely lacking. For example, we know that several complexes are recruited to sites of DNA damage, but the recruitment mechanism remains obscure. Other proteins and protein complexes have also been shown to be recruited to damaged foci (Wang *et al.,* 2000a), and spatial and temporal issues need to be resolved to gain a better understanding of the hierarchy by which this occurs. Moreover, although we know the CLC–CSC and the ATR–ATRIP/Rad3–Rad26 complexes are at damage foci, and are required for the activation of the eventual effector, Chk1, where within the nucleus Chk1 activation occurs remains a mystery. It is entirely possible that there are multiple "flavors" of these complexes, with lesion, temporal, and spatial-specific functions.

Understanding how the G_2 checkpoint is maintained and how the cell is able to recognize ongoing DNA repair is emerging as an important area of research. Although recent research on Rad18 suggests a link between DNA repair and checkpoint maintenance (Verkade *et al.,* 1999), little is known about the mechanisms behind this process. Additionally, Rad18 is an essential gene in yeast, suggesting it plays a role in normal cellular processes, but the essential function is not understood. Perhaps once the essential function of Rad18 has been elucidated, it will provide some insight into the link between DNA repair and checkpoint maintenance.

There is a high conservation of the G_2 checkpoint in eukaryotic cells, but ultimately whatever effect is seen in one organism should be confirmed in human cells. Only by fully understanding the various genes involved in the G_2 checkpoint and what roles, if any, they play in normal cellular processes can we hope to create a new generation of drugs that will exploit the G_2 checkpoint as a potential target for anticancer therapies. By understanding how the G_2 checkpoint functionally interacts with other signal transduction pathways, such as G_1/S checkpoints, such drugs may prove to be very important developments as cancer therapies become more and more individualized. Finally, the conservation in checkpoint signaling that has been found thus far has beautifully demonstrated the advances that can be made when a combined effort is made in several systems, each with its experimental advantages. As we move from "the age of the genome" to an age of "postgenomics," such parallel multisystem approaches will enable us to understand complex biological problems at a stunning rate and depth.

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Chromosomes of the Budding Yeast *Saccharomyces cerevisiae*

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The mitotic chromosomes of the baker's yeast, *Saccharomyces cerevisiae,* cannot be visualized by standard cytological methods. Only the study of meiotic bivalents and the synaptonemal complex and the visualization of chromosome-sized DNA molecules on pulsed-field gels have provided some insight into chromosome structure and behavior. More recently, advanced techniques such as *in situ* hybridization, the illumination of chromosomal loci by GFP-tagged DNA-binding proteins, and immunostaining of chromosomal proteins have promoted our knowledge about yeast chromosomes. These novel cytological approaches in combination with the yeast's advanced biochemistry and genetics have produced a great wealth of information on the interplay between molecular and cytological processes and have strengthened the role of yeast as a leading cell biological model organism. Recent cytological studies have revealed much about the chromosomal organization in interphase nuclei and have contributed significantly to our current understanding of chromosome condensation, sister chromatid cohesion, and centromere orientation in mitosis. Moreover, important details about the biochemistry and ultrastructure of meiotic pairing and recombination have been revealed by combined cytological and molecular approaches. This article covers several aspects of yeast chromosome structure, including their organization within interphase nuclei and their behavior during mitosis and meiosis.

KEY WORDS: *Saccharomyces cerevisiae,* Chromosome, Mitosis, Meiosis, Interphase, Nucleolus.

I. Introduction

In eukaryotes, genetic material is organized as linear pieces of DNA complexed with proteins, which at least for some parts of the cell cycle, notably during division, condense into rod-shaped chromosomes that can be detected microscopically. The structure of chromosomes seems to be conserved among fungi, plants, and animals, with the known exception of dinoflagellate chromosomes (Bhaud *et al.,* 2000 and references therein), although the DNA content of chromosomes can vary among species by more than three orders of magnitude. Also the chromosomes of the fission yeast *Schizosaccharomyces pombe,* which shares with *Saccharomyces cerevisiae* a DNA content at the low end of the eukaryotic range, seem to conform to the typical pattern, although they have not been studied in much detail (Robinow and Hyams, 1989; Scherthan *et al.,* 1994). In *S. cerevisiae,* by contrast, mitotic chromosomes in the classic cytological sense have not been detected.

The elusive nature of budding yeast chromosomes seems to have its basis in the fact that they are relatively crowded in the nucleus where they remain confined even during division. Moreover, yeast with a mere 13 Mb per haploid genome seems to pack its chromatin only moderately to achieve a form that is suitable for transport during mitosis. Thus, although yeast is a convenient model system for many different aspects of cell biology, its full potential could not be exploited owing to the lack of accompanying cytological examination. However, budding yeast chromosomes can be made visible in the guise of bivalents during pachytene, when 16 rod-shaped structures emerge that represent the 32 chromosomes of a diploid cell. They also leave their traces in the form of bands on pulsed-field electrophoresis gels, with each band representing the DNA of an entire chromosome. Finally, chromosomes can be glimpsed in interphase nuclei as more or less distinct domains of chromatin after painting with a fluorescent chromosome-specific DNA probe.

This article will review ways in which yeast chromosomes can be studied cytologically and how this information helps highlight parallels and differences in the chromosomal organization and dynamics during mitosis and meiosis of budding yeast and organisms with more complex genomes. Moreover, questions concerning the suprachromosomal nuclear organization and its influence on nuclear metabolism and gene expression will be addressed. In spite of yeast's poor cytology, this is a field in which remarkable progress has been made over the past few years and in which yeast together with vertebrates and *Drosophila* has contributed most to our knowledge. Also, the discovery of the mechanisms for chromosome condensation and sister chromatid cohesion has been significantly promoted by studies in yeast.

II. Chromosome Structure and Division

A. The Visualization of Yeast Chromosomes

In *S. cerevisiae* there is no conventional cytology in the sense of examining condensed metaphase chromosomes. All that can be discriminated during the cell cycle is the change in nuclear morphology at mitosis and meiosis and the separation of nuclei during cell budding and sporulation. There were sporadic attempts to prepare and count metaphase chromosomes in squash or smear preparations (Kater, 1927; Wintersberger *et al.,* 1975) but it must be assumed that the structures observed were artificially produced aggregates of chromatin because they did not match the chromosome number and relative sizes that are known today. A major obstacle to the study of chromosomes is the fact that mitosis in yeast takes place within the intact nuclear membrane. Also the low degree of metaphase chromosome condensation may hamper their visualization. Cytology in yeast has thus been limited to the analysis of meiotic chromosomes (which do appear as distinct rod-shaped structures—Kuroiwa *et al.,* 1984) and the study of synaptonemal complexes (SCs) that are associated with the meiotic chromosomes during prophase of meiosis I (Figs. 1 and 2). More recently, it relies on the *in situ* staining of chromosomes or regions of chromosomes by fluorescence methods.

Sites along chromosomes can be visualized by fluorescence *in situ* hybridization (FISH). DNA from the region to be depicted is amplified, labeled with a fluorescent tag, denatured, and applied to nuclei with denatured DNA on a slide. Under appropriate conditions, complementary DNA strands will reassociate and the labeled sequence will bind to the corresponding chromosomal regions and elicit a fluorescent signal that can be detected using a fluorescence microscope (Fig. 3a). This technique has revolutionized yeast cytology (Scherthan *et al.,* 1992; Loidl *et al.,* 1994; Weiner and Kleckner, 1994; Guacci *et al.,* 1994). However, probing of individual chromosomal loci provides little information on the behavior of the whole chromosome, such as orientation and compaction state. This disadvantage can be overcome by using probes for regions all along a chromosome and by highlighting entire or large parts of chromosomes by "chromosome painting" (Scherthan *et al.,* 1992; Trelles-Sticken *et al.,* 1999) (Fig. 3b). A related method for the delineation of individual chromosomes is genomic *in situ* hybridization (GISH). A genomic DNA probe from *S. paradoxus* can be used to label selectively an *S. paradoxus* chromosome in a strain of *S. cerevisiae,* where it substitutes for its homeolog (A. Lorenz *et al.,* 2002) (Fig. 3c). However, if this approach is used, the possibility cannot be excluded that foreign chromosomes may behave differently from authentic chromosomes. The same is true for the study of yeast artificial chromosomes (YACs), which can be the same size as authentic yeast chromosomes and can be easily

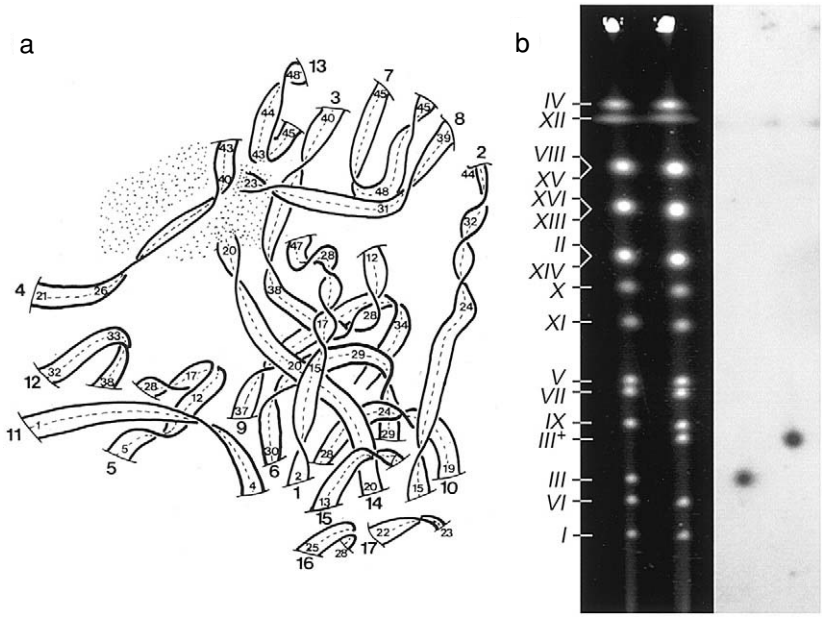


FIG. 1 (a) Reconstruction of serial sections of a yeast SC complement. The 17 SC segments correspond to 16 bivalents as segments 8 and 14 are part of the same SC, which is interrupted by the nucleolus. The small digits indicate section numbers. From Byers and Goetsch (1975b), reproduced with permission from the authors. (b) Electrophoretic karyotype of yeast. Chromosome-sized DNA molecules are separated by pulsed-field gel electrophoresis. There is good correspondence between relative chromosome sizes in kilobases, the relative lengths from the SC and pachytene karyotype (see Fig. 3a), and the band distribution in the gel. The second lane shows a strain with a chromosome *III* carrying a duplication of an ~100-kb segment (Loidl and Nairz, 1997). Southern hybridization with DNA probes for all 16 linkage groups (here *LEU2* for chromosome *III*) allows the assignment of bands to chromosomes. Chromosome numbers are shown on the left. In the strain shown (SK1), chromosomes *VIII* and *XV, XIII* and *XVI,* and *II* and *XIII* are of similar sizes and migrate as a single band. The migration of chromosome *XII,* which bears the NOR, is not proportionate to its size.

delineated by FISH with DNA from the species from which they are derived (Loidl *et al.,* 1995). Another useful cytological marker is the ~0.5- to 2-Mb-long *RDN* tract (the nucleolus organizing region—NOR) on chromosome *XII,* which can be selectively stained with a probe against ribosomal DNA (rDNA) (Guacci *et al.,* 1994, 1997b). The distribution and orientation of NOR regions within the yeast nucleus provide important information on the nuclear architecture (Fig. 3e–h).

Another important tool to study certain aspects of yeast chromosome behavior is labeling with green fluorescent protein (GFP). A GFP-Lac repressor fusion protein is expressed in yeast cells and binds to an array of chromosomally integrated Lac operator sites. The resulting GFP fluorescence signal at this chromosomal region can be detected in living cells, allowing chromosome dynamics

to be studied (Robinett *et al.,* 1996; Straight *et al.,* 1996). A similar system, using GFP-tetracycline repressor molecules, which bind to chromosomally integrated Tet operator repeats, was introduced by Michaelis *et al.* (1997) (Fig. 3d).

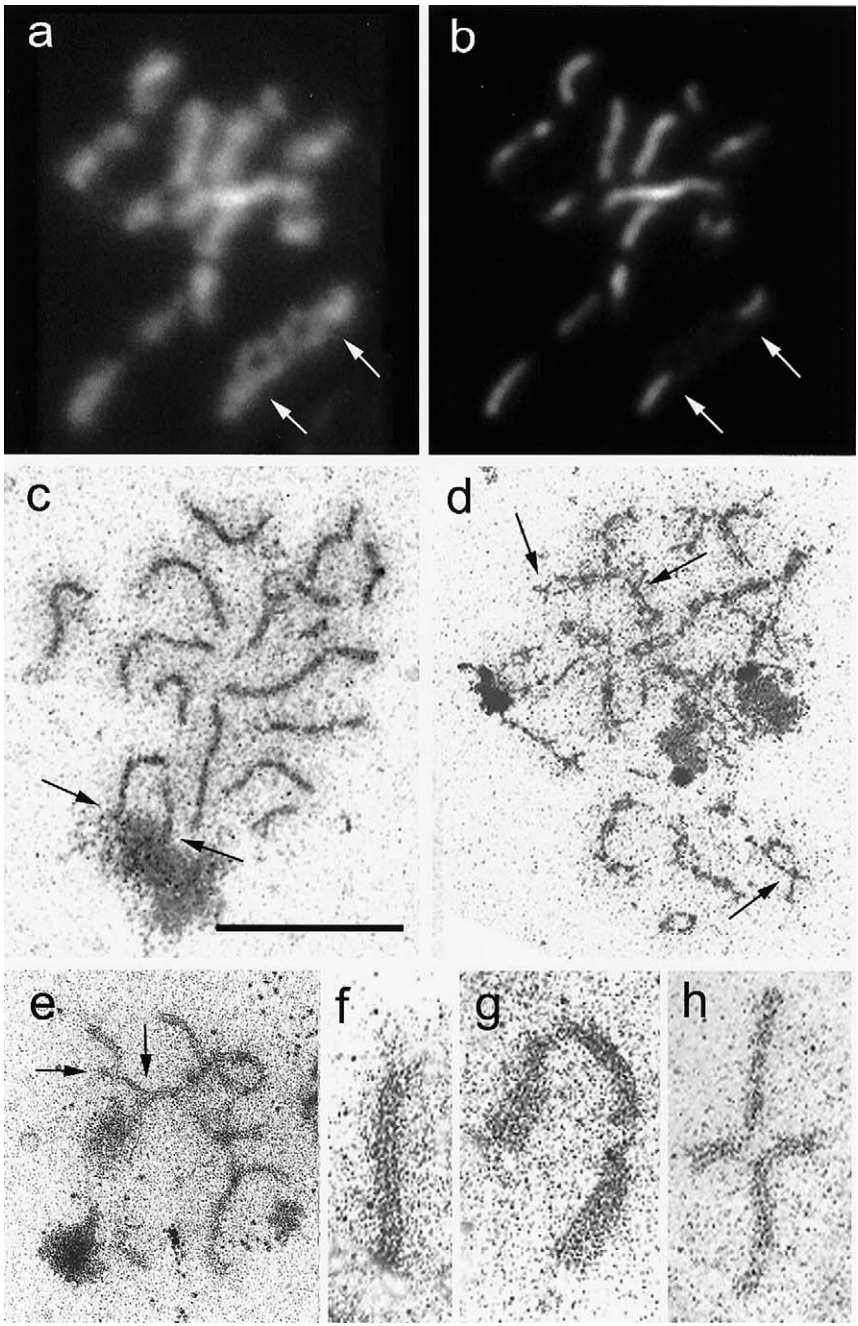
Yeast pachytene bivalents are an excellent subject for cytological investigation. Pachytene is the only stage in which condensed yeast chromosomes (in wild-type cells normally present as synapsed pairs) have been visualized after staining with the DNA-specific fluorescent dye diamidinophenylindole (DAPI) (Kuroiwa *et al.,* 1984, 1986). One reason for this may be the presence of the SC axes (see Section II.E.1) whose robustness possibly helps to preserve chromosome integrity in cytological preparations. Even in the *spo11* mutant in which only unsynapsed axial elements are formed, condensed univalent chromosomes were observed (Giroux *et al.,* 1989). But more importantly, pachytene is the only stage in the yeast's life cycle during which chromosomes are not attached to the spindle pole body or the spindle (see Sections II.C.1 and III.D) and thus are sufficiently separated to be visualized individually.

The presence of an SC in *S. cerevisiae* was first shown in electron microscopic images of ultrathin sections (Engels and Croes, 1968; Moens and Rapport, 1971a). Later, complete SC complements were reconstructed by serial sectioning (Byers and Goetsch, 1975b; Moens and Ashton, 1985) (Fig. 1a). Serial sectioning is very tedious, however, and the meiotic cytology of yeast was clarified only after whole mount SC spreading was successfully introduced (Dresser and Giroux, 1988; Loidl *et al.,* 1991). The compacted SC-associated chromatin can be highlighted by DNA-specific fluorescent dyes (Fig. 2a) although it tends to appear somewhat fuzzy. Because SC appears as a distinct structure all along paired homologs in pachytene, it is a good marker for tracing chromosomes for some applications. It can be labeled by a silver staining procedure for proteins (Dresser and Giroux, 1988; Loidl *et al.,* 1991) or by immunostaining of its various components (Klein *et al.,* 1992; Sym *et al.,* 1993) (Fig. 2).

Byers and Goetsch (1975b) constructed a karyotype by plotting the chromosomes according to their decreasing SC length. They found that chromosome sizes differ gradually, which is in good agreement with the physical sizes from the genome project and with the even distribution of bands of chromosome-sized DNAs in pulsed-field electrophoresis gels (Carle and Olson, 1985) (Fig. 1b). SC analyses have also proved useful in the detection of chromosomal rearrangements and abnormal karyotypes (see Section II.B) and they have helped to characterize many meiotic recombination and/or pairing mutants in *S. cerevisiae* where SC formation is incomplete or aberrant (Kupiec *et al.,* 1997).

B. The Karyotype

Because of its advanced genetics and molecular biology, the budding yeast is one of the very few organisms in which the construction of a genetic linkage map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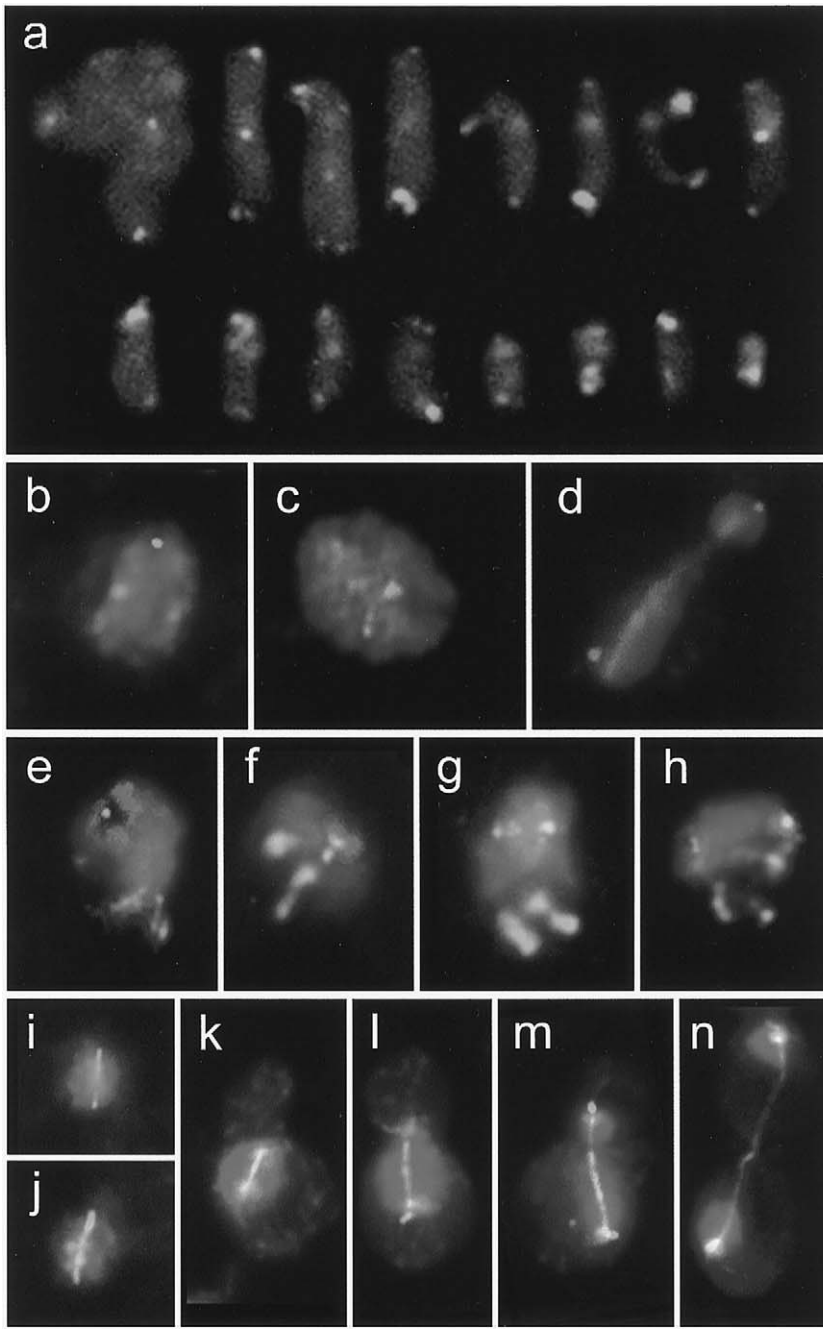
(Mortimer and Schild, 1980) and a physical chromosome map (Cherry *et al.,* 2002) has proved superior to and essentially replaced cytological analysis for the assessment of its chromosomal status. The karyotype of *S. cerevisiae* has 16 metacentric/submetacentric to subtelocentric chromosomes with DNA contents ranging from up to 230 to 3000 kb. In addition, many strains carry a 2-μm plasmid, which was genetically detected as a 17th linkage group. The largest chromosome, number *XII,* carries the NOR. Relative chromosome sizes, as studied by electrophoretic karyotyping, can vary considerably between different wild and cultivated strains, due to translocations, ancient duplications, and partly also the presence of variable copy numbers of Ty retrotransposons (Naumov *et al.,* 1992; Camasses, 1996; Ibeas and Jimenez, 1996; Casaregola *et al.,* 1998; Seoighe and Wolfe, 1998; Rachidi *et al.,* 1999) (Fig. 1b). Most of the karyotype instability is contributed by chromosome *XII* as it can easily gain or lose rDNA repeats by mitotic intrachromosomal recombination (Loidl and Nairz, 1997; Carro and Pina, 2001). Wild populations are mostly diploid, but laboratory strains can be maintained as haploid cells. Aneuploidy and polyploidy are common in industrial strains (Hadfield *et al.,* 1995).

Genome and chromosome mutations can be cytologically detected and their meiotic pairing behavior studied in SC sections and spreads. For example, trisomic, triploid, and tetraploid yeast strains were investigated by Byers and Goetsch (1975b), Moens and Ashton (1985), and Loidl (1995). It was found that homology recognition and alignment involve all three or four chromosome sets, whereas synapsis occurs mostly between pairs of homologs in the tetraploid. In the triploid and in trisomic strains, both triple synapsis (Fig. 2g) and II+I pairing with switching of synapsed partners do occur. Also in the tetraploid, switching between synapsed partners takes place occasionally and leads to quadrivalent formation. Likewise, translocation quadrivalents were formed by synapsis between the homologous portions in a translocation heterozygote (Loidl *et al.,* 1998) (Fig. 2h). It seems though that the detection of minor chromosomal rearrangements is limited

FIG. 2 The visualization of meiotic yeast chromosomes and meiotic pairing. (a) Condensed yeast chromosomes (bivalents) at the pachytene stage of meiosis (DAPI staining). Sixteen bivalents are formed by the 32 chromosomes of diploid cells. However, due to end-to-end associations there are usually fewer bivalents discernible. In the NOR, chromosomes are not synapsed (arrows). (b) Immunostaining of components of the SC (in this case Zip1p) also delineates the bivalents in pachytene. In the NOR (arrows) there is a gap in the Zip1 array. (c) SCs are formed between homologous chromosomes at pachytene and represent the 16 bivalents. The nucleolus appears as a large patch of medium-density material. The SC associated with it is discontinuous (arrows) (Ag staining, electron microscopy). (d) Bivalents in diplotene when homologs remain associated only at chiasmata (arrows) after their partial repulsion (Ag staining, electron microscopy). (e) Abnormal synapsis occurs in cases of missing or insufficient homologies. Here, SC formation in a haploid strain is shown. Pairing partner switches (arrows) indicate that synapsis involves nonhomologous regions. (f) Wild-type bivalent showing two lateral elements plus the central element. (g) Pachytene trivalent in a triploid. Three lateral elements plus two central elements can be seen. (h) Quadrivalent as formed in tetraploid meiosis and translocation heterozygotes. Scale bar in (c) = 10 μm in (a–e).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by the capability of the SC to accommodate structural differences between pairing chromosomes. This phenomenon, in which duplication/deletion buckles are straightened by differential shortening of the two axes and inversion loops are obliterated by conversion of homologously to heterologously synapsed tracts, was first observed in mice and was called synaptic adjustment by Moses *et al.* (1978). Moens and Ashton (1985) failed to detect in reconstructed SC sections a heterozygous inversion in chromosome *III*. Likewise, Dresser *et al.* (1994) did not detect on spread SCs an inversion loop in a yeast strain that was heterozygous for an inversion encompassing an ~270-kb-long tract. In our laboratory, a strain with a structurally different pair of chromosomes *III* was tested for the presence of an asymmetric SC. In this strain, one of the chromosomes was ~700 kb longer due to the presence of seven tandemly arranged extra copies of the interval between the *MAT* and *HMR* locus (Loidl and Nairz, 1997). In spite of this considerable

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Fig. 3. (a) Pachytene karyotype. Sixteen bivalents arranged according to size. The largest bivalent can be identified as number *XII* by the nucleolus that forms a large bulge. Centromeres (red) and telomeres (green) are labeled by FISH with a pan-centromeric and a pan-telomeric probe, respectively (Jin *et al.,* 1998). (b, c) Differential staining of chromosomes in interphase. (b) Painted left arms of chromosome *XI* (red) in a diploid interphase nucleus. The SPB (immunostained with an antitubulin antibody—orange) serves as a landmark to define intranuclear chromosome positions. The arms show a Rabl-like orientation with one end (the centromere) near the SPB and the telomeres pointing toward the opposite pole of the nucleus (courtesy of J. Fuchs). (c) Detection of an individual chromosome by GISH. A single chromosome *IV* of *S. paradoxus* (green) delineated with *S. paradoxus* genomic DNA is present in an *S. cerevisiae* interphase nucleus. Complementary FISH with *S. cerevisiae* genomic DNA marks the remainder of the chromatin (red). (d) Detection of chromosomal sites with integrated *tet*O repeats by the binding of TetR–GFP. Here, a centromere-near locus (green) is highlighted. The separated sister centromeres migrate at the tips of the mitotic spindle (orange) in anaphase. (e–h) Diploid nucleus in different stages of mitosis. Centromeres of all chromosomes are highlighted by FISH labeling with a pan-centromeric probe (red). The rDNA tracts (NORs) are shown in green. (e) In interphase the centromeres are arranged in a ring around the SPB (orange). The space within the centromere ring is not stained with DAPI, i.e., it is devoid of DNA. The NORs occupy the opposite region of the nucleus. (f) Late in G_1 the SPB duplicates as a prelude to mitosis. The NORs appear more condensed than in the previous stage. (g) At a later stage the SPBs are separated and the centromeres occupy the space between them. This stage might be interpreted as the equivalent of prometaphase or metaphase in higher eukaryotes. (h) In anaphase to telophase the SPBs are even further apart and the centromeres are grouped in two clusters around them. Sister chromatids are separate in both NORs. (i–n) Mitotic spindle (green) and centromeres (red) at different stages of mitosis in well-preserved cells. (i) Centromeres form a single cluster while a short spindle is formed. This stage corresponds to the nucleus shown in (g). (j) Sometimes centromeres can be seen in two separate clusters that are distant from the poles of a short spindle. (k) All centromeres are separated and are at the poles of the metaphase spindle when the dividing nucleus is still completely inside the mother cell. Anaphase A movement of centromeres [from a median position as shown in (j) toward the poles] has occurred. (l, m) Further separation of chromatids occurs by the elongation of the mitotic spindle (anaphase B) after the passage of one spindle pole through the budneck. (n) Telophase. The two chromatin masses are completely separated. SPBs and spindles are immunostained with antibodies against tubulin. Chromatin is stained blue by the DNA-specific fluorescence dye DAPI. The pale blue staining in the cytoplasma in i–n is due to mitochondrial DNA. (See also color insert.)

structural heterozygosity, the corresponding SC was mostly inconspicuous; only the additional chromatin of the multiplicated chromosome region projected as a lateral bulge (J. Loidl, unpublished result).

C. Chromosome Structure

1. The Centromere

The centromere is the site of kinetochore formation, where the chromosome associates with microtubules and assembles molecular motors that effect its movement along the spindle (He *et al.,* 2001; Jones *et al.,* 2001). It is the major site of sister chromatid cohesion (Blat and Kleckner, 1999; Megee and Koshland, 1999; Tanaka *et al.,* 1999) and plays a special role in sister cohesion in meiosis (see Section II.C.5). Moreover, it contains receptors for both spindle attachment of kinetochores and tension in the spindle microtubules, which trigger the spindle checkpoint as long as chromosomes are not properly attached to the division spindle or a proper spindle is not formed (Biggins and Murray, 2001; Fraschini *et al.,* 2001; Stern and Murray, 2001). At least in metazoans, the centromeric region serves an additional role as an anchoring site for chromosomal passenger proteins. These chromosomal passengers detach from the centromeres at anaphase and remain in the midzone of the spindle, and function in cytokinesis. A similar behavior might be attributed to the Ipl1 protein of yeast. It serves a role in the bipolar attachment of sister kinetochores, but has to be detached from the centromeric region, once tension is removed at the kinetochores upon the destruction of cohesion at the onset of anaphase (Tanaka *et al.,* 2002). Whereas in the mitotic chromosomes of higher eukaryotes the centromere forms the primary constriction, no morphological feature has yet been observed in yeast chromosomes at the site of the centromere.

Yeast centromeres are special in that they are associated with nuclear microtubules throughout the entire cell cycle (Byers and Goetsch, 1975a; Jin *et al.,* 2000). This implies that in interphase more or less active kinetochores are present whereas in multicellular eukaryotes the centromere organizes a functional kinetochore only in the G_2 phase. By comparision with fission yeast or higher eukaryotic regional centromeres, the budding yeast centromeres are small and have a simple DNA sequence composition. Yeast centromere DNA consists of three conserved sequence domains, CDEI to CDEIII, with a total length of $\sim$125 base pairs (Hegemann and Fleig, 1993). It encompasses a single nucleosome, containing a modified histone H3, Cse4p, and is able to interact only with a single microtubule (Sullivan *et al.,* 2001). In higher eukaryotes the ability of a wide variety of poorly conserved DNA motifs to organize kinetochores and the occurrence of neocentromeres have given rise to the concept of centromere selection as an epigenetic phenomenon, i.e., a centromere is a centromere because it has been one during the previous cell cycle (Choo, 2000; Sullivan *et al.,* 2001). Yeast centromeres seem to be notably

different in this repect as kinetochore formation is closely linked to a specific DNA sequence.

2. The Telomere

In yeast, telomeres consist of ~300 base pairs of $C_{1-3}A/TG_{1-3}$ repeats. A variety of proteins involved in transcriptional regulation and repair are particularly abundant at telomeres (Zakian, 1996; Shore, 1998). Internal to the telomere repeats, there reside two classes of heterogeneous middle repetitive sequences, called X and Y′. The adjacent subtelomeric region of ~20 kb features low gene densities, reduced levels of transcription and recombination (Gottschling *et al.,* 1990; Pryde and Louis, 1997; Su *et al.,* 2000), and late DNA replication (Ferguson and Fangman, 1992). Whereas reduced transcription and late replication are correlated with the subtelomeric position of a sequence (see Section III.C.2, 3), this does not seem to be the case for reduced recombination. Sequences removed out of the spatial context with the telomere maintain a reduced recombination rate, which indicates that it is a property of the subtelomeric DNA sequences themselves and does not require proximity to a telomere. Reduced recombination possibly evolved by selection against distal chiasmata, which are inefficient in sustaining bivalents (Su *et al.,* 2000).

The special DNA sequence composition and protein endowment of telomeres allow them to serve three important functions. First, they are the substrate for telomerase, a reverse transcriptase, that adds telomere sequence repeats to the chromosome ends. This process compensates for the DNA loss at the primer-binding site during DNA synthesis. Second, the special structure of telomeres protects them from being mistaken as broken DNA and ligated by the cell's DNA repair machinery. Third, telomeres congregate in a small area of the nuclear envelope in the meiotic prophase of most organisms. This is believed to promote homologous synapsis (see Section II.E.1). Yeast telomeres have the additional property of being anchored to the nuclear envelope during most stages of the cell cycle. This is important in maintaining an orderly interphase chromosome arrangement and in establishing a silencing domain at the nuclear periphery (see Section III.C).

3. The Nucleolus Organizing Region (NOR)

The yeast NOR contains the ~500–2000 kb *RDN1* locus on chromosome *XII,* which consists of 50–200 copies of rRNA genes in tandem arrangement (Cherry *et al.,* 2002). The NOR is surrounded by the nucleolus, in which rRNA is processed and stored. In most organisms, the nucleolus is resolved and the nucleolar proteins are partitioned to daughter nuclei by depositing on chromosomes during mitosis and meiosis (Paweletz and Risueno, 1982), but its position on a chromosome is marked by the secondary constriction. In yeast, the nucleolus remains

intact during mitosis and becomes dumbbell shaped and partitioned between the incipient daughter nuclei (see Byers, 1981). In the pachytene stage of meiosis, the nucleolus is associated with bivalent *XII,* and the portion of the bivalent that passes through the nucleolus remains unsynapsed (Byers and Goetsch, 1975b; Kuroiwa *et al.,* 1986) (Figs. 1a and 2a–c). Consequently, meiotic recombination within the NOR is low (Petes and Botstein, 1977). The failure of the NOR to synapse is occasionally observed in other organisms, and it may help prevent unequal recombination events between rDNA tandem repeats that would be detrimental to the cell. Like in mitosis, also during meiosis I the nucleolus becomes constricted and its halves are partitioned to the halves of the dividing nucleus. In meiosis II, however, nucleoli are excluded from the spores (Byers, 1981).

4. Chromosome Condensation

a. The Compaction of Mitotic and Meiotic Chromosomes In interphase, DNA is not present in its extended form, but is packaged into chromatin with the help of histones and nonhistone structural proteins. This packaging possibly helps to confer "tidiness" in the nucleus. There is some uncertainty about the nature of yeast chromatin. Although there exists a yeast histone H1 homolog that may play a similar role to linker histones, it is not essential and seems to occur only at restricted locations in the chromatin, with a notable affinity to rDNA sequences (Freidkin and Katcoff, 2001). In most other eukaryotes, chromatin is organized, according to the standard textbook model, as a 30-nm solenoid that is folded into a 250- to 300-nm interphase chromatin fiber (see Pienta and Coffey, 1984; Earnshaw, 1988). During division, additional chromosome shortening takes place because of the need to accommodate chromatids in the separating daughter nuclei.

Guacci *et al.* (1994) calculated the compaction ratio for yeast chromatin in interphase by measuring the distances of FISH signals of physically mapped sequences. They found that interphase chromatin is compacted by a factor of ~80 compared to native B-form DNA. This value is very similar to the compaction ratios of 70–100 estimated from FISH studies of interphase chromosomes in vertebrate cells (Lawrence *et al.,* 1988; Trask *et al.,* 1989). The compaction ratio for chromatin in nocodazole- and *cdc20*-arrested cells, which are in a stage that may be roughly equivalent to mitotic metaphase in higher eukaryotes, was at most two times that of interphase chromatin (Guacci *et al.,* 1994). For higher eukaryotes, values from the literature for metaphase chromosome compaction vary considerably from 5- to 10-fold to 20- to 100-fold over interphase chromatin (Trask *et al.,* 1993; Lewin, 2000). Thus, as a rough estimate, yeast metaphase chromosome condensation is one order of magnitude less. The slight additional increase from interphase to metaphase chromosome condensation may be one reason that yeast mitotic chromosomes have not so far been observed cytologically as distinct units (Peterson and Ris, 1976; Gordon, 1977).

Deriving chromosome compaction from measuring distances between distinct FISH signals has the obvious shortcoming that the course of chromatin between two

FISH markers is uncertain. To reduce the influence of meandering chromatin tracts, Guacci *et al.* (1994) used markers separated by relatively short physical distances. On the other hand, markers that are too close may underestimate chromosome condensation as their distance will depend on the folding of the chromatin fiber between them, which is not proportional to overall chromosome shortening. If the markers are situated within a common large loop or one turn of a chromosomal coil, their distance will not be reduced to the same degree as the length of the chromatid [Lawrence *et al.* (1988) for experimental evidence and discussion]. Moreover, chromosome condensation in chemically arrested and *cdc* mutant cells might be abnormal. Therefore, in our laboratory entire chromosome arms were painted (Fig. 3b) and their lengths were measured in cells simultaneously immunostained for the mitotic spindle as marker for the stage of the cell cycle (J. Fuchs *et al.,* in preparation). In general, these measurements confirmed the values for interphase and metaphase chromosome compaction given by Guacci *et al.* (1994).

Guacci *et al.* (1994) estimated the interphase length of the longest chromosome arm (1084 kb) to be over 4 μm. Because the mitotic spindle elongates to 6-10 μm (Byers and Goetsch, 1975a; Winey *et al.,* 1995), some arms would be longer than half the length of the anaphase spindle and run the risk of being cleaved at cytokinesis (Schubert and Oud, 1997). However, mitotic condensation by a factor of only two would be sufficient to confine chromosome arms to incipient daughter nuclei (Guacci *et al.,* 1994). This calculation does not take into account chromosome arm *XIIR,* which, with the NOR included, can be up to 3 Mb (corresponding to 900 μm DNA), and given a compaction ratio of 80×2, would measure over 5 μm at mitosis. Thus, given the inaccuracies of measurements, it is unclear whether the rDNA tracts just fit into half nuclei, or if they have to assume a degree of compaction higher than the other chromosome regions. Additional condensation of the rDNA was in fact suggested by a study that showed by *in vivo* localization that condensin (a protein complex with a role in mitotic chromosome condensation—see below) is overrepresented in the rDNA region (Freeman *et al.,* 2000). Moreover, there is evidence that linker histone preferentially associates with rDNA (Freidkin and Katcoff, 2001), which could also cause a higher degree of compaction.

A little more is known about meiotic than mitotic chromatin organization. In pachytene, chromatin loops that are anchored in the axial/lateral elements can be seen to project from both sides of the SC. From the width of the chromatin halo around sectioned yeast SCs, the size of these loops was estimated to be $\sim$1 μm, which corresponds to 7 μm or 20 kb of DNA on the assumption that the loops consist of nucleosome strings (Moens and Pearlman, 1988). Yeast pachytene bivalents are astonishingly long in relation to their DNA content. Byers and Goetsch (1975b) measured SCs in reconstructed serial sections and found the average total length of the SC complement was about 28 μm. This value was later confirmed in spread SCs (Loidl *et al.,* 1991). If the $\sim$12-megabasepairs (Mbp) DNA of budding yeast (excluding the rDNA) extend over 28 μm total pachytene SC length (excluding the NOR), then the DNA/SC ratio is 429 kb/μm. For comparison, the haploid human genome of 3300 Mbp and a DNA contour length of 1100 mm corresponds

to a total pachytene bivalent length of 245 μm [average of measurements by Holm and Rasmussen (1977) and Solari (1980)]. This is a DNA/SC ratio of 13,500 kb/μm, and it means that in humans chromatin is $\sim$30 times more compacted along pachytene SCs than in yeast. If yeast pachytene chromosomes had the same degree of condensation as human pachytene chromosomes, the total length of the whole complement of 16 bivalents would be only about 1 μm. Doing light microscopic cytology on them would be impossible!

Byers and Goetsch (1975b) calculated the compaction ratio of 1C genomic DNA in pachytene chromosomes based on a total SC length of 28 μm as 1:100. Moens and Ashton (1985) arrived at 1:477 and 1:318 for the longest and the shortest chromosome, respectively. A recalculation using 12 Mbp as the haploid genome size and a factor of 3030 bp/μm DNA length (i.e., 3960 μm DNA) produces a compaction ratio of 1:$\sim$140. [The corresponding value for humans is 1:$\sim$4500; for rough estimates of values from other organisms see Loidl (1994).] Thus it seems that chromosome condensation along the length axis at pachytene and at mitotic metaphase (1: $\sim$160, see above) is similar and different chromosome compaction is not a likely explanation of why pachytene but not mitotic metaphase chromosomes are visible after staining with DAPI.

b. Models of Chromosome Condensation According to the radial loop/scaffold model of chromosome structure (Earnshaw, 1988), mitotic chromosome condensation is brought about by the attachment of scaffold associated regions (SARs) in the DNA to a proteinaceous core. This chromosome scaffold is defined by the protein fraction that remains after the eluation of histones (Paulson and Laemmli, 1977; Gasser *et al.,* 1989). It consists mainly of topoisomerase II (topo II) and structural maintenance of chromosomes (SMC) group proteins (Heck, 1997; Ball and Yokomori, 2001). Accordingly, topo II was found to decorate the cores of yeast meiotic chromosomes (Klein *et al.,* 1992). SARs were found to be spaced at intervals from <3 kb to 112 kb intervals along the DNA in *Drosophila,* and they are enriched in specific DNA sequence motifs (Amati and Gasser, 1988; Gasser *et al.,* 1989). If an array of adjacent SARs binds side by side to the core, then the intervening DNA regions (in the shape of the 30-nm solenoid) would form lateral loops causing considerable longitudinal compaction of the chromosome. Evidence that SAR-defined loops actually correspond to cytologically visible chromatin loops came from the observation that typical SAR DNA sequences localized near the axes of human chromosomes (Bickmore and Oghene, 1996).

Although topo II as a constituent of the chromosome scaffold has been implicated in mitotic chromosome condensation, a mutant in topo II did not show a defect in chromosome condensation (as inferred from the cytological appearance of the rDNA tract) in yeast (Lavoie *et al.,* 2002). On the other hand, condensin, a protein complex consisting of two SMC proteins and three other components, is essential for both the establishment and maintenance of the condensed state of chromosomes (Lavoie *et al.,* 2002). It was speculated that condensin constitutes

the part of the scaffold with which the SARs associate. However, a preferential interaction of cohesin proteins with SAR DNA sequences could not be established so far. Therefore the mechanism of condensin-mediated chromosome compaction is not yet clear. Moreover, normal condensation of yeast chromosomes requires cohesin, a protein complex similar to condensin [Lavoie *et al.* (2002) and references therein] and better known for its role in sister chromatid cohesion (see Section II.C.5).

In animals and plants, the chromatids of metaphase chromosomes appear coiled upon certain pretreatment procedures (Ohnuki, 1968; Rattner and Lin, 1985). Therefore, the coiling of an ~300-nm unit (possibly the core-lateral loop structure) has been proposed as the ultimate step in the metaphase chromosome compaction process (Pienta and Coffey, 1984). This interpretation is corroborated by the observation that closely adjacent sister chromatid exchanges cause the exchange of ~300-nm-wide chromatid subunits (Schvartzman *et al.,* 1978). In yeast, metaphase chromosomes seem to lack the final condensation step due to coiling. Coiling was proposed to be a consequence of the contraction of a proteinaceous core from which 30-nm chromatin loops extend to one side only (Stack and Anderson, 2001). As in a spiral cord, coiling is imposed on a noncontractile flexible structure by the contraction of a contractile string to which it is linked side by side. According to this model, only chromatids in which the core is localized laterally can undergo coiling. This requirement is not fulfilled by pachytene bivalents, which are therefore uncoiled (Stack and Anderson, 2001). From the similar lengths of uncoiled pachytene and metaphase chromosomes in yeast (see Section II.C.4.1), it might be concluded that metaphase chromosomes are not coiled either.

5. Sister Chromatid Cohesion

Sister chromatid cohesion is a process that prevents the precocious partition of sister DNA molecules immediately after replication. If sister chromatids would fall apart before they orient toward the opposite spindle poles, missegregation would occur. Moreover, the association between sister DNA molecules supports mechanisms of recombinational DNA damage repair, which use the sister molecule as template (Nasmyth, 2001).

Two mechanisms contributing to sister chromatid cohesion have been identified originally in budding yeast, and later confirmed for higher eukaryotes. One is by DNA catenation, as newly synthesized sister DNA molecules are intertwined at multiple sites. These links are resolved by DNA topoisomerase II prior to anaphase (Holm, 1994). In addition, sister chromatid cohesion is mediated by chromatid-linking proteins (CliPs) that were initially identified as antigens reacting with an autoimmune serum (Rattner *et al.,* 1988). In yeast, a first hint of the existence of CliPs came from the study of mutants with a high incidence of chromosome loss and premature separation of sister chromatids (Strunnikov *et al.,* 1995; Guacci *et al.,* 1997b; Michaelis *et al.,* 1997). A number of chromosomal proteins (in yeast

Ctf7p, Scc2p, and Scc4p) have been identified as factors that are required to establish cohesion at S phase concomitantly with replication. Another group of proteins, central for sister chromatid cohesion, forms a complex, the cohesin, that is highly conserved from yeast to humans. Cohesin is the molecular glue between sisters (Hartman *et al.,* 2000; Ball and Yokomori, 2001; Uhlmann, 2001). Chromatin immunoprecipitation (ChIP) assays have revealed that it is enriched around the centromeres (Blat and Kleckner, 1999; Megee and Koshland, 1999; Tanaka *et al.,* 1999). This localization is in accordance with the prominent role of cohesion in centromere coorientation. Two components of the cohesin complex, Smc1p and Smc3p, are members of the SMC protein family, of which other members have been demonstrated to play a role in chromosome condensation and contribute to the condensin protein complex (see Losada and Hirano, 2001) (see Section II.C.4.2). The cell-cycle-regulated resolution of sister chromatid cohesion in yeast via the APC-dependent cleavage of the cohesin-component Scc1/Mcd1p has been thoroughly studied by Kim Nasmyth and his group (Nasmyth *et al.* 2000; Nasmyth, 2001).

In yeast there exists a functional link between the mitotic condensation and cohesion of chromatids. Condensin and cohesin protein complexes are similar not only in sharing members of the SMC protein family, but also by the function in chromosome condensation of the cohesin components Mcd1/Scc1p and Pds5 (Guacci *et al.,* 1997b; Hartman *et al.,* 2000). It was speculated that cohesin has the ability both to link and pull together sites along one and the same DNA molecule and to connect different DNA molecules coming from two sister chromatids. This would lead to longitudinal compaction of the chromatin thread by the looping out of intervening DNA tracts, and, at the same time, to the cohesion of sisters (Guacci *et al.,* 1997b; Heck, 1997; Gottschling and Berg, 1998). In *Xenopus* eggs, on the other hand, immunodepletion of cohesin complex members did not notably impair the formation of condensed, rod-shaped chromosomes, whereas cohesion was affected (Losada *et al.,* 1998). Moreover, direct physical interaction between cohesin and condensin protein complexes has not yet been demonstrated, and in *Xenopus* mitotic chromosomes, they immunolocalize to different regions (the interchromatid gap vs. the chromatids) (Losada and Hirano, 2001). This seeming contradiction is explained by a model in which the loops generated by cohesin-mediated longitudinal condensation are further compacted with the help of condensin. If cohesin-dependent condensation were impaired, chromosome compaction would rely completely on the condensin-dependent shortening of the intervening DNA tracts (Hartman *et al.,* 2000).

An alternative model of the involvement of cohesin in yeast chromosome condensation was put forward by Lavoie *et al.* (2002). They proposed that the sites at which cohesin is bound to the chromatin fiber could serve as boundaries, restricting condensin activity to defined domains and ensuring the formation of regular arrays of condensin-generated loops.

D. The Mitotic Division

Although the dynamics of spindle formation and cytokinesis during the mitotic cell cycle have been known for some time, the fact that yeast performs an endonuclear mitosis and the lack of a notable mitotic chromosome condensation (see Section II.C.4.1; Peterson and Ris, 1976; Gordon, 1977) precluded the analysis of chromosome behavior. Electron microscopic studies of the yeast cell cycle have revealed that the first visible event in cell division is the duplication of the spindle pole body (SPB), the microtubule-organizing organelle in the nuclear membrane, late in the G_1 phase (Pringle and Hartwell, 1981). Shortly later, a bud emerges from the cell wall opposite the duplicated SPB. There is some uncertainty as to when S phase takes place with respect to the time of budding. Although it appears from some studies that bud emergence and the beginning of DNA synthesis are almost concomitant, others have indicated that buds appear during S phase. On the other hand, almost all budded cells in an exponentially growing population are able to divide in the presence of hydroxyurea, which is an inhibitor of DNA synthesis. This would indicate that DNA replication is essentially completed by the time of bud emergence (Pringle and Hartwell, 1981). While the bud is growing, the daughter spindle pole bodies separate and move along the nuclear membrane to opposite positions of the nucleus. Between them there extends a bundle of intranuclear microtubules, whose elongation probably drives this movement. Astral microtubules then pull or push the nucleus halfway through the budneck into the daughter cell, which has grown from the bud to about half the size of the mother. In the course of this movement, the nucleus assumes an oblong shape. On the basis of staining with DAPI, it was assumed that in these longitudinal nuclei the mitotic segregation of chromosomes takes place (Pringle and Hartwell, 1981).

Recent studies using FISH probes and GFP tags to various chromosomal regions provided evidence that mitotic chromosome movement starts much earlier. During interphase, centromeres maintain a close association with the SPB (Goh and Kilmartin, 1993; Guacci *et al.,* 1997a; Jin *et al.,* 1998, 2000), whereas they occupy the region between daughter SPBs soon after the separation of the SPBs. However, they do not congregate into a metaphase plate (Straight *et al.,* 1997). Winey *et al.* (1995) studied mitotic spindles in three-dimensional (3-D) reconstructions of electron micrographs. They discriminated kinetochore and continuous microtubules and found that most of the former did not reach the middle between the two spindle poles even in very short spindles. This also led to the conclusion that there was no metaphase plate and that separation of a centromere can start when it is anywhere on the spindle, well before the nucleus enters the isthmus between the mother and the bud. The very early separation of sister centromeres was confirmed by Goshima and Yanagida (2000) who found their bipolar orientation at the time of bud emergence. This was at a time immediately after separation of the SPBs. It is not quite certain whether at that time S phase is complete (see above)

but as centromere DNA replicates early in yeast (McCarroll and Fangman, 1988), it is possible that centromere separation takes place as early as middle or late S phase.

Observations on living cells showed that early centromere separation is transient in many cases and centromeres perform several separation and reassociation ("breathing") cycles before they finally separate. This suggests that cohesion between sisters is mediated by elastic structures (He *et al.,* 2000; Tanaka *et al.,* 2000). However, Goshima and Yanagida (2001) observed breathing of centromere 15 only in a subset of cells and concluded that the brief reassociation that occasionally occurred is functionally irrelevant for sister chromatid separation and that the force opposing the pulling by the kinetochore microtubules is generated by noncentromeric sister chromatid cohesion. On the other hand, the observation that cohesins, the proteins that are essential for sister chromatid cohesion, are most abundant in the centromeric regions of budding yeast (Blat and Kleckner, 1999; Megee and Koshland, 1999; Tanaka *et al.,* 1999) suggests that this region primarily contributes to cohesion. In another *in vivo* study, Pearson *et al.* (2001) found that centromere breathing is accompanied by the oscillation of centromeres between the two poles of the spindle and that the final anaphase separation starts when the centromeres are anywhere between the two poles of the short mitotic spindle. This is different from mitosis in plants and animals in which chromosomes attach to the spindle and move to a region that is equidistant from the two poles. Only after all chromosomes have assembled at this so-called metaphase plate do sister chromatids start to disjoin.

The *in vivo* studies by He *et al.* (2000), Tanaka *et al.* (2000), Goshima and Yanagida (2001), and Pearson *et al.* (2001) revealed that in yeast anaphase movement takes place while the nucleus is still entirely in the mother cell. Also with FISH probes for centromeres, centromere separation can be seen to occur in nuclei within the mother cell, shortly after the elongation of the intranuclear microtubules that drives the SPBs apart (Fig. 3i–k). There exist two mechanisms by which anaphase separation of sister chromatids can occur. First, they can move poleward by the shortening of kinetochore microtubules, which is called anaphase A. Second, chromatids can migrate together with the spindle poles due to the elongation of the spindle (anaphase B). Because spindle elongation in yeast is considerable (up to $\sim$10 μm, see Winey *et al.,* 1995) it may be assumed that anaphase B plays the major role in this organism. However, the breathing and oscillating movement of centromeres along the short spindle (see above) suggests that shortening of kinetochore microtubules is involved and also contributes to the final separation of centromeres (Guacci *et al.,* 1997a; Straight *et al.,* 1997). The existence of early anaphase A movement is also evident from the rapid regrouping of centromeres around the two sister SPBs in the presence of only moderate spindle elongation (Fig. 3e–h, j, and k). Whether there is a contribution of kinetochore microtubule shortening to late anaphase movement is less clear (Guacci *et al.,* 1997a; O'Toole *et al.,* 1999).

Labeling of centromeric and telomeric chromosome regions with GFP showed that chromosomes are stretched during anaphase as the separation of centromeres is not immediately followed by the passive movement of telomeres (Straight *et al.,* 1997). Instead, the distance between the centromere and telomere of a chromosome increases. Only when sister centromeres have separated for a considerable distance do sister telomeres also separate and snap to opposite poles (Straight *et al.,* 1997; Pearson *et al.,* 2001). This elastic behavior of chromosome arms is also apparent after chromosome painting, which shows that compacted metaphase chromosomes stretch during anaphase and become shortened again in telophase (J. Fuchs *et al.,* in preparation).

By integrating the observations mentioned above, chromosome behavior during mitosis can be summarized as follows (Fig. 4). During interphase, centromeres are attached to the SPB via intranuclear microtubules (Figs. 3e and 4a). Shortly after SPB duplication, when the sister SPBs are separated by a short distance, the centromeres start to oscillate between them, and sister centromeres may separate transiently (Fig. 4b and c). This process is reminiscent of prometaphase chromosome movements known from other organisms. Due to the early onset of these mitotic activities, immediately after S phase or with DNA replication still in progress, a classical G_2 stage of interphase is absent (Fig. 4d). A stage analogous to the metaphase plate in plant and animal mitosis, when all centromeres congregate midway between the spindle poles, is missing. At some point, sister centromeres will finally separate and move to opposite poles (Anaphase A—Fig. 4e). Anaphase A and B seem to begin simultaneously, when the nucleus resides in the mother cell, with anaphase B continuing when the nucleus is stretched and extends through the budneck (Fig. 4f). Chromatids are considerably stretched at late anaphase suggesting that cohesion between distal regions of sister chromatids is maintained until very late. Upon its sudden release under the tension of the spindle, chromatids rapidly recoil toward the poles (Fig. 4g).

E. Chromosomes in Meiosis

1. The Meiotic Prophase: Bouquet Formation and Pairing

During meiotic prophase the cognition of homologous chromosomes, their intimate pairing via the SC, and crossing over take place. Although there is still little information on how homologous chromosomes recognize and approach each other (Loidl, 1990), the molecular processes of recombination and the biochemistry of the SC are better understood. In fact, most of what we currently know about these aspects of meiosis was first discovered in budding yeast. One of the most important insights was that the first step in most if not all meiotic recombination events is the formation of programmed DNA double-stranded breaks (DSBs) (Sun *et al.,* 1989; Cao *et al.,* 1990) and the identification of Spo11p, a type II topoisomerase, that

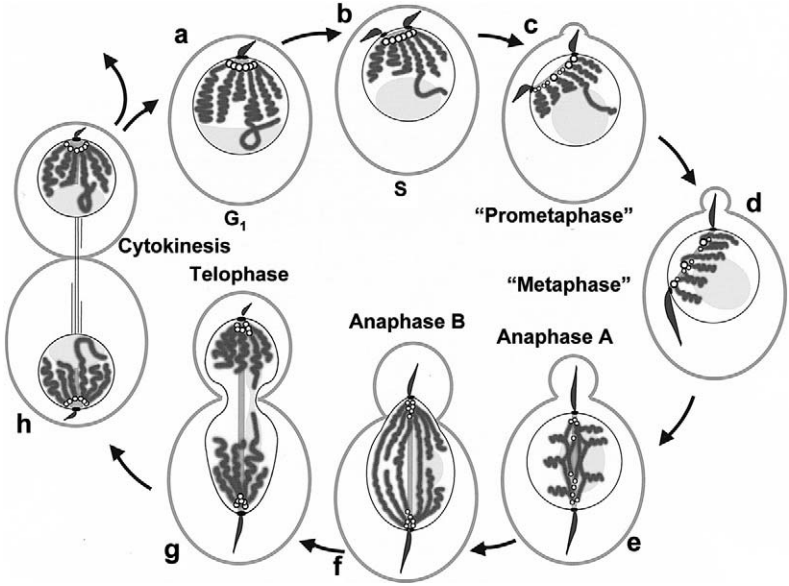

FIG. 4 Interpretation of chromosome behavior during yeast mitosis. This scheme compiles observations on the mitotic spindle (see Segal and Bloom, 2001) and DAPI-stained chromatin, supplemented with data from chromosome labeling by FISH, chromosome painting, and GFP (see Fig. 2; for references see text). (a) During interphase, centromeres form a ring around the SPB to which they are attached via intranuclear microtubules. (b) At around S phase when the duplicated SPBs have begun to separate, chromosomes start to condense about two-fold relative to their interphase lengths. (c, d) Shortly after the initiation of SPB separation, the centromeres occupy the space between sister SPBs and start to oscillate between them. This movement may be accompanied by transient splitting of sister centromeres. The oscillation of centromeres between the spindle poles is reminiscent of the prometaphase stage in plants and animals. In *S. cerevisiae,* however, this movement does not result in the assembly of the centromeres in the middle between the spindle poles ("metaphase plate") before they split. Instead, sister centromeres separate irrespective of their position on the spindle. (e) In early anaphase sister centromeres separate by movement to the opposite poles (anaphase A) while the nucleus still resides in the mother cell. (f) Further separation of the sister chromatids is driven by the elongation of the spindle (anaphase B) while the nucleus stretches and squeezes through the budneck. The chromatids become stretched. (g) When the arms finally separate in telophase, chromatin rapidly recoils toward the poles. (h) During cytokinesis, chromosomes resume interphase organization.

catalyzes them (Bergerat *et al.,* 1997; Keeney *et al.,* 1997). In yeast, single-stranded DNA tracts are formed at DSBs and invade double-stranded homologous DNA to form heteroduplices that are early intermediates in meiotic recombination. They precede the formation of the SC and are necessary for its formation [see Hunter and Kleckner (2001) and references therein for the temporal relationships between DNA events and SC development]. Because molecular recombination is beyond the scope of this article, the reader is referred to several recent articles on this topic (Roeder, 1997; Zickler and Kleckner, 1999; Keeney, 2001).

Zickler and Kleckner (1998) attempted to stage yeast prophase nuclei by their appearance in DAPI-stained preparations. However, a more reliable classification of nuclei as leptotene, zygotene, pachytene, or diplotene can be made according to the degree of SC formation as visualized by silver staining or immunostaining of SC components, notably Zip1p. Moreover, the distances of homologous loci seen by FISH are indicative of the state of chromosome pairing (Scherthan *et al.,* 1992). One of the first microscopically visible events upon the induction of meiosis in yeast is the roughly parallel alignment of homologous chromosomes (as represented by FISH signals) at a distance of $\sim$1–2 μm (Scherthan *et al.,* 1992). This association also occurs in mutant strains that are incapable of SC formation (Loidl *et al.,* 1994) and it resembles the presynaptic alignment of homologs that has been described for a number of other organisms (Loidl, 1994). The nature of the presynaptic alignment is unclear. It probably reflects the primary homologous encounter of regions along two chromosomes. Originally, it was proposed that a homology-searching mechanism via DNA might involve strand invasion and transient heteroduplex formation (Loidl, 1990). On the other hand, the matching of intact DNA molecules was considered as a less time- and energy-consuming way for repeated transient homology testing events during genome-wide homology search (Kleckner and Weiner, 1993; Camerini-Otero and Hsieh, 1993; Loidl, 1994). In yeast, where strand exchanges at multiple sites along pairs of chromosomes precede and initiate the formation of SCs (Sym *et al.,* 1993) it is difficult to separate the functions of homology recognition and initiation of recombination; they might but need not spatially coincide. However, support for presynaptic alignment occurring independently of DNA strand exchange at DSB sites comes from *Caenorhabditis elegans*. Although Spo11-induced DSBs are not necessary for homologous synapsis in this organism, presynaptic alignment does occur (Pasierbek *et al.,* 2001).

In most higher organisms, meiotic chromosome synapsis is preceded by the assembly of all chromosome ends in a limited area on the inner nuclear surface (Dernburg *et al.,* 1995 and Zickler and Kleckner, 1998). This so-called "bouquet" arrangement exists also in *S. cerevisiae* (Scherthan, 2001). Whereas in vegetative yeast nuclei telomeres associate in a few aggregates at the nuclear periphery (Klein *et al.,* 1992; Gotta *et al.,* 1996; Laroche *et al.,* 1998), they congregate in a region around the SPB in meiosis (Trelles-Sticken *et al.,* 1999, 2000). Simultaneous FISH with a pan-telomeric and a pan-centromeric probe demonstrated that at first telomeres move toward the centromere cluster, and then centromeres lose contact with the SPB such that the orientation of chromosome arms with respect to the SPB reverses. Telomere clustering is most compact at zygotene, when first stretches of homologous chromosomes are connected by the synaptonemal complex. At pachytene, when synapsis is complete, the cluster becomes dissolved (Trelles-Sticken *et al.,* 1999).

Bouquet formation is dependent on *NDJ1/TAM1* (Trelles-Sticken *et al.,* 2000), which encodes a telomere-associated protein with a role in chromosome pairing

and segregation (Chua and Roeder, 1997; Conrad *et al.,* 1997). Abrogation of the bouquet in *S. cerevisiae* leads to a notable delay in chromosome synapsis but it does not completely prevent meiosis. Therefore it was suggested that the bouquet, although it is not essential, confers a selective advantage by permitting rapid sporulation under naturally occurring conditions (Trelles-Sticken *et al.,* 2000). It is conceivable that telomere-mediated chromosome movement brings about homologous contacts and/or that general telomere clustering in the bouquet brings corresponding chromosome regions into close proximity, which promotes the establishment of homologous contacts (Loidl, 1990; Rockmill and Roeder, 1998). It is also possible that the bouquet arrangement may facilitate synapsis by imposing a roughly parallel orientation of chromosome arms and thereby resolving entanglements between chromosomes (interlocking) (Zickler and Kleckner, 1998; Scherthan, 2001). It has been proposed that the bouquet is an intensification of the Rabl orientation (see Section III.D) at the preceding interphase (Fussell, 1987; Aragón-Alcaide *et al.,* 1997). However, this does not seem to be the case in yeast or in other organisms, as the chromosome arms turn around by 180 degrees during the transition from the Rabl configuration to the bouquet and telomeres at first face the SPB-distal pole of the nucleus and then assemble near the SPB (Zickler and Kleckner, 1998; Loidl, 2000).

Following the early steps in meiotic recombination and concomitantly with the arrangement of chromosomes in the bouquet, the SC is initiated. Synapsis probably starts at the sites of DSBs (Agarwal and Roeder, 2000) and proceeds along homologous pairs in a zipper-like fashion. The ultrastructure of the SC is remarkably similar between yeast and other organisms (Loidl, 2000), whereas its protein components are only weakly conserved (Roeder, 1997). The function of the SC is still not fully clarified. The classic view of meiosis had been that the SC establishes and stabilizes the association between homologs that allows the exchange of DNA sequences, but the sequence of meiotic events as observed in yeast forced the abandonment of this concept (Hawley and Arbel, 1993). The minor role of the SC in chromosome pairing is also evident from the fact that it is indifferent to homology. If homology recognition has failed or pairs of homologous chromosomes are not present, as is the case in haploids, the SC is formed between nonhomologous chromosome regions (Loidl *et al.,* 1991) (Fig. 2e). Currently it is believed that the SC is important for the maturation of recombination intermediates into chiasmata and for chiasma interference (Roeder, 1997).

SC-associated recombination nodules were detected in electron microscopic images of sectioned yeast nuclei (Byers and Goetsch, 1975b; Schmekel, 2000). In other organisms they were found to come in two types: early and late nodules, the former probably marking the sites where enzymes involved early in recombination assemble and the latter being the ultrastructural manifestations of crossovers. In yeast, two classes of SC-associated dots, probably corresponding to early and late nodules, can be discriminated by immunostaining. One contains DSB-associated

proteins (Gasior *et al.,* 1998) and the other contains late recombination enzymes (Ross-Macdonald and Roeder, 1994).

2. The Meiotic Divisions

As in mitosis, the condensation of chromosomes causes their transformation from the transcriptionally active form into the compact transport form. Most of this condensation seems to be completed by the end of pachytene. In diplotene, the intimate synapsis of homologous chromosomes is resolved and the bivalents are held together only at chiasmata. Unlike many other organisms, in yeast the lateral elements of the SCs are still maintained in this stage. This allows the visualization of diplotene bivalents in strains, which do not rush through meiosis as quickly as the widely used strain SK1 (Fig. 2d). But by the subsequent stage, which corresponds to diakinesis or metaphase I, and during which chromosomes reach their highest compactedness in higher eukaryotes, they are no longer visible as individual structures in yeast either by silver or by DAPI staining. In yeast, the degree of chromosome compaction seems to remain roughly the same between pachytene and the onset of the first meiotic division (J. Fuchs *et al.,* in preparation). As previously mentioned, pachytene is the only stage in which centromeres are not clustered, but immediately after they reassemble in the vicinity of the doubled spindle pole body and remain tightly associated with the poles of the spindles throughout both meiotic divisions (Klein *et al.,* 1999; Buonomo *et al.,* 2000).

Although chromosome dynamics during mitosis and the two meiotic divisions is superficially similar, there is a profound difference in that in the first meiotic division homologous centromeres rather than sister centromeres separate. This behavior is dictated by the primary role of meiosis: the haploidization of the diploid chromosome set. The formation of bivalents is necessary to predetermine pairs of homologs for disjunction, and chiasmata in cooperation with the cohesion of sister chromatids in chiasma-distal regions serve the role of linking the homologs (Maguire, 1974). If cohesion is lost distally to the chiasmata, homologs are ready to separate at meiosis I, but sister chromatids of each homolog migrate together as they remain connected by cohesion in and/or around the centromere (Buonomo *et al.,* 2000). Moreover, a special, not yet fully understood protein endowment with monopolins ensures the coordinated capture of microtubules extending to one and the same pole by the two sister centromeres (Tóth *et al.,* 2000). Prior to the second meiotic division, cohesion is released in the centromeric regions, and this allows the separation of sister chromatids.

The stepwise release of sister chromatid cohesion first from the chromosome arms and then from the centromeres, which helps to coordinate the first, reductional, and the second, equational, division, is a special meiotic requirement. Klein *et al.* (1999) showed in yeast that this is met by Rec8p, a meiotic version of the cohesin component Scc1/Mcd1p (see Section II.C.5). Although meiosis-specific versions

of sister chromatid cohesion proteins have been characterized in higher eukaryotes, it is not yet clear how the differential release of cohesion along the arms and between the centromeres is regulated (Nasmyth, 2001).

Meiosis in the yeast is unusual in that the second meiotic division begins before the nuclei have completely separated at the end of meiosis I, i.e., two spindles form within a single intact nucleus (Moens and Rapport, 1971b). A portion of the nucleus containing most of the nucleolar material is not incorporated by the developing ascospores (Brewer *et al.,* 1980; Byers, 1981).

III. Chromosomes in Interphase

As we have seen in the previous sections, the smallness and poor visibility of chromosomes make yeast an unfavorable subject for cytological studies on dividing nuclei. Interphase chromosomes, on the other hand, are equally unamenable to direct cytological detection in yeast and higher eukaryotes, with the exception of polytene chromosomes. The smallness of the yeast genome becomes an advantage when the simplicity of the genome facilitates the study of genes with a role in nuclear architecture or the influence of interphase chromosome organization on gene expression. This made yeast an equally useful and in some cases even superior experimental system for the study of nuclear architecture.

A. The Importance of Interphase Nuclear Order

Interphase nuclei are not like a bag with chromosomes arbitrarily stuffed into it, but their topology is highly ordered and regulated. One possible reason for the maintenance of order is to ensure that chromosomes do not become too entangled during interphase. This would facilitate their timely congregation at the spindle equator at the onset of mitosis. Another demand may be the gathering of genes that need to be transcribed in concert. Replication is also temporally coordinated and takes place in specially determined regions. Possibly, the available pool of replication enzymes is most economically utilized by concentrating it locally. A notable case of nuclear compartmentalization is the handling of rRNA in a reserved area, the nucleolus. Nuclear compartments may be dynamic and vary between cell types or developmental stages. In higher eukaryotes, evidence is accumulating that large-scale changes in gene activity during development and differentiation are accompanied or influenced by alterations of the topological organization of genes within the nucleus (Marshall *et al.,* 1997a; Lamond and Earnshaw, 1998; Cockell and Gasser, 1999; Francastel *et al.,* 2000). Also in yeast there exist developmental programs, such as mating and sporulation, that call for a coordinated and global regulation above the transcriptional activity of individual genes. Not much is known

about development-related changes of chromosomal topology in yeast, but there is evidence for cell-cycle-regulated reorganization within the interphase nucleus.

The following discussion is divided into two parts. The first part deals with nuclear compartments and forms of chromosome arrangements that have been shown to exist both in higher eukaryotes and in yeast. These are the interphase chromosome territories, the nuclear periphery and the nucleolus as specialized domains, and the distinction between a centromeric and a telomeric pole of the nucleus. The second part concerns various types of nonrandom positioning of chromosomes relative to each other, which are known from a variety of organisms, but whose existence in yeast is under debate.

B. Chromosome Territories

In conventionally stained microscopic preparations, interphase nuclei appear as a relatively homogeneous chromatin mass and it is not apparent if and to which degree the decondensed chromosomes are intermingled and entangled. Cremer and co-workers addressed this question in an elegant series of experiments on animal cell cultures. They induced small lesions in interphase nuclei by means of a microbeam and studied the distribution of affected chromosomal regions (which they identified by the incorporation of radioactively labeled nucleotides during repair synthesis) at subsequent metaphase. By this they could see that the damage was limited to small areas on one or few metaphase chromosomes, which suggested that the exposed region of the interphase nucleus was occupied by chromatin belonging to only one or a few chromosomes (Cremer *et al.,* 1982a,b). This led to the concept of well-separated interphase chromosome territories and their role in the functional compartmentalization of the cell nucleus (Cremer *et al.,* 1993) as opposed to nonterritorial models of chromosome organization (Comings, 1968). The introduction of *in situ* hybridization methodology confirmed the existence of chromosome territories for a wide range of animal and plant species (Manuelidis, 1985; Lichter *et al.,* 1988; Manuelidis and Borden, 1988). Genes are preferentially located at or near the surface of chromosome territories, such that newly synthesized RNA may be deposited directly into the interchromatin space (Kurz *et al.,* 1996; Verschure *et al.,* 1999).

Haber and Leung (1996) questioned the existence of chromosome territories in yeast on the grounds that simultaneously induced double-stranded DNA breaks showed no preference for repair by religation of the broken ends within the chromosome over interchromosomal recombination events. However, the detection of dense, mutually exclusive areas of staining by chromosome painting and GISH (Scherthan *et al.,* 1992; J. Fuchs, A. Lorenz, and J. Loidl, unpublished results) (see Section II.A; Fig. 3b and c) provides evidence for the territorial organization of interphase nuclei in budding yeast. In fact, a recent investigation of Cre-induced mitotic recombination events suggested that intrachromosomal events are favored

over interchromosomal events (Burgess and Kleckner, 1999). It is therefore conceivable that spatial proximity is not relevant to the choice of partners for the type of recombination studied by Haber and Leung (1996). Moreover, whereas Haber and Leung (1996) based their objection to chromosome territories on the assumption that they are static, it is now known that although chromosomes are confined to nonoverlapping territories, there is sufficient mobility to allow interactions between them (Heun *et al.,* 2001c) (see Section III.F).

In animal nuclei, the interchromatin space was found to consist of a network of nuclear channels that connects the nuclear interior and the nuclear pores, and it was proposed that they serve for the trafficking of mRNAs and molecules involved in nuclear metabolism (Cremer *et al.,* 1993; Bridger *et al.,* 1998; Visser *et al.,* 2000). These channels are not easily detected as gaps between chromatin masses, but in cells expressing vimentin with a nuclear localization signal, filamentous intranuclear arrays of the protein were formed and delineated the interchromosomal domain compartment (Bridger *et al.,* 1998). In yeast nuclei, there is a chromatin-poor or chromatin-free area in the vicinity of the SPB that appears as a dark spot after chromatin staining with DAPI (Jin *et al.,* 2000). Other lacunae or nuclear channels have not yet been demonstrated, but because there is evidence that the transcriptionally active regions are in the interior of the nucleus (see Section III.C.3), an interchromatin transportation route, comparable to nuclear channels, between the transcription sites and the nuclear surface is likely to exist.

C. Suprachromosomal Nuclear Organization and the Differential Activation of Genes

Heterochromatin is a hostile environment for active genes. It was first observed in *Drosophila* that if a gene was brought into the vicinity of heterochromatin, its transcription was variably repressed. This phenomenon was termed position effect variegation (PEV) (Henikoff, 1990). Likewise, genes that are transcriptionally silenced are sequestered to pericentric heterochromatin in developing human lymphocytes (Brown *et al.,* 1997; Carmo-Fonseca, 2002). When a similar silencing effect, the telomere position effect (TPE), was discovered in telomere-near regions of *S. cerevisiae,* this similarity (which also extends to late replication and repetitiveness) prompted *S. cerevisiae* telomeres (or more precisely the subtelomeric chromatin) to be defined as heterochromatic (Gottschling, 1992). Subtelomeric yeast chromatin and heterochromatin also share the presence of hypoacetylated histones and reduced accessibility by restriction enzymes (de Bruin *et al.,* 2001). It was shown that the TPE depends on the presence of the silencing factors Sir2p, Sir3p, and Sir4p together with Rif1p, Rif2p, and Rap1p at the telomeres (Bourns *et al.,* 1998). In mutants where the colocalization of these silencing factors with telomeres is lost, telomere proximal silencing is abolished (Gotta *et al.,* 1996).

In yeast, not only telomeres but also the silent mating type loci and the rDNA repeats are associated with proteins that confer transcriptional silencing. It was shown that the telomeres and the silent mating type locus *HML* (Laroche *et al.,* 2000) are situated at the nuclear periphery. rDNA tracts have a silencing effect on Pol II-transcribed genes in their vicinity (see Section III.C.4), and they are nestled against the nuclear envelope in interphase nuclei (own observations). Hence one might ask if there is a causal relationship between the peripheral nuclear localization and the silenced state of a chromosomal region. If so, does some property of the nuclear periphery actively promote silencing or are silenced regions relegated to the periphery just to get them out of the way? In the following, the role of the nuclear positioning of chromosomal loci in terms of their activity and replication timing will be discussed.

1. Telomeres Cluster at the Periphery of Nuclei

Klein *et al.* (1992) found by immunostaining that Rap1 protein is situated at the ends of meiotic chromosomes and that in interphase nuclei it is present as peripheral dots. Later it was confirmed by FISH with probes against telomere-near sequences that the localization of Rap1p reflects the peripheral distribution of the telomeres. It was also shown that telomeres form clusters as there are fewer signals than the 32 telomeres present in haploid nuclei (Gotta *et al.,* 1996). It is not known if telomere clusters include nonrandom groups of telomeres (like homologous telomeres or the telomeres of the same chromosome) but because there are often four or more telomeres as part of a cluster, they must primarily consist of nonhomologous telomeres. Their association is possibly related to the high rate of ectopic recombination between subtelomeric sequences (Pryde *et al.,* 1997). Moderate clustering of telomeres (although not at the nuclear periphery) was subsequently also found in human cells where it is more frequent in stationary or differentiated cells than in cycling cells (Nagele *et al.,* 2001).

Is there a structure that anchors yeast telomeres to the nuclear periphery? It was shown that the Ku70 subunit of the Ku protein heterodimer, which is abundant at telomeres, interacts with the myosin-like proteins Mlp1 and Mlp2, which in turn interact with Nup145p, a nucleoporin. This suggests a physical link between nuclear pore complexes and telomeres. Accordingly, the deletion of *MLP1* and *MLP2* and a mutation in *NUP145* led to the mislocalization of telomeres. Therefore, it was proposed that extensions from the nuclear pore complexes formed by Mlp proteins tether chromosome ends to the nuclear periphery through interaction with Ku70p (Galy *et al.,* 2000). On the other hand, Tham *et al.* (2001) demonstrated that the Ku complex is not essential for linking a specific telomere (of the left arm of chromosome *VII*) to the periphery. This rules out the model in its simplest form and suggests that some telomeres may be more dependent on Ku than others.

It should also be noted that in mammalian meiosis, telomeres tend to attach to regions of the nuclear envelope, which are poor in nuclear pores (Scherthan *et al.,* 2000), and that the association of chromosome ends with the nuclear envelope during formation of the meiotic bouquet in yeast does not require Ku70p (H. Scherthan, personal communication).

2. The Nuclear Periphery Constitutes a Silent Chromatin Domain

Numerous reports from a variety of organisms demonstrate the preference of certain chromosomes or chromosome regions to adopt a peripheral or internal region within the nucleus. Boyle *et al.* (2001) and Cremer *et al.* (2001) noted that in spherical human cells the most gene-dense chromosomes concentrate at the center of the nucleus, whereas the more gene-poor chromosomes are located toward the nuclear periphery. [In flat nuclei of amniotic fluid cells and fibroblasts where the central region is not remote from the periphery, this relationship does not exist (Cremer *et al.,* 2001).] Moreover, it seems to be a general rule that transcriptionally silenced and/or late replicating chromosomes or chromosome regions, which are often heterochromatic, tend to be located at the periphery of the nucleus. This was first found for the inactive X chromosomes in females of humans and other mammals. In cultured human cells, the Barr body formed by the inactive X is adjacent to the nuclear envelope in 75–80% of interphase cells (Walker *et al.,* 1991 and references therein). It was later confirmed for a variety of dividing cell types from mammals that the peripheral nuclear compartment contains predominantly transcriptionally silent late replicating chromosomal regions or loci, whereas active early replicating regions preferentially localize to the internal compartment (Ferreira *et al.,* 1997; Bridger *et al.,* 2000; Nogami *et al.,* 2000b; Li *et al.,* 2001).

Also in budding yeast, there exists a relationship between the peripheral position of chromosomal regions and the repression of transcription. Deletion of *HDF1* and *HDF2,* the genes encoding the subunits of the Ku protein heterodimer, disrupts the peripheral localization of telomeres and abolishes silencing (Laroche *et al.,* 1998). Likewise, disabling the anchoring proteins Mlp1, Mlp2, and Nup145 led to the mislocalization of telomeres and to the derepression of a subtelomeric reporter gene (Galy *et al.,* 2000). Andrulis *et al.* (1998) showed that, conversely, perinuclear localization helps to establish silencing of an *HMR* locus with a defective silencer. A silencer is a *cis*-acting element that recruits the silencing factors Sir2p, Sir3p, and Sir4p and confers silencing to an adjacent region (Cheng and Gartenberg, 2000). The modified silencer in the experiment of Andrulis *et al.* (1998) bound the GAL4 DNA-binding domain fused to an integral membrane protein, which targeted the silencer to the nuclear periphery and led to the silencing of a nearby reporter gene. Because it was shown that the other silent mating type locus, *HML,* is situated at the nuclear periphery (Laroche *et al.,* 2000), it is possible that the defective silencer used by Andrulis *et al.* (1998) had lost the ability to tether the

HMR locus to the periphery and that by restoring the natural position silencing was regained.

By using a system for reversible derepression of a subtelomeric marker, Tham *et al.* (2001) and Feuerbach *et al.* (2002) studied the connection of the repressed status with the nuclear position of an individual telomere. The transcription of a telomere-close *URA3* gene was induced by growth in medium lacking uracil. Whereas Tham *et al.* (2001) found that the telomere maintained its peripheral position, Feuerbach *et al.* (2002) noted that 40% of telomeres were outside the silent domain. Thus, a majority of telomeres loses the TPE under selective conditions, indicating that localization near the nuclear periphery is not sufficient for silencing. On the other hand, as was noted above, peripheral localization is necessary for TPE because a mutant situation in which telomeric silencing was maintained in the absence of peripheral localization has not yet been encountered (Tham *et al.,* 2001) (see Fig. 5). Therefore it is reasonable to assume that telomeres assemble at the periphery of nuclei not because of the transcriptionally repressed state but rather because the localization of telomeres is critical for telomeric repression of transcription (Fig. 5).

This poses the question of whether the nuclear periphery is a silencing domain because it hosts the telomeres with their associated silencing factors, or if silencing at the periphery is independent of the presence of telomeres. This question was addressed by Feuerbach *et al.,* (2002). They removed telomeres from the periphery by the deletion of proteins that anchor telomeres to the nuclear pores (Galy *et al.,* 2000) and found that a nuclear envelope-tethered *HMR-TRP1* reporter construct underwent significant derepression. From this they concluded that removal of telomeres from the periphery deconstructs the peripheral silencing domain. In another experiment, Feuerbach *et al.* (2002) used a reporter construct consisting of an *HML* locus with intact silencers with an *ADE2* reporter gene next to it, which was ectopically inserted in a telomere-distant chromosomal region and therefore derepressed. When they disassembled the peripheral silent domain by deleting the telomere anchors, silencing at the ectopic site was partially reestablished. This suggests that by relocalizing telomeres away from the nuclear periphery to the nuclear lumen the silencing property is redistributed, presumably by the dispersal of Sir2p.

Thus, by taking into account all available evidence, it appears that silencing at the periphery is caused by the cooperative effect of silencing factors that are associated with telomere clusters at the nuclear envelope and that other loci with a potential of being silenced benefit from the high local concentration of Sir factors (Cockell and Gasser, 1999).

The concept of a nucleus with silenced gene loci and noncoding portions of the genome at the periphery and active genes buried inside is somewhat counterintuitive. One would rather tend to assume that transcribing regions are doing better in receiving transcription-activating signals and exporting mRNA to the cytoplasm if they are close to the nuclear surface. As one possible reason, it was proposed that the

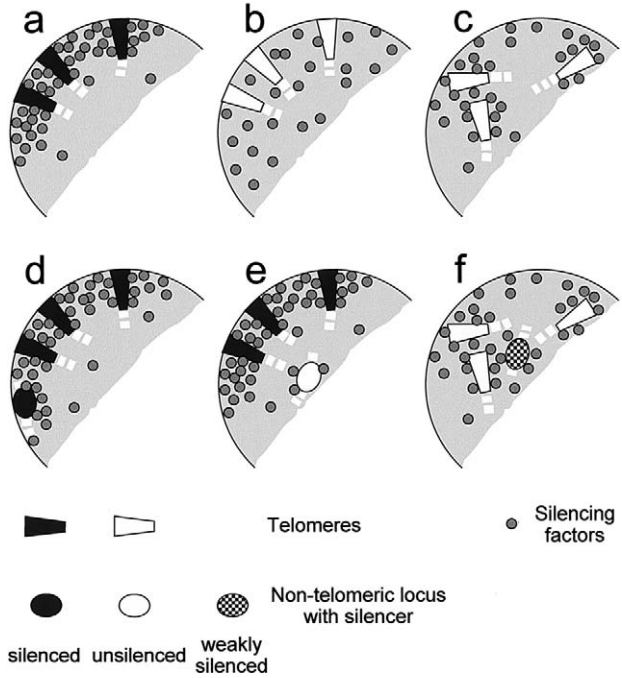

FIG. 5 Relationship between transcriptionally repressed status and peripheral location and its interpretation. (a) Telomeres attached to the nuclear envelope show the TPE. (b) Telomeres that are situated at the periphery but are unable to recruit silencing factors do not confer silencing to nearby genes (Gotta *et al.,* 1996). (c) Telomeres that are detached from the nuclear envelope do not exert the silencing effect (Laroche *et al.,* 1998; Galy *et al.,* 2000). (d) A nontelomeric reporter construct that resides at or is brought to the periphery is transcriptionally silent. It possibly benefits from the high local concentration of silencing factors. (e) Silencing of a reporter construct is derepressed if it is not at the periphery. (f) A nonperipheral reporter construct is partially repressed upon the detachment of telomeres from the nuclear envelope (Feuerbach *et al.,* 2002). All these situations can be explained by a model according to which the nuclear periphery is a region in which silencing factors are highly concentrated due to the presence of telomeres that recruit silencing factors (Cockell and Gasser, 1999). (c) If telomeres are not situated at the periphery, the density of silencing factors associated with each individual telomere may be insufficient to produce the TPE. (d) Silencing of transcriptionally repressed regions such as the *HML* and *HMR* loci may benefit from the high concentration of silencing factors at the nuclear periphery. (e) If a locus is internally located, silencing factors may be titrated away by the telomeres, even if it has an intact silencer. (f) If telomeres are not tethered to the nuclear envelope, silencing factors may be more evenly distributed in the nucleus, allowing limited transcriptional suppression of loci in the nuclear interior.

less vital parts of the genome at the periphery serve as a safeguard against mutagens, chromosome-breaking agents, and viruses (Hsu, 1975). Moreover, it is conceivable that concerted transcriptional regulation and the sharing of transcription factories (Lamond and Earnshaw, 1998; Cook, 1999) are favored for genes that are in close contact by their confinement to the nuclear interior.

3. Late Replication Origins Are Located at the Periphery

In the budding yeast it was found that replication origins near telomeres generally are activated late in S phase (Fangman and Brewer, 1992). Heun *et al.* (2001a) reported that late-firing replication origins are transiently located at the periphery of nuclei during G_1, whereas early-firing origins are randomly localized within the nucleus throughout the cell cycle. This was true not only for origins that occupy telomere-near regions on chromosomes (and could be assumed to be recruited to a peripheral position by their physical association with a telomere—see Section III.C.1), but also for late origins that lie far from telomeres. However, peripheral nuclear localization is not sufficient to confer delayed initiation, as early origins were found at internal and peripheral sites in equal frequencies. Obviously, a sequence domain that flanks the minimal core sequence of the origin is equally important for the timing of replication at a given origin. When the minimal core sequence (containing the autonomously replicating sequence) originating from a late-firing origin was transferred to a plasmid, it became early firing. However, if it was transferred together with the flanking sequences, it remained late firing. Moreover, when a normally early-firing origin was inserted into this sequence domain, it was shifted to late activation (Friedman *et al.,* 1996). Heun *et al.* (2001a) proposed a model in which the DNA elements flanking late-firing origins promote association with the nuclear periphery in early G_1, which is necessary for the establishment of a late-firing chromatin state. Because of the contrast between the dynamic late origin localization and the more stable association of telomeres with components of the nuclear envelope (see Section III.C.1), Heun *et al.* (2001a) proposed the existence of at least two pathways for targeting chromosomal regions to the nuclear periphery.

In addition to the recruitment of late-firing origins to the nuclear periphery, there appears to exist an additional level of spatial organization of replication. For higher eukaryotes, it has been shown that replication forks tend to assemble in replication factories, rather than each having a set of replication proteins on its own (Cook, 1999). Thus it seems that instead of the replication forks wandering along the chromosomes, the chromosomes are fed through replication factories that are anchored in the nuclear matrix. By marking the sites of DNA synthesis *in situ* by incorporation of bromodeoxyuridine, Heun *et al.* (2001c) showed that in yeast, replication takes place in a limited number of foci.

4. The Nucleolus

The nucleolus is a nuclear compartment that forms around the sites of rDNA repeats, which are therefore called the nucleolus organizing region (NOR) (see Section II.C.3). Within the nucleolus, rDNA transcription by RNA polymerase I (Pol I), processing of the transcripts, and ribosome assembly take place. Unlike higher eukaryotes, the *S. cerevisiae* NOR contains also 5 S rDNA, which is transcribed by Pol III. The nucleolus also plays a role in gene regulation and in cell-cycle control by serving as a site of both recruitment and exclusion of regulatory

complexes (Garcia and Pillus, 1999). Yet it is not essential for growth under normal laboratory conditions (Nierras *et al.,* 1997).

The yeast interphase nucleolus is a crescent-shaped structure that occupies up to one-third of the volume of the nucleus. It is located opposite the SPB, and it makes extensive contact with the nuclear envelope (Gordon, 1977; Oakes *et al.,* 1998; Yang *et al.,* 1989; Léger-Silvestre *et al.,* 1999) (Fig. 6). Although its peripheral position might be assumed to facilitate the transport of ribosomal components between the nucleolus and the cytoplasm, it was shown that gold-labeled ribosomal precursor RNA was also present near nuclear pores on the nonnucleolus side of the nucleus. This suggested that preribosomal subunits are exported to the cytoplasm through all the pores (Léger-Silvestre *et al.,* 1999). Moreover, in most higher organisms, the nucleolus is not in direct contact with the nuclear envelope. When Nop1p, the yeast nucleolar fibrillarin homolog, was overexpressed in *S. cerevisiae,* the nucleolus lost its peripheral localization without producing a discernible growth phenotype (de Beus *et al.,* 1994). Thus, it seems that the association of the nucleolus with the nuclear envelope is not a requirement for its function in ribosome synthesis. It was, however, noted that Pol II transcription of some reporter genes that were integrated into rDNA is partially silenced (Bryk *et al.,* 1997; Fritze *et al.,* 1997; Smith and Boeke, 1997; Smith *et al.,* 1998). Unlike the TPE on silencing, nucleolar silencing is dependent only on *SIR2* (Fritze *et al.,* 1997; Smith *et al.,* 1998). Smith *et al.* (1998) observed that *SIR4,* which is essential for silencing at the silent mating-type loci *HML* and *HMR* and at telomeres, even inhibits silencing in rDNA. They

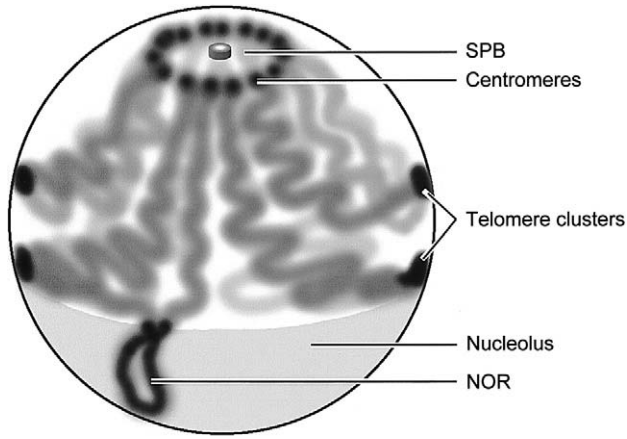

FIG. 6 Model of the chromosomal organization within a yeast interphase nucleus. Centromeres form a ring around a chromatin-free zone with the SPB in its center. The chromosome arms project away from the centromeric pole and their ends are fused into a few clusters at the nuclear envelope. The rDNA tract forms a loop in the region opposite to the centromeric pole and organizes the crescent-shaped nucleolus. For details and references see text.

proposed a model by which Sir4p at telomeres normally recruits the silencing factor Sir2p (Landry *et al.,* 2000) and titrates it out of the nucleolus. Accordingly, Gotta *et al.* (1997) had shown that the deletion of *SIR4* causes redistribution of Sir2p to the nucleolus. As most of the Sir2p is associated with telomeres and is therefore present in the outher shell of the nucleus, the need of the nucleolus to obtain its share of the Sir2p pool might cause its position at the periphery of the nucleus in yeast. Interestingly, also in mutants in which functional Pol I is missing and rDNA is expressed from a Pol II promoter on a plasmid, the nucleolus lost its peripheral localization (Oakes *et al.,* 1998). This would rather suggest that silencing of Pol II transcription is the cause for peripheral localization and it would be interesting to see if silencing was maintained in nucleoli that are internally localized due to Nop1p overexpression.

There were two hypotheses put forward for why Pol II-mediated transcription is silenced in nucleoli. One is that Sir2-mediated chromatin modification is required to exclude the machinery for homology searching and recombination from rDNA. Transcriptional silencing could be a side effect of the avoidance of potentially dangerous (since often unequal) mitotic and meiotic recombination (Smith and Boeke, 1997; Davis *et al.,* 2000). Alternatively, repression of Pol II transcription could be a mechanism to suppress transcription of rDNA by Pol II. It was found that *S. cerevisiae* has the inherent ability to transcribe rDNA by Pol II (Vu *et al.,* 1999). This Pol II pathway (possibly an evolutionary relic), if unsuppressed, might upset regulation of ribosome synthesis.

D. Centromere–Telomere Polarization

Another aspect of order found within interphase nuclei is the roughly parallel arrangement of chromosome arms with centromeres assembled near one pole of the nucleus and the chromosome ends (the noncentromeric ends in the case of telocentric chromosomes) extending toward the opposite pole. This kind of chromosome arrangement is termed Rabl orientation after Carl Rabl who first reported it in nuclei of salamander larvae (Rabl, 1885). Since then it has become widely observed in a variety of plants and animals and it has been interpreted as a remnant of the orientation imposed on chromosomes by the preceding anaphase movement (Fussell, 1987; Dong and Jiang, 1998; Jin *et al.,* 1998; Zickler and Kleckner, 1998).

In budding yeast, centromeres form a cluster around the SPB. This was first suggested by the local accumulation of the centromere protein Ndc10 in interphase nuclei (Goh and Kilmartin, 1993). Later, centromere clustering was confirmed by Guacci *et al.* (1997a) using FISH probes against centromeric regions of various chromosomes. FISH labeling of the whole set of centromeres with a pan-centromeric FISH probe showed that centromeres are arranged as a ring, with the SPB at its hub (Jin *et al.,* 1998, 2000) (Fig. 3e). Moreover, chromosome arms

show a tendency to extend toward the opposite side of the nucleus (Fig. 6) and the intranuclear distance of a chromosomal locus from the centromeric pole is roughly proportional to its genomic distance (in base pairs) from the centromere. Chromosome painting was recently used to confirm this orientation of chromosome arms (J. Fuchs *et al.,* in preparation) (Fig. 3b). Thus, morphologically and probably also by its mode of origin, the polarized interphase arrangement in yeast resembles the Rabl orientation.

In yeast, the maintenance of centromere clusters depends on intact kinetochores and microtubules. It was found to be sensitive to nocodazole, a microtubule inhibitor, and it was reduced in a temperature-sensitive mutant of *NDC10* upon shift to nonpermissive temperature (Jin *et al.,* 2000). Moreover, although in living cells centromere-near GFP-marked loci showed only limited diffusional movement, it was increased after nocodazole treatment (Marshall *et al.,* 1997b). This suggests that centromere clusters are maintained by their physical connection with the SPB via intranuclear microtubules that persist in interphase (Byers and Goetsch, 1975a; Goh and Kilmartin, 1993). Whereas yeast centromere clusters are highly stable and disintegrate only after nuclei have ceased dividing for several days (Jin *et al.,* 1998), in other organisms Rabl orientation is presumably lost gradually as soon as centromeres become detached from microtubules. For instance, in *Drosophila* it was shown that the Rabl orientation prevails in nuclei of embryos when interphases are short and chromosomes have little time to relax from anaphase orientation, but deteriorates in mitotically less active cells (Dernburg *et al.,* 1996). In human lymphocytes, centromeres, unlike telomeres, were preferentially electroeluted from agarose-embedded nuclei (Weipoltshammer *et al.,* 1999). This indicates that centromeres do not stably attach to the nucleoskeleton and hence their distribution may quickly randomize in interphase.

Also in *S. pombe,* centromeres are clustered during interphase and there are parallels between centromere clustering in *S. cerevisiae* and *S. pombe* that go beyond the similarity to the Rabl orientation in multicellular eukaryotes. Also in *S. pombe,* centromere clusters are disrupted only at the onset of mitosis and reestablished at anaphase (Funabiki *et al.,* 1993). The clustered interphase centromeres move in concert with the cytoplasmic microtubules, which likely reflects their association with the SPB. Treatment with microtubule-inhibiting drugs indicated that clustering and movement are dependent on intact cytoplasmic microtubules (Goto *et al.,* 2001; Pidoux *et al.,* 2000). For both *S. cerevisiae* and *S. pombe* it was shown that when clusters were experimentally disrupted, a Rabl-like configuration can be formed *de novo* without a preceding mitosis (Jin *et al.,* 2000; Goto *et al.,* 2001). This indicates that in these organisms Rabl orientation is not a mere consequence of anaphase chromosome arrangement but that it is actively maintained, which suggests a function during interphase or subsequent division. It might be assumed that the maintenance of centromere clusters either is important to support nuclear compartmentalization and/or that it is advantageous for the

rapid resumption of mitosis by rendering unnecessary the *de novo* establishment of centromere–microtubule contact at the onset of mitosis.

E. Order of Chromosomes Relative to Each Other

A much debated question in cytology concerns the existence and nature of nonrandom positioning of chromosomes relative to each other. Examples for such arrangements are the nonrandom positioning of the parental chromosome sets relative to each other in diploids, mostly visualized as genome separation, somatic pairing (in yeast more correctly called vegetative pairing) of homologous chromosomes in diploids, and the nonallelic interaction of specific chromosomes or chromosome regions.

In most past reports, the nonrandom arrangement of chromosomes in interphase nuclei was inferred from observations of the nonrandom arrangement in ring-shaped prometaphase or metaphase chromosome rosettes, or spread metaphase plates (Avivi and Feldman, 1980). However, in these stages, the influence of mechanical sorting of the chromosomes within the mitotic spindle according to their sizes or strength of spindle attachment on the position of individual chromosomes within the ring or plate cannot be ruled out (Mosgöller *et al.,* 1991). Size-dependent sorting of chromosomes in the metaphase plate is frequently observed and is particularly obvious in species with bimodal karyotypes (White, 1973). Only recently, the use of *in situ* hybridization techniques has allowed the direct observation of spatial relationships in interphase nuclei. In the following section I will summarize and evaluate studies that are relevant to the question of interphase chromosome arrangement in the budding yeast.

1. Parental Chromosome Sets Are Not Separated in Diploid Yeast Nuclei

The separation of paternal and maternal chromosome sets within nuclei was described for somatic cell hybrids of animals (Rechsteiner and Parsons, 1976; Zelesco and Marshall-Graves, 1988) and plants (Gleba *et al.,* 1987), for differentiated tissues of plant sexual hybrids (Schwarzacher *et al.,* 1989; Leitch *et al.,* 1991; Castilho *et al.,* 1999), and for at least some differentiated cell types of mouse (*Mus musculus* × *M. spretus*) hybrids (Mayer *et al.,* 2000a). Parental chromosome separation is not limited to the cells of hybrid organisms or cultured hybrid cells. It was also observed to be maintained for several cell generations in the early embryo following the fusion of gametes (Odartchenko and Keneklis, 1973; Mayer *et al.,* 2000b). In these cases it might reflect the differential expression of the parental chromosome sets and it often accompanies the heterochromatinization and/or elimination of one (mostly the paternal) of the genomes (White, 1973).

Lorenz *et al.* (2002) tested to see if genome separation occurs in the nuclei of hybrids of the two related species *S. cerevisiae* and *S. paradoxus*. They simultaneously *in situ* hybridized hybrid nuclei with differently labeled *S. cerevisiae* and *S. paradoxus* genomic DNA probes. It was found that the two chromosome sets became intermingled immediately after the formation of hybrid zygotes and remained unseparated for all generations during vegetative growth. Because there is no spatial separation of the genomes even in the hybrid, it is reasonable to assume that separation of the parental chromosome sets does not exist in nonhybrid cells either.

2. Somatic Pairing: Does It Exist in Yeast?

Somatic pairing of homologous chromosomes was first observed in Diptera by N. M. Stevens (1908) and has since been confirmed for other genera in this order (Grell, 1969). For many decades there has been a debate among cytologists as to whether somatic pairing is a common phenomenon, albeit being less evident in other organisms (Avivi and Feldman, 1980; Comings, 1980; Therman and Denniston, 1984; Jin *et al.,* 1998). Also, more recent reports from a variety of organisms and cell types are highly controversial. Riesselmann and Haaf (1999) showed preferential somatic pairing of the imprinted region on mouse chromosome 7 during the S phase of the cell cycle and proposed that trans-interactions between oppositely imprinted chromosome regions may be important for the maintenance of imprinting. Likewise, homologous association of oppositely imprinted chromosomal loci was reported for human T lymphocytes by LaSalle and Lalande (1996), but Nogami *et al.* (2000a) did not observe this arrangement of the same pair of loci in a different cell type. Somatic pairing was also reported for human Sertoli cells (Chandley *et al.,* 1996) and anther tapetum cells of wheat (Aragón-Alcaide *et al.,* 1997). In these cases it is possible to speculate that pairing may be induced in nonmeiotic cells in the context of a generative tissue due to the expression of pairing genes elicited by diffusible factors. In strong contrast, no evidence for somatic pairing or any other nonrandom associations between homologous or nonhomologous chromosomes was obtained from carefully performed 2-D and 3-D studies on human fibroblasts and lymphocytes (Cremer *et al.,* 2001 and references therein). Although an increased frequency of homologous associations was observed for gene-dense human chromosomes, such as chromosome 19, Cremer and Cremer (2001) explained this finding by their preferential location in the nuclear interior.

Also for yeast the existence of nonmeiotic homologous pairing was proposed. It was found to occur in a high proportion of premeiotic nuclei by Weiner and Kleckner (1994). Loidl *et al.* (1994) also observed the juxtaposition of homologous regions under premeiotic starvation conditions. These reports were based on a comparison of the frequency or intimacy of associations between FISH signals at allelic and nonhomologous chromosomal sites: The predominance of associations of homologous over heterologous FISH signals was taken as evidence for somatic

(or correctly vegetative) pairing. Evidence for homologous pairing in interphase nuclei of vegetatively growing diploid cultures was subsequently provided by cytological and noncytological assays (Keeney and Kleckner, 1996; Burgess *et al.,* 1999; Burgess and Kleckner, 1999). Keeney and Kleckner (1996) observed a cooperative effect on DNase I sensitivity if the same two alleles of the DNase-sensitive site were present on two homologs, and Burgess and Kleckner (1999) found that Cre-induced recombination between two *lox*P constructs was higher when present at allelic chromosomal sites. One explanation for these effects could be direct, physical interaction, i.e., vegetative pairing, between the sites involved.

On the other hand, several studies from our laboratory have led us to cautiously reevaluate the phenomenon of vegetative pairing in budding yeast. First, the original reports on somatic pairing in yeast were compromised by not taking into account the centromere–telomere polarization (see Section III.D). This causes loci with the same genomic distance (in base pairs) from the centromere to occupy the same latitude of the nucleus with respect to the centromeric pole (Jin *et al.,* 1998) and therefore allelic loci will on average be in closer proximity than two randomly selected loci. FISH with probes to distal regions of short and long chromosomes thus will result in homologous signals being closer together than nonhomologous signals; but this will be merely a function of relative arm lengths. Thus, only if comparison is made between allelic and nonhomologous loci at identical centromere distances could the influence of Rabl orientation be compensated and a mechanism for the active colocalization of homologous chromosomes or chromosome regions possibly be established.

However, even after correction for the contribution of the Rabl orientation to the association of homologous loci, a very small preference for homologous over heterologous associations seems to remain (Burgess *et al.,* 1999; Burgess and Kleckner, 1999; A. Lorenz, J. Fuchs, and J. Loidl, unpublished results). Several possible explanations for this preference, which do not invoke homology-dependent vegetative pairing, are listed below.

1. It is conceivable that subregions along a chromosome undergo different degrees of condensation. This would compromise the above-mentioned compensation of the Rabl effect, as loci on nonhomologous chromosomes could occupy different latitudes of the nucleus even if they had the same genomic distances from their respective centromeres. In fact, it was noted by Dekker *et al.* (2002) that yeast chromosomes comprise AT- and GC-rich domains (isochores) of about 50–100 kb, which might differ with respect to chromatin condensation.

2. Because in yeast telomeres are located at the nuclear periphery (see Section III.C.1), loci on short arms would be located near the nuclear surface, whereas intercalary regions on long arms at a similar latitude would tend to occupy the interior of the nucleus. This would create a tendency of arms of similar lengths (and thus of homologs) to colocalize. Probably also specific intercalary regions are at least transiently attached to the nuclear envelope. This could specify chromosomal regions

(again comprising homologs) with a preferential internal distribution, which are, as a consequence, closer together than any randomly selected pair of chromosomal sites (compare Cremer and Cremer, 2001).

3. Because in cytological assays the presence of a single FISH signal is taken as indicative of the association of homologous probed loci, FISH signal loss is a factor that may contribute significantly to the overestimation of homologous associations. It was determined that ~10% of a probed locus did not produce a detectable FISH signal in standard preparations (A. Lorenz, personal communication).

Although these observations and considerations are not strong evidence against vegetative pairing, they may illustrate that the observation of seeming vegetative pairing is prone to methodological errors and should emphasize the need for exploring alternative explanations.

One of the potential advantages of somatic/vegetative pairing would be in the recombinational repair of DNA damage by utilizing the homologous chromosome as template. Although the sister chromatid is usually preferred in this respect, the homolog is utilized in unreplicated (G_0/G_1) cells (Kadyk and Hartwell, 1992). This repair would be expected to be more efficient if homologous chromosomes are permanently juxtaposed. Alternatively, recombinational repair by the homolog would be possible only if it happened to lie in the vicinity by accident, or if lesions would trigger a homology search mechanism. The question of whether exposure to ionizing radiation affects the relative spatial location of homologous chromosomes was therefore examined. Dolling *et al.* (1997) tested this in human skin fibroblasts and lung endothelial cells. They found that homologous chromosome domains were repositioned closer to each other within interphase nuclei after exposure to radiation. On the other hand, Skalníková *et al.* (2000) and Jirsova *et al.* (2001) found no obvious induction of somatic pairing upon irradiation but a general restructuring of nuclei, best described as shrinkage. However, recombinational repair constitutes only a minor repair pathway in vertebrates, whereas in yeast it predominates. But even in yeast, global vegetative pairing was not notably induced upon irradiation of G_0 cells (J. Fuchs and J. Loidl, unpublished results) and it must be concluded that only loci that have undergone lesions, associate with their homologs during the repair process.

3. Specific Interchromosomal Interactions

There are studies based on higher eukaryotic cells that certain chromosomes interact with each other preferentially, or that there is even a fixed spatial order between nonhomologous chromosomes. A very common example is the juxtaposition of nucleolus-organizing chromosomes due to the fusion of nucleoli. In some cases interchromosome trans-associations were claimed to cause the fixed order of chromosomes within a haploid set, as for example in the gametes of the Turbellarian *Polychoerus* (Costello, 1970). A regular arrangement of nonhomologous

chromosomes was also claimed to occur in human fibroblasts and HeLa cells by Nagele *et al.* (1995). These authors reported that the relative positions of chromosomes within radial arrays (rosettes) at prometaphase were nonrandom in that they were segregated into two tandemly linked haploid sets. Within each set chromosomes had a fixed positional order. However, a reinvestigation by Allison and Nestor (1999) did not confirm this order. Ashley and Wagenaar (1974) found that in the plant *Ornithogalum virens* chromosomes are associated end to end in somatic and generative cells. For this organism it was also proposed that these associations would involve specific nonhomologous chromosomes such that all chromosomes of a haploid set would be arranged in a fixed order within a ring (Ashley, 1979).

In yeast, clustered centromeres at interphase are arranged in a ring around the SPB (see Section III.D) that is reminiscent of prometaphase rosettes. This prompted the question of whether any specific order existed between chromosomes in the ring (Jin *et al.,* 2000). In diploids, centromeres of homologous chromosomes were labeled by FISH and their angular separation within the ring was measured. If homologous chromosomes had a tendency to be positioned at opposite positions in the ring, then this would indicate the existence of a tandem arrangement of the two haploid chromosome sets with a fixed position of chromosomes within each set, as had been proposed for human and *Ornithogalum* chromosomes. However, such an arragement was not observed by Jin *et al.* (2000).

Although a situation as found in *Ornithogalum,* with highly specific associations involving pairs of telomeres, may be exceptional, chromosome ends appear to be sticky in general. For instance, subtelomeric regions of human chromosomes were found to be frequently associated (Stout *et al.,* 1999). A similar phenomenon may be telomeric associations in yeast (see Section III.C.1). In this case it is not clear which telomeric or subtelomeric regions or sequences are responsible for interactions and if these associations are sequence specific and involve specific telomeres, or if telomeres associate randomly.

Another indication of the interaction of specific chromosome regions in higher eukaryotes involves chromosomal rearrangements with characteristic breakpoints that are found in many cancers. The best known example is the translocation between chromosomes 9 and 22, which produces the Philadelphia chromosome found in chronic myelogenous leukemia. In radiation-associated papillary thyroid cancer, an inversion of chromosome 10 is frequent with breakpoints near the *RET* and *H4* genes, which are 30 Mb apart. Nikiforova *et al.* (2000) found by FISH that the regions of chromosome 10 that harbor these two loci are juxtaposed in 35% of normal human thyroid cells but significantly less often in other cell types. These recurrent chromosomal rearrangements suggest that they tend to involve chromosomal sites that are already in close contact before damage occurs, implying that the proximity of these regions may be of functional significance. An example for recurrent nonallelic interactions in yeast is the recombination events between the *MAT* and the *HML* and *HMR* loci, which are the basis of mating type interconversion. It is likely that the associations that underlay these recombinations

occur only transiently upon the induction of a DSB by the HO endonuclease (Herskowits *et al.,* 1993) and normally will escape cytological detection.

There is accumulating evidence that associations of specific chromosome regions can be mediated by proteins with sequence-specific DNA binding. The *Drosophila* protein *zeste* can self-associate and can thereby possibly spatially link chromosomal loci to which it binds (Bickel and Pirrotta, 1990). A similar linking effect could be exerted by the sequence-specific DNA-binding members of the mammalian Ikaros family of transcription factors that recognize related DNA sequences and are capable of dimerizing with themselves and other family members (Brown *et al.,* 1997)

A related phenomenon of ectopic interactions between repetitive sequences was reported for budding yeast by Aragón-Alcaide and Strunnikov (2000). They used the *lac*O/LacI–GFP system, which is based on the transgenic expression of the bacterial lac repressor protein fused to GFP. The fusion protein binds to tandem repeats of a chromosomally integrated *lac*O sequence and makes them visible in the microscope (Straight *et al.,* 1996) (see Section II.A). When Aragón-Alcaide and Strunnikov (2000) studied the intranuclear distribution of pairs of *lac*O inserts at allelic and ectopic loci, they found that even ectopic *LacO* repeats were frequently associated. For detection they used a truncated LacI molecule that is unable to tetramerize and to physically link two *lac*O sequences. Therefore they concluded that transgenic *lac*O inserts had the capability of interacting directly with each other.

In a similar experiment using the *tet*O/TetR–GFP system, Fuchs *et al.* (2002) observed the frequent association of *tet*O repeats irrespective of their chromosomal positions. However, in strains in which the TetR gene was not present, *tet*O loci (then visualized by FISH to the *tet*O sequence) were no longer associated. Therefore it is likely that the association of *tet*O sequences requires TetR and hence does not reflect an inherent property of tandem repeats per se and that *lac*O associations could in a similar, not yet fully understood way, be mediated by nontetramerizing LacI molecules.

The linkage of *tet*O repeats at ectopic chromosomal sites by TetR was sufficiently strong to perturb the normal polarized orientation of chromosome arms (Fuchs *et al.,* 2002), and LacI-mediated *lac*O associations are sufficient to link sister chromatids and thereby to substitute for sister chromatid cohesion (Straight *et al.,* 1996). This demonstrates that protein-mediated specific chromosome associations can be quite robust. Although ectopically expressed LacI and TetR molecules are an artificial system, they may serve as a model for the role of DNA sequence-specific binding proteins in mediating interchromosomal interactions that could help to establish or maintain a nonrandom interphase chromosome arrangement.

Recently, a method was described by which the frequency of interaction between any two chromosomal loci can be determined. It employs the stabilization of DNA–DNA associations in isolated nuclei by formaldehyde cross-linking and the restriction digest and ligation of cross-linked DNA fragments. Upon the reversal

of cross-linking, it can be determined by quantitative polymerase chain reaction (PCR) with specific primer pairs, which DNA sequences are ligated with high frequency and thus are presumably interacting frequently in the sampled nuclei (Dekker *et al.,* 2002). This method may be useful in revealing the existence of specific interactions between chromosomes in yeast and other organisms.

F. Interphase Chromosome Dynamics

As was discussed above, certain chromosome regions tend to occupy specific domains within the nucleus. This raises the question whether this organization is static and persists throughout mitosis or whether these chromosome regions are placed at these positions anew in each cell cycle. If this was the case, then it might be asked if the microtubule-generated anaphase movement is sufficient to deliver chromosome regions to their designated nuclear addresses, or if there exist mechanisms for actively placing chromosomes during interphase.

Anaphase movement would probably be sufficient to place the clustered centromeres near the nuclear periphery in the proximity of the spindle pole and the telomeres in the opposite compartment of the nucleus. Rabl orientation results from the trailing of chromosome arms at mitosis and is stabilized by intranuclear microtubules that are retained during interphase. If the clustering of centromeres is disrupted, it can even be reconstituted by virtue of intranuclear microtubules (Jin *et al.,* 2000) (see Section III.D). The association of telomeres with the nuclear envelope is probably maintained during intranuclear mitosis and hence does not require restoration at interphase. Thus, a considerable share of interphase chromosome order is maintained or generated during mitosis.

However, there is evidence that differently activated or replicated genes shuttle between the nuclear periphery and interior throughout interphase (Heun *et al.,* 2001a). [For examples of cell-cycle-dependent relocalization between the nuclear interior and periphery in vertebrate cells, see Vourc'h *et al.* (1993), Hulspas *et al.* (1994), Croft *et al.* (1999), Bridger *et al.* (2000), and Tumbar and Belmont (2001).] Such movement can be hardly imagined as mediated by the intranuclear spindle. Marshall *et al.* (1997b) and Heun *et al.* (2001b) studied interphase chromosome movements in living yeast cells by GFP tagging of inserted *lac*O sequences (see Section II.A). Both groups found that chromatin undergoes significant motion within the nucleus. This movement can be quite rapid with ≥ 0.5 μm within 3–10 sec (Heun *et al.,* 2001c). However, movements were constrained in a way that is consistent with a suprachromosomal nuclear architecture in which chromosomes cannot move completely freely. Centromeres and telomeres showed less mobility than interstitial regions. Marshall *et al.* (1997b) found that chromatin diffusion was insensitive to metabolic inhibitors and proposed that it results from Brownian motion rather than active motility. On the other hand, Heun *et al.* (2001b) found that reduction of the intracellular ATP level largely suppressed the movement. Both

groups agreed that microtubule-destabilizing drugs do not inhibit the movements, which are therefore unlikely to be mediated by microtubule-dependent motors.

Another case of extensive chromosome motion, which is unrelated to anaphase movement, is the formation of the bouquet in meiotic prophase (see Section II.E.1). In bouquet formation, many chromosomes perform almost a U turn, as during interphase the telomeres (especially of long chromosome arms) tend to be near the SPB distal pole, whereas in the bouquet stage they gather near the SPB. So far it has not been possible to inhibit this movement by microtubule inhibitory drugs, and mutants for microtubule-associated proteins showed no impaired bouquet formation (H. Scherthan, personal communication).

IV. Summary and Conclusions

In spite of the poor amenability of *Saccharomyces cerevisiae* to cytological investigation, there has been a considerable increase in our knowledge about the structure and behavior of yeast chromosomes in the past decade. Many insights, especially on meiotic chromosome pairing and the architecture of interphase nuclei of eukaryotes, have been gained from the study of yeast. On the other hand, the behavior of chromosomes during mitotic and meiotic divisions is less understood and is in some part conjectured from analogies with organisms that are cytologically more favorable objects. However, with respect to chromosome condensation and movement, notable differences seem to exist. There is justified hope that with further improvements in the staining of living cells and in time-lapse confocal microscopy some of the remaining secrets of yeast chromosomes will be unveiled in the near future.

In the following, the major conclusions that emerged from cytological observations about yeast chromosomes over the past few years are summarized.

- As in higher eukaryotes, interphase chromosomes in yeast occupy their own nuclear territory that does not overlap (on a microscopic scale) with other chromosome's territories. It is not yet known if the formation of compact territories is limited to certain substages of interphase.
- The arrangement of chromosomes within interphase nuclei is not random. There are several layers of suprachromosomal nuclear organization.

  Telomeres and other chromosomal regions that permanently or transiently show reduced transcriptional activity occupy the periphery of nuclei. The nuclear periphery is a silencing domain by virtue of the telomeres that are attached to the nuclear envelope and enrich this area for silencing factors associated with them. It is not yet completely clear if there is one or more mechanisms for the tethering of telomeres to the nuclear envelope. Telomere attachment to the nuclear envelope seems to be different in mitosis and meiosis.

Centromeres are clustered throughout most parts of the cell cycle in the vicinity of the spindle pole bodies. They often form a ring with the spindle pole body at its hub. As in higher eukaryotes, centromere clustering is primarily a consequence of the assembly of centromeres at the poles of the mitotic spindle during late anaphase/telophase. However, yeast chromosomes that transiently have lost this order can regain centromere clustering without progressing through mitosis due to to their permanent attachment to intranuclear microtubules.

Chromosome arms project out of the centromere cluster, and although they may meander through the nucleus, their general direction is toward the centromere-distal pole of the nucleus. This polarized orientation resembles the Rabl orientation known from higher eukaryotes.

- There is little evidence for additional modes of higher order chromosomal organization, such as somatic pairing, juxtaposition of specific nonallelic chromosome sites, or parental chromosome separation, which have been reported to occur in several eukaryotes. However, chromosomally inserted transgenic sequences can cause the spatial association of host chromosomes due to the interaction with sequence-specific DNA-binding proteins.
- Although centromeres and telomeres are anchored in the nuclar periphery, they have some limited freedom to move within their assigned domains; for intercalary regions this movement is more extensive. It is not yet clear if interphase chromosome movements are thermal or mediated by a cellular mechanism.
- In yeast mitosis and meiosis there is a weak but notable chromosome condensation cycle. In contrast to higher eukaryotes, yeast mitotic metaphase chromosomes seem to lack the final compaction step imposed by the coiling of an axis with lateral chromatin loops. Also in other respects, yeast mitosis deviates somewhat from the consensus mitotic process as known from higher eukaryotes:

  There is no clear G_2 phase between the completion of replication and the onset of mitotic activities. This is partly explained by peculiarities of yeast mitosis (shared with other fungi) that may help to accelerate the cell cycle: There is no nuclear envelope breakdown (mitosis is endonuclear) and centromeres need not capture microtubules as they are permanently connected. As a consequence, late replication of distal chromosome regions and anaphase centromere migration might even overlap.

  In mitosis, yeast chromosomes do not assemble in a metaphase plate but there is some oscillation of centromeres between opposite spindle poles as tension builds up. When sister centromeres have reached the opposite poles in late anaphase, cohesion in distal arm regions is maintained for some time, which causes the chromatids to stretch before they finally let go and rapidly recoil.

- In meiosis, a typical bouquet is formed and a phase of presynaptic alignment (distance pairing) precedes intimate synapsis at pachytene. Zygotene to pachytene seems to be the only stage in the life cycle of the budding yeast, when centromere clustering is relaxed or even released, whereas telomeres remain in contact with the nuclear envelope.

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Proteinases and Their Inhibitors in the Immune System

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The most important roles of proteinases in the immune system are found in apoptosis and major histocompatibility complex (MHC) class II-mediated antigen presentation. A variety of cysteine proteinases, serine proteinases, and aspartic proteinases as well as their inhibitors are involved in the regulation of apoptosis in neutrophils, monocytes, and dendritic cells, in selection of specific B and T lymphocytes, and in killing of target cells by cytotoxic T cells and natural killer cells. In antigen presentation, endocytosed antigens are digested into antigenic peptides by both aspartic and cysteine proteinases. In parallel, MHC class II molecules are processed by aspartic and cysteine proteinases to degrade the invariant chain that occupies the peptide-binding site. Proteinase activity in these processes is highly regulated, particularly by posttranslational activation and the balance between active proteinases and specific endogenous inhibitors such as cystatins, thyropins, and serpins. This article discusses the regulation of proteolytic processes in apoptosis and antigen presentation in immune cells and the consequences of therapeutic interference in the balance of proteinases and their inhibitors.

KEY WORDS: Proteinases, Inhibitors, Apoptosis, Immune cells, Antigen presentation.

I. Introduction

Proteolytic enzymes or proteases are enzymes that have a wide range of physiological functions in virtually all cell types and species ranging from digestion of food to specific processes such as zymogen activation. Two classes of proteases exist, proteinases that cleave amide bonds within a protein and peptidases

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TABLE I
Overview of Proteinases and Their Inhibitors That Play a Role in the Immune System

Major group	Members
Proteinases	
Cysteine	Papain-like: cathepsin B, C, F, H, J, K, L, N, S, T, U, V/L2, W, X/Z, papain, calpain, legumain
	Caspases-1–14
Serine	Cathepsin A, G
	Granzymes
Aspartic	Cathepsin D, E
Inhibitors	
Cystatins	Stefin/cystatin A and B
	Cystatin C, D, S, SN, and SA
	Low- and high-molecular-weight kininogen and T kininogen
	Glycosylated cystatins E/M, F/leukocystatin
	Calpastatin
Thyropins	p31 and p41 variants of the invariant chain
Caspase	Bcl-2, Fas-associated death domain-like IL-1-converting enzyme inhibitory proteins (FLIP), inhibitors of apoptosis proteins (IAPs), and heat shock proteins (Hsp27, 70, and 90)
Serpins	Proteinase inhibitor (PI)-1, 6, and 9/SPI-6, squamous cell carcinoma antigen (SCCA-2), monocyte/neutrophil elastase inhibitor (M/NEI), cytokine response modifier (Crm)A/PI-2

that cleave one, two, or three end-chain amino acids of a protein. Proteinases are categorized on the basis of what compound is used to generate a nucleophile that is required to attack the carbonyl-carbon of an amide bond. Based on this classification, five major groups of proteinases have been identified: cysteine proteinases, serine proteinases, aspartic proteinases, metalloproteinases, and a group of unclassified proteinases. Proteinases that play a role in the immune system are mainly cysteine proteinases, but serine proteinases and aspartic proteinases are also involved (Table I).

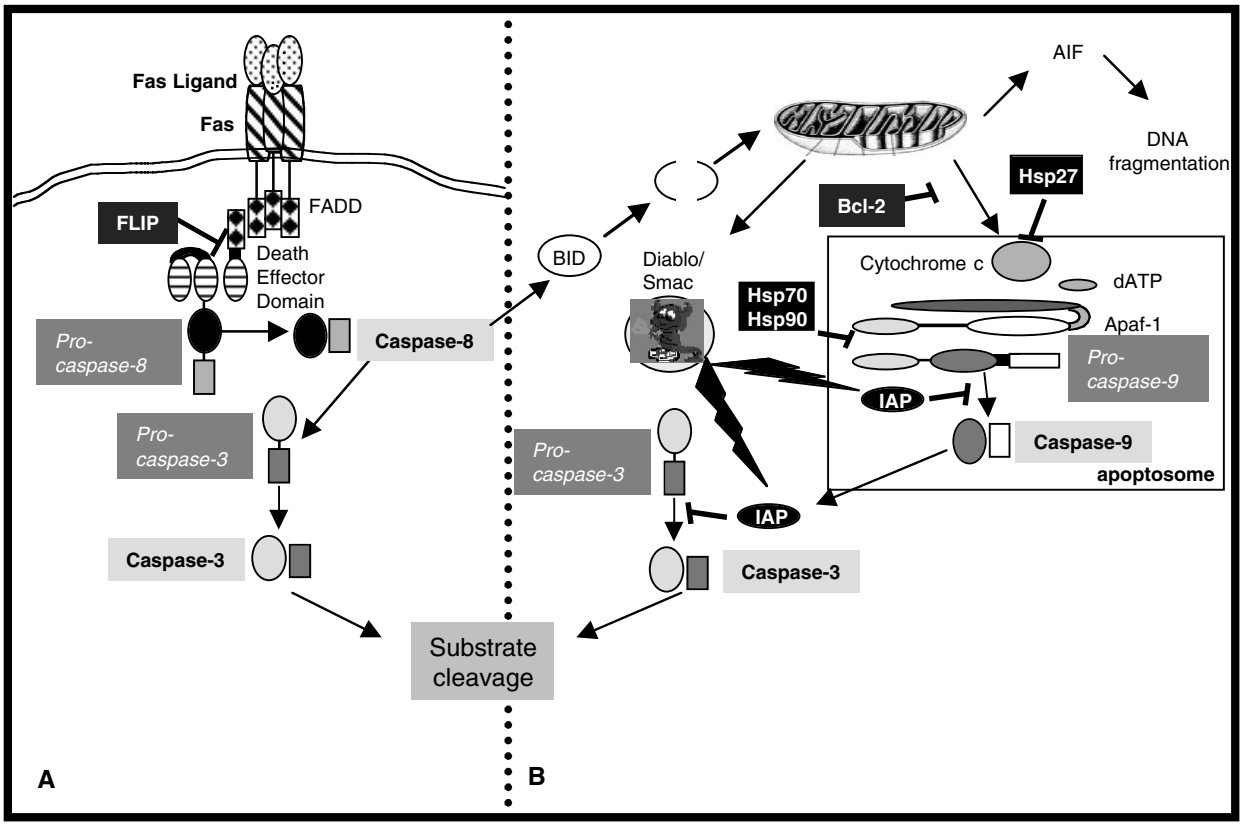
The majority of cysteine proteinases are cathepsins, calpains, and caspases. Cathepsins are proteinases originally considered as intracellular enzymes, usually active in lysosomes. In the last decades, a whole family of cathepsins has been described, including cathepsins A, B, C (also referred to as dipeptidyl peptidase I), D, E, F, G, H, J, K, L, N, S, T, U, V/L2, W, and X/Z. Most cathepsins are related to papain, the first cysteine proteinase that was recognized as such, and hence are members of the papain-like family of cysteine proteinases. Calcium-dependent papain-like cysteine proteinases or calpains belong to the papain-like family as well. They do not play a major role in the immune system. However, they have recently been linked to apoptosis and therefore are discussed here briefly. Caspases are different from the papain-like proteinases (Drenth *et al.,* 1968; McDonald and Barrett, 1986; Salvesen *et al.,* 1987; Murachi, 1989; Chapman *et al.,* 1997; Linnevers *et al.,* 1997; Adachi *et al.,* 1998; Barret *et al.,* 1998; Nagler and Menard,

1998; Santamaria *et al.,* 1998a,b; Wang *et al.,* 1998; Bromme *et al.,* 1999; McGrath, 1999; Tisljar *et al.,* 1999; Wex *et al.,* 1999; Turk *et al.,* 1997, 2001). The caspase family currently consists of 14 members and they are the most extensively studied executioners of apoptosis. Besides their role in apoptosis, they are also involved in cytokine maturation. Legumain, a lysosomal asparagin-specific cysteine endopeptidase, is involved in antigen presentation.

Cathepsins such as cathepsins A and G are serine proteinases; of these cathepsin G is involved in regulation of apoptosis because it can activate caspases. Another group of serine proteinases, the granzymes, are essential mediators of the cytolytic machinery in cytotoxic T cells (CTLs) and natural killer (NK) cells and are discussed here as well. Aspartic proteinases such as cathepsins D and E are important participants in the antigen presentation route. Some cathepsins are expressed in a tissue-specific fashion. For instance, cathepsin S is expressed in lymphatic tissue and is involved in invariant chain (li) degradation of major histocompatibility complex (MHC) class II molecules in antigen-presenting cells (Turnsek *et al.,* 1975; Kirschke *et al.,* 1986; Riese *et al.,* 1996; Villadangos *et al.,* 1997). Cathepsin W, or lymphopain, is expressed in $CD8^+$ CTLs and NK cells (Linnevers *et al.,* 1997; Brown *et al.,* 1998), cathepsin J in murine placenta (Tisljar *et al.,* 1999), cathepsin K in osteoclasts (Tezuka *et al.,* 1994; Bromme *et al.,* 1996; Gelb *et al.,* 1996), and cathepsin V in thymus and testis (Adachi *et al.,* 1998; Santamaria *et al.,* 1998a; Bromme *et al.,* 1999).

Activity of proteinases is tightly controlled to prevent unwanted damage to cells and tissues. Regulation is achieved by a series of factors such as local pH, redox potential, their synthesis as inactive precursors, targeting to specific cellular compartments such as endosomes or lysosomes, and binding to specific inhibitors (Chapman *et al.,* 1997). Naturally occurring inhibitors of cysteine proteinases are the cystatin superfamily, thyropins, inhibitor of apoptosis proteins (IAPs), Fas-associated death domain-like interleukin-1 (IL-1)-converting enzyme (FLICE) inhibitory proteins (FLIPs), heat shock proteins (Hsps), members of the Bcl-2 family, and serpins (Abrahamson, 1994; Potempa *et al.,* 1994; Lenarcic and Bevec, 1998; Tschopp *et al.,* 1998; Budihardjo *et al.,* 1999; Beere *et al.,* 2000; Bruey *et al.,* 2000; Krammer, 2000; Saleh *et al.,* 2000).

Cystatins are natural inhibitors of papain-like cysteine proteinases. The cystatin superfamily can be divided into four families. Family 1, the stefins, consists of stefin/cystatin A and B and is characterized by the lack of disulfide bridges. These molecules are not synthesized as preproteins with a signal peptide sequence and are thus not secreted. Family 2 members are secreted cystatins C, D, S, SN, and SA, containing two intrachain disulfide bonds. Family 3 consists of low-molecular-weight (MW) kininogens, high MW kininogens, and T-kininogens. Family 4 members are secreted glycosylated cystatins E/M and F/leukocystatin sharing little homology with family 2 members (Calkins and Sloane, 1995; Turk *et al.,* 1997; Halfon *et al.,* 1998; Ni *et al.,* 1998). Recently, the thyropin family, a new family of inhibitors of cysteine proteinases, has been described, containing a thyroglobulin type-1 domain (Lenarcic and Bevec, 1998). IAPs, Hsps, FLIPs, and Bcl-2 members


Fas Ligand
Fas
FADD
FLIP
Death
Effector
Domain
Pro-
caspase-8
Caspase-8
Pro-
caspase-3
Caspase-3
Substrate
cleavage
A
B
BID
Diablo/
Smac
AIF
DNA
fragmentation
Hsp27
Bcl-2
Cytochrome c
dATP
Hsp70
Hsp90
Apaf-1
Pro-
caspase-9
IAP
Caspase-9
apoptosome
Pro-
caspase-3
IAP
Caspase-3

all are involved, either directly or indirectly, in the regulation of caspase activity. Serine proteinases such as cathepsin G and granzymes are inhibitable by members of the serine proteinase inhibitor (serpin) superfamily (Potempa *et al.,* 1994).

In this article we discuss various functions of cysteine, serine, and aspartic proteinases and their inhibitors, the cystatins, thyropins, caspase inhibitors and serpins in immune system. We focus on the role of these proteinases and inhibitors in two major processes in the immune system that require proteolysis, i.e., apoptosis and antigen presentation. The potential consequences of therapeutical interference in the balance of proteinases and their inhibitors in various human diseases and animal disease models are discussed as well.

II. Proteinases and Their Natural Inhibitors in Apoptosis

Apoptosis is a sophisticated mechanism to eliminate cells in an ordered way without spilling intracellular contents into the cellular environment. Spilling is prevented by keeping the plasma membrane barrier function intact. However, neighboring cells recognize subtle changes such as phosphatidylserine (PS) exposure at the extracellular side of the plasma membrane. This results in efficient capture and digestion of apoptotic cells. Typical morphological characteristics of apoptosis include cell shrinkage, membrane blebbing, and chromatin condensation (Kerr *et al.,* 1972; Wyllie, 1980). These changes are the consequence of catabolic actions inside cells and are mediated by hydrolytic enzymes such as caspases that destroy cytoskeletal proteins, nuclear proteins, and DNA repair enzymes and endonucleases that fractionate DNA. Presently, 14 members of the caspase family are known and this number is still growing. Many caspases play a role in either cytokine activation or apoptosis. Caspase activation is generally considered a crucial step in apoptosis. Two major pathways have been described that lead to caspase activation and subsequent apoptosis (Fig. 1): a death receptor

FIG. 1 Two major pathways exist that induce apoptosis: (A) a death receptor (DR)-dependent and (B) a mitochondrion-dependent pathway. Fas-induced apoptosis is described as an example of DR-mediated apoptosis. Trimerization of Fas by its ligand results in the activation of caspase-8. This activation can be prevented by FLIP. Stress signals or growth factor withdrawal can activate the mitochondrion-dependent apoptotic pathway. Cytochrome *c* is released from mitochondria resulting in the activation of caspase-9. Cytochrome *c* release can be prevented by Bcl-2. Two routes of FasL-induced apoptosis can be identified. Type I apoptosis results in the rapid activation of caspase-8 with subsequent activation of caspase-3 and substrate processing. Type II apoptosis requires amplification via the mitochondrial pathway. BID processing is required for full activation of the apoptotic cascade. Two other factors released from mitochondria are Diablo/Smac and apoptosis-inducing factor (AIF). Diablo/Smac binds to inhibitors of apoptosis (IAP) thereby preventing caspase inhibition. AIF translocates to the nucleus where it can cleave DNA into large fragments. Finally, executioner caspases such as caspase-3, -6, and -7 become activated and essential substrates are cleaved, leading to destruction of a cell.

(DR)-dependent and a mitochondrion-dependent pathway. Recently, an endoplasmic reticulum (ER)-dependent pathway has been described as well (Nakagawa *et al.,* 2000).

DRs belong to a subgroup of the tumor necrosis factor receptor (TNF-R) superfamily. So far, the following DRs have been described: Fas/Apo-1/CD95, TNF-R1, DR3/Apo3/WSL-1/TRAMP/LARD, DR4/TRAIL-R1, DR5/TRAIL-R2/TRICK2/KILLER, and DR6 (Ashkenazi and Dixit, 1999). Fas is the most extensively studied DR (Nagata, 1997, 1999). Stimulation of Fas at the cell surface results in the formation of a death-inducing signaling complex (DISC). The C-terminal domain of an adaptor molecule, Fas-activated death domain (FADD/MORT), is attached to the cytoplasmic death domain of Fas. The N-terminal domain of FADD contains a death-effector domain (DED) that interacts with the DED domain of procaspase-8/FLICE, resulting in the activation of caspase-8 and subsequently caspase-3 (Kischkel *et al.,* 1995; Boldin *et al.,* 1996; Muzio *et al.,* 1996; Medema *et al.,* 1997). However, it has recently been demonstrated that TNF-induced apoptosis in fibrosarcoma cells is caspase independent. This form of DR-induced apoptosis was enhanced in the presence of pan-caspase inhibitors. In contrast, small molecular cathepsin B inhibitors, cystatin A and antisense cathepsin B mRNA rescued these cancer cells from TNF-induced apoptosis, clearly showing that cathepsin B can also function as a dominant execution proteinase in DR-mediated apoptosis (Foghsgaard *et al.,* 2001).

The mitochondrion-dependent pathway is activated by growth factor withdrawal or irradiation. These exogenous challenges induce cytochrome *c* release from mitochondria, which causes the formation of a protein–protein complex, called the apoptosome, composed of apoptotic protease activating factor (Apaf)-1, cytochrome *c* (Apaf-2), dATP, and procaspase-9 (Apaf-3). This leads to caspase-9 activation, followed by activation of other caspases. This pathway can be inhibited by proteins of the Bcl-2 family that block cytochrome *c* release and hence prevent activation of caspase-9 (Arch and Thompson, 1999; Budihardjo *et al.,* 1999; Kroemer and Reed, 2000). Several other factors have been shown to interfere with the formation of the apoptosome and caspase-9 activation. These include Hsp27, which interacts with cytochrome *c*, and Hsp70 and Hsp90, which interact with Apaf-1 (Beere *et al.,* 2000; Bruey *et al.,* 2000; Saleh *et al.,* 2000). Two other factors involved in apoptosis that can be released from mitochondria are Diablo/Smac and apoptosis-inducing factor (AIF) (Susin *et al.,* 1999; Du *et al.,* 2000; Verhagen *et al.,* 2000). Diablo/Smac can promote apoptosis by binding to members of the IAP family, thereby preventing caspase inhibition.

ER-dependent apoptosis has recently been described as well. It is associated with activation of procaspase-12 that resides in the ER (Nakagawa *et al.,* 2000). Calpain is implied as an activator of caspase-12 (Nakagawa and Yuan, 2000). These three routes result in activation of executioner caspases such as caspase-3, -6, and -7 that cleave a variety of substrates. Fas-induced apoptosis can follow two different pathways. So-called type I apoptosis is characterized by rapid DISC formation

and high levels of active caspase-8 (Fig. 1A). Type II apoptosis is characterized by low levels of DISC and low caspase-8 activity. The latter form of apoptosis requires the cleavage of BID, which triggers an amplification signal via the mitochondrial pathway (Fig. 1B) (Scaffidi *et al.,* 1998). Both forms of apoptosis can be inhibited by FLIPs. FLIPs were first identified as viral products that interfere with DR-mediated elimination of infected cells. Cellular homologs of viral FLIPs (cFLIPs) have been found as well. At least two splice variants have been described, a small and long isoform, $cFLIP_S$ and $cFLIP_L$, respectively, which are both capable of inhibiting Fas-induced apoptosis by blocking caspase-8 activation (Irmler *et al.,* 1997; Thome *et al.,* 1997; Tschopp *et al.,* 1998). A major function of executioner caspases is activation of endonucleases (Wyllie, 1980). Caspase-activated endonucleases include caspase-activated DNase (CAD) or DNA fragmentation factor (DFF45). In addition, chromatin condensation factor, Acinus, induces chromatin condensation without DNA fragmentation after activation by caspase-3 (Liu *et al.,* 1997; Enari *et al.,* 1998; Sakahira *et al.,* 1998; Sahara *et al.,* 1999). Caspase-independent mechanisms of DNA fragmentation have been described as well. For instance, apoptosis-inducing factor (AIF) can translocate from mitochondria to the nucleus, where it cleaves DNA into large fragments (Susin *et al.,* 1999).

Recently, lysosomal cysteine proteinases and aspartic proteinases have been linked to apoptosis. For instance, leakage from digitonin-permeabilized lysosomes may cause apoptosis directly or via activation of caspases. Cathepsin B can both activate a variety of caspases and induce nuclear fragmentation directly in isolated nuclei in a caspase-independent way (Ishisaka *et al.,* 1998; Schotte *et al.,* 1998; Vancompernolle *et al.,* 1998; Isahara *et al.,* 1999). It has been proposed that lysosomal proteinases contribute to apoptosis by the cleavage of BID, followed by an amplification signal via the mitochondrial pathway but not by direct cleavage of procaspases (Salvesen, 2001; Stoka *et al.,* 2001). Furthermore, lysosomal enzymes of macrophages are involved in the degradation of nucleosomes of apoptotic cells. This degradation can be inhibited by cathepsin inhibitors (Odaka and Mizuochi, 1999). The role of proteinases and their inhibitors in apoptosis occurring in, or induced by, B cells, T cells, NK cells, neutrophils, monocytes, and DCs is increasingly appreciated and is therefore discussed in more detail in the following sections.

A. Regulation of Apoptosis in B and T Lymphocytes

During their life span, B and T lymphocytes encounter various episodes of selection. In the bone marrow, B cells with specificities against self-antigens are eliminated (LeBien, 2000). Selection of the T cell repertoire occurs in the thymus. This subject has been reviewed recently and will not be discussed in detail (Anderson *et al.,* 1999; Krammer, 2000; Williams and Brady, 2001). Briefly, high-affinity T cell receptor (TCR)/self-peptide-MHC interactions result in negative selection, whereas low-affinity TCR/self-peptide-MHC interactions result

in positive selection. In the absence of TCR/self-peptide-MHC interaction, cells die by neglect. After these selection episodes, the basic selection mechanism is survival of highly specific B or T cells and, as a consequence, apoptosis of less specific cells. Furthermore, to switch off an immune response, B and T cells must be eliminated.

B cells can undergo apoptosis at several stages of their life span. In general, B cells recognize antigens with their B cell receptor (BCR). This leads to adequate B cell activation only when growth factor and costimulation signals are present. Activation of BCRs in the absence of essential helper stimuli, or wrong order of activating events, leads to apoptosis. BCR-induced apoptosis of CD40-stimulated memory B cells requires a caspase-independent phase upstream of mitochondria and a caspase-dependent phase downstream of mitochondria. The latter is mediated by caspase-9 and activation of effector caspases such as caspase-3. Mitochondria connect BCR-induced apoptosis to effector caspases independently of caspase-8. This is remarkable, because Fas-induced apoptosis in CD40-stimulated memory B cells requires activation of caspase-8 (Berard *et al.,* 1999). BCR-induced apoptosis in the immature B cell lymphoma WEHI-231 requires activation of calpain. In turn, calpain can activate caspase-7, thereby bypassing caspase-8 or the mitochondrial pathway. Calpain activity can be inhibited by its specific inhibitor calpastatin. Interestingly, calpastatin is upregulated by crosslinking of CD40 resulting in inhibition of apoptosis in these B lymphocytes (Ruiz-Vela *et al.,* 1999). The role of calpastatin in BCR-induced apoptosis has been further extended in a reconstitution assay in which long-term cultured pre-B cells were transplanted in nonobese/SCID mice. In this model, it has been demonstrated that calpastatin expression does not interfere with B cell differentiation. However, it resulted in intrinsic resistance against BCR induced in IgM-positive B cells (Ruiz-Vela *et al.,* 2001). Activation of calpain is also involved in the regulation of apoptosis induced by TCR triggering, dexamethasone, the calcium ionophores A23187 and ionomycin, and the adenylate cyclase activator forskolin. Inhibition of calpain in these experimental settings blocks DNA fragmentation, suggesting an upstream regulatory role of calpains either at the level of caspases or endonucleases (Sarin *et al.,* 1993, 1995; Squier and Cohen, 1997).

After binding of an antigen to their BCR, B cells can internalize and process antigens and present them to T cells as peptides on MHC class II molecules. Signals can be transmitted to B cells via MHC class II antigens. Recently, it has been suggested that apoptosis in B cells can be induced via their MHC class II molecules. This alternative form of apoptosis seems to be caspase independent (Drenou *et al.,* 1999). Caspase-independent cell death has also been observed when apoptosis is induced by CD47 ligation on B cell chronic lymphocytic leukemia. Cytoplasmic features of apoptosis (cell shrinkage, decrease in mitochondrial membrane potential, and phosphatidylserine exposure) were observed, whereas nuclear features (chromatin condensation, DNA fragmentation, and processing of poly ADP-ribose polymerase) were absent under these conditions. Furthermore, caspases-3, -7, -8, and -9 remained in their precursor form (Mateo *et al.,* 1999).

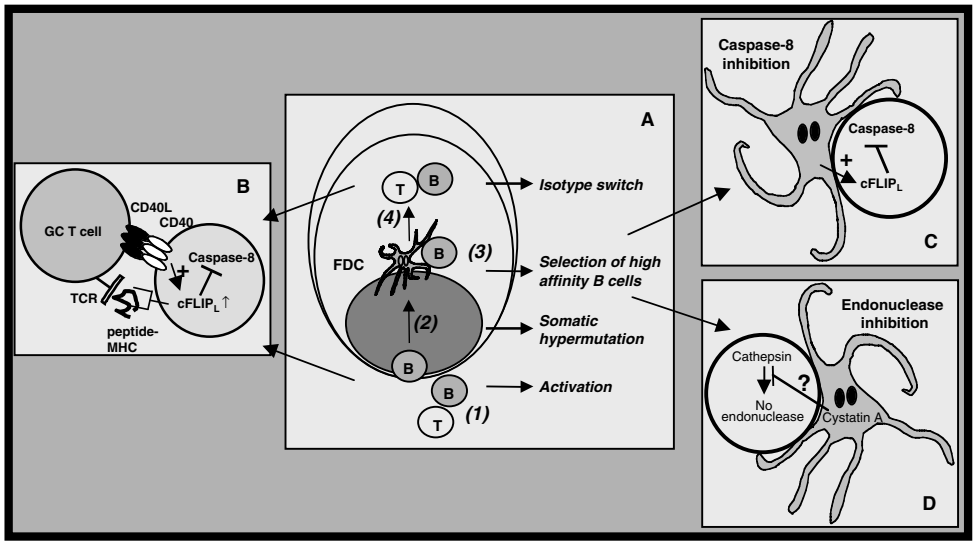


FIG. 2 Regulation of germinal center (GC) B cell apoptosis during GC reactions. (A) Schematic overview of the GC reaction. (1) Antigen (Ag)-specific B cells become activated by Ag-specific T cells. (2) B cells will diversify their B cell receptor (BCR) repertoire in the dark zone. (3) High-affinity BCR B cells are rescued by follicular dendritic cells (FDC). (4) B cells with relevant BCR specificities become isotype-switched plasma cells and memory cells. (B) GC B cells are rescued by CD40L-derived signals from $CD4^+$ T cells. Due to upregulation of $cFLIP_L$, caspase-8 activation is prevented. (C) GC B cells are rescued by FDCs. FDCs induce $cFLIP_L$ and prevent activation of caspase-8. (D) FDCs also switch off endonuclease activity in GC B cell nuclei. This endonuclease activity is present in the absence of caspase activity and is most likely regulated by a cathepsin.

During germinal center (GC) reactions in secondary lymphoid follicles (Fig. 2A), B cells undergo several steps that lead to the formation of high-affinity memory B cells and plasma cells (MacLennan, 1994; Liu and Arpin, 1997; Lindhout *et al.,* 1997; van Eijk *et al.,* 2001a). Antigen-specific B cells bind antigen and become activated by T cells that have been activated by antigen-presenting cells. Following activation, B cells undergo somatic hypermutation of their variable region genes encoding for BCRs, which results in a variety of BCR affinities. It is believed that follicular dendritic cells (FDCs) select the high-affinity B cells by switching off the apoptotic machinery in these cells. It is suggested that T cells check the B cells once more and after "approval" by these T cells, B cells can continue their journey in the GC (van Eijk *et al.,* 2001a).

GC B lymphocytes undergo rapid apoptosis *in vitro,* unless they receive survival signals from FDCs or CD40L on $CD4^+$ T cells. GC B cell apoptosis is characterized by rapid degradation of $cFLIP_L$ and subsequent activation of caspase-8 and caspase-3. Apoptosis is accelerated *in vitro* when Fas is ligated, but most likely FasL is not required. It seems that Fas aggregation occurs spontaneously and that GC B cell apoptosis is activated by $cFLIP_L$ decay (Hennino *et al.,* 2001; van Eijk

et al., 2001a,b). Interestingly, freshly isolated intact GC B cells already have endonuclease activity in their nuclei in the absence of caspase activity (Lindhout *et al.,* 1995; van Eijk and de Groot, 1999), suggesting that this endonuclease differs from the recently described caspase-dependent endonuclease CAD/DFF45 or the chromatin condensation factor Acinus (Liu *et al.,* 1997; Enari *et al.,* 1998; Sakahira *et al.,* 1998; Sahara *et al.,* 1999). We have recently demonstrated that this endonuclease activity is controlled by a yet unidentified cathepsin, different from cathepsins B, F, K, L, and S, positioned downstream of caspase-3 (van Eijk and de Groot, 1999). Induction of the GC B lymphocyte phenotype implies leakage of a cathepsin-like proteinase from the lysosomal compartment, leading to endonuclease activation either by removal of its inhibitor or processing of a proendonuclease. However, it cannot be ruled out yet that this cathepsin fragments DNA itself.

Lysosomal leakage has been linked with apoptosis before (Ishisaka *et al.,* 1998). Of note, $cFLIP_L$ degradation in GC B cells occurs independently of cathepsin or caspase activation. Other proteinases cannot be excluded to be involved at the moment. Both CD40L- and FDC-derived signals result in maintenance of $cFLIP_L$ (Fig. 2B and C), thereby preventing caspase-8 activation (Hennino *et al.,* 2001; van Eijk *et al.,* 2001a,b). In addition to their caspase silencing capacity, FDCs also block endonuclease activity in nuclei of GC B cells when they are attached to these cells (Fig. 2D) (Lindhout *et al.,* 1995; van Eijk and de Groot, 1999). Interestingly, FDCs contain large amounts of cystatin A, a natural cathepsin inhibitor (Rinne *et al.,* 1983). In the immune system, cystatin A is also expressed in thymic cells and granulocytes (Davies and Barrett, 1984; Soderstrom *et al.,* 1994). We propose that this cystatin A is involved in the regulation of GC B cell endonuclease activity. Inhibition of apoptosis by cystatin A was also found in a heptoma cell line (Jones *et al.,* 1998). In that model, cystatin A inhibited cathepsin B downstream of caspases. It has also been demonstrated that cystatin A inhibits virus-induced apoptosis in a fish cell line (Bjorklund *et al.,* 1997). Other cystatins, such as cystatin B, have also been linked to apoptosis, implying that cathepsins indeed contribute to apoptosis (Pennacchio *et al.,* 1998). In conclusion, cysteine proteinases other than caspases such as cathepsins appear to be very important mediators of apoptosis in B and T lymphocytes.

B. Neutrophils and Monocytes

Neutrophils and monocytes contain many proteolytic enzymes, including the serine proteinases cathepsin G, neutrophil elastase, proteinase 3, and azurocidin (Hanson *et al.,* 1990). They are mainly localized in azurophilic granules. After degranulation of neutrophils, cathepsin G is detectable at the cell surface (Lomas *et al.,* 1995; Owen *et al.,* 1995). Cathepsin G plays a role in blood clotting, responses to a variety of bacteria, lymphocyte activation, enhancing cytotoxicity of T cells and NK cells, tissue remodeling, and cleavage and inactivation of neutrophil chemoattractants such as TNF-α, IL-1, and IL-8 (MacIvor *et al.,* 1999). Maturation of cathepsin G

by posttranslational processing requires cathepsin C activity. Inhibition of cathepsin C impairs activation of cathepsin G (McGuire *et al.,* 1993).

Mice lacking cathepsin G are not defective in bacterial killing (MacIvor *et al.,* 1999), but show increased susceptibility to fungal infections despite normal neutrophil development and recruitment (Tkalcevic *et al.,* 2000). Although neutrophil function seems to be intact in cathepsin G-deficient mice, neutrophils are found in excessive numbers in wounds (Abbott *et al.,* 1998; MacIvor *et al.,* 1999), suggesting that cathepsin G is involved in degradation of chemoattractant(s) for neutrophils (Abbott *et al.,* 1998). It has been shown recently that cathepsin G is indeed involved in chemokine inactivation (Fig. 3). Cathepsin G, secreted by

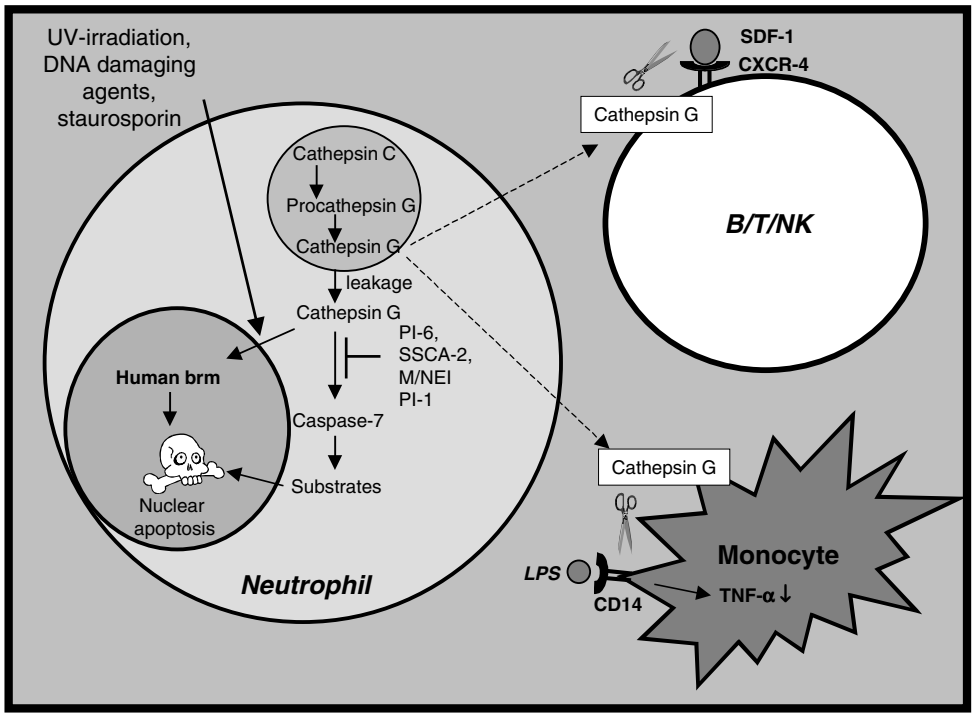


FIG. 3 Cathepsin G-induced apoptosis in neutrophils and inactivation of stromal-derived factor (SDF)-1 on B, T, and NK cells and CD14 on monocytes. Cathepsin C activity is required to get activated cathepsin G. Cathepsin G may leak into the cytoplasm of a neutrophil or monocyte, causing unwanted activation of caspase-7 and subsequent apoptosis. Unwanted apoptosis after leakage of cathepsin G into the cytoplasm is counteracted by the presence of a set of serpins of which proteinase inhibitor (PI)-6 is the most powerful inhibitor and squamous cell carcinoma antigen (SCCA)-2, monocyte/neutrophil elastase inhibitor (M/NEI), and PI-1 are less potent. Cathepsin G may also contribute to nuclear apoptosis by processing human brahma (brm) protein as a result of UV irradiation, DNA-damaging agents, or staurosporin. Secreted cathepsin G may have an immune regulatory role as it is involved in chemokine (SDF-1) inactivation on B, T, and NK cells and lipopolysaccharide (LPS) desensitization as a result of CD14 processing on monocytes.

neutrophils or monocytes, was found to be associated with the cell surface of CXCR4-positive cells such as B and T lymphocytes and NK cells. CXCR4 is the receptor for the chemokine stromal-derived factor (SDF)-1. SDF-1 is rapidly inactivated by N-terminal cleavage by cathepsin G (Delgado *et al.,* 2001). Human neutrophil cathepsin G is also capable of downregulating CD14 on human monocytes (Fig. 3). In this way, monocytes become insensitive to lipopolysaccharide (LPS) stimulation, as indicated by decreased TNF-α synthesis (Le-Barillec *et al.,* 2000). Both examples suggest an important antiinflammatory role of neutrophil-derived cathepsin G. Recently, it has been demonstrated that cathepsin G is involved in apoptosis of neutrophils (Fig. 3), because it can activate recombinant procaspase-7 (Zhou and Salvesen, 1997). When cathepsin G is introduced in the cytoplasm, it can induce morphological changes that are characteristic for apoptosis (Bird, 1999). In addition, cathepsin G contributes to nuclear phenomena of apoptosis by cleavage of the human brahma (brm) protein induced by UV irradiation, DNA damaging agents, or staurosporin in leukemic precursor NB4 cells. The brm protein is part of a polypeptide complex that is believed to regulate conformation of chromatin. In contrast, caspases-3, -6, and -7 were not able to process human brm (Biggs *et al.,* 2001).

Under normal conditions, cathepsin G is compartmentalized in granules. However, it may enter the cytoplasm when errors occur during packaging, degranulation, or phagocytosis (Bird, 1999). Serpins can inhibit activity of misdirected cathepsin G after leakage into the cytoplasm. Expression of the serpin PI-6 is restricted to the cytoplasm of neutrophils and PI-6 is not released via the conventional secretory pathway (Scott *et al.,* 1996, 1999). PI-6 is a potent inhibitor of cathepsin G and hence may serve as a safeguard to prevent self-induced apoptosis by cathepsin G leakage into the cytoplasm (Fig. 3). In addition to PI-6, other serpins have been described that inhibit cathepsin G activity. Two other ovalbumin serpins, squamous cell carcinoma antigen-2 (SCCA-2) and monocyte/neutrophil elastase inhibitor (M/NEI), also inhibit cathepsin G, but to a lesser extent (Remold-O'Donnell *et al.,* 1989, 1992; Sugimori *et al.,* 1995; Schick *et al.,* 1997). Yet another serpin, SPI-1, produced by rabbit pox virus, forms stable complexes with cathepsin G. It has been demonstrated that SPI-1 may function as an inhibitor of caspase-independent apoptosis (Moon *et al.,* 1999).

In conclusion, cathepsin G seems to have an important antiinflammatory role by chemokine inactivation and inhibition of LPS signaling. Furthermore, tight regulation of cathepsin G activity by serpins is important to prevent unwanted self-induced apoptosis that may occur upon cathepsin G leakage from granules into the cytoplasm.

C. Apoptosis Induced by Cytotoxic T Cells and NK Cells

Cytotoxic cells such as CTLs and NK cells use two different contact-dependent mechanisms to kill their targets. One mechanism depends on DR-mediated

apoptosis by actions of FasL and Fas or TNF and TNF-R. A second mechanism depends on exocytosis of granules that results in the action of a pore-forming protein, perforin, in combination with secretion of granzymes, a powerful set of serine proteinases. An additionally secreted protein is granulysin (Pena *et al.,* 1997). Granulysin has antimicrobial activities and can activate a death pathway that can be inhibited by bcl-2 and acts distinctly from Fas or granzymes, but depends on AIF (Kaspar *et al.,* 2001; Pardo *et al.,* 2001). $CD8^+$ CTLs and NK cells preferentially use the granule exocytosis pathway, but also the FasL/Fas pathway. In addition to $CD8^+$ CTLs, $CD4^+$ Th1 and Th2 CTL subsets have been described. Th1 CTLs kill their targets by action of FasL/Fas, whereas Th2 subsets are cytotoxic by exocytosis of granules (Trapani, 1998). FasL/Fas interactions result in the formation of DISC, followed by activation of caspase-8 and additional caspases, which cleave a broad range of substrates, ultimately leading to destruction of the target cell (Fig. 4).

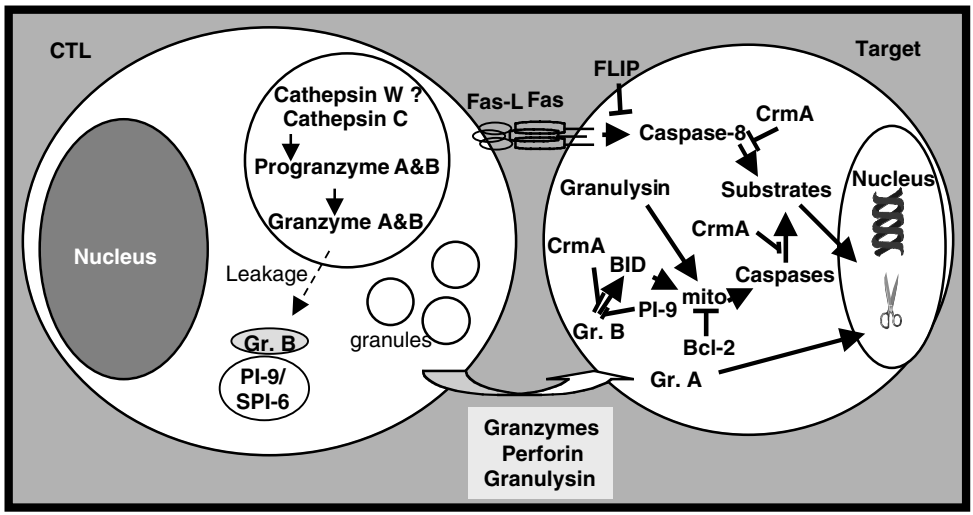


FIG. 4 CTLs can kill target cells by two cell contact-dependent mechanisms: DR induced and granule mediated. DR-induced apoptosis results in activation of caspase-8, which can be blocked by FLIP and the serpin CrmA. Granule-mediated apoptosis proceeds through granzyme B, which can induce apoptosis by processing of BID, followed by amplification of the apoptotic signal via the mitochondrial pathway, resulting in caspase activation. Granzyme A induces DNA fragmentation independent of caspases. Granulysin can induce apoptosis via the mitochondrial pathway as well. Bcl-2 may inhibit this mitochondrial pathway. Apoptosis of CTLs as a result of leakage of granzyme B into their cytoplasm is prevented by serpins such as PI-9/SPI-6, which are present in the cytoplasm and are not secreted. Activation of granzymes requires activity of cathepsin C. Cathepsin C knockout mice contain only the inactive proform of granzyme A and B and are defective in CTL killing. Humans that carry mutations in the gene encoding cathepsin C do not show a generalized T cell immune deficiency. It may very well be that in the human system, cathepsin W compensates for loss of cathepsin C to activate granzymes.

So far, five granzymes, namely granzyme A, B, H, K, and M, have been identified in the granule exocytosis pathway that is used by human cytotoxic cells. Granzymes A–G have been found in mice (Kam *et al.,* 2000). Granzymes A and B are the most extensively studied granzymes and are discussed here in more detail. The combined action of the pore-forming molecule perforin and granzyme B is responsible for the induction of apoptosis in target cells (Blink *et al.,* 1999). The mechanism of action involves receptor-mediated endocytosis of granzyme B. Secreted granzyme B binds to the mannose 6-phosphate/insuline-like growth factor II receptor and requires the action of perforin to be released into the cytoplasm of a target cell (Froelich *et al.,* 1996; Motyka *et al.,* 2000). Granzyme B, a serine proteinase with caspase activity, can cleave caspases-3, -7, -8, -9, and -10. In this way, the apoptotic cascade is activated and amplified (Froelich *et al.,* 1998). Recently, it has been shown that granzyme B does not trigger caspase activation directly, but activates the mitochondrial pathway by rapid processing of BID. The importance of the mitochondrial pathway in granzyme B induction of apoptosis was strengthened by studies in which bcl-2 was overexpressed, which prevented full activation of caspases (Heibein *et al.,* 2000; Sutton *et al.,* 2000). In contrast to granzyme B, which requires amplification by caspases, granzyme A induces an alternative pathway that leads to DNA damage, independently of caspase activation. This possibly provides an apoptotic back-up pathway that remains functional in the presence of viral caspase-blocking antiapoptotic proteins such as the serpin cowpox virus serpin cytokine response modifier A (CrmA) (Andrade *et al.,* 1998; Beresford *et al.,* 1999; Shresta *et al.,* 1999).

Granzymes are produced as preproenzymes with a leader sequence. Proteolytic removal of this leader sequence produces proenzymes. Activation of granzymes requires further cleavage of terminal dipeptide domains (McGuire *et al.,* 1993). Active granzymes are stored in lysosomes (Podack *et al.,* 1991). Lysosomal cathepsin C (dipeptidyl peptidase I) can activate granzymes *in vitro* and is present in the secretory granular compartment of CTLs (Fig. 4) (Kummer *et al.,* 1996; Pham *et al.,* 1998). It has recently been demonstrated that cathepsin C knockout mice contain normal levels of granzymes A and B, but all in the preform. These findings demonstrate that cathepsin C is required for activation of granzymes A and B and granule-mediated apoptosis (Pham and Ley, 1999).

Recently, a novel papain-like proteinase family member, cathepsin W or lymphopain, has been cloned. Cathepsin W belongs to the cathepsin F-like subgroup and is expressed only in NK cells and $CD8^+$ CTLs (Linnevers *et al.,* 1997; Brown *et al.,* 1998; Wex *et al.,* 1998, 1999). This cathepsin may also be involved in the apoptosis-inducing cytolytic pathway used by CTLs to kill their target cells. Interestingly, humans lacking functional cathepsin C show no signs of general T cell immunodeficiency indicating that compensatory mechanisms exist (Toomes *et al.,* 1999). Possibly, cathepsin W is able to replace cathepsin C to activate granzyme A and B. Regulation of cathepsin W activity remains to be elucidated. It has been suggested that cystatin F, or leukocystatin, a specific inhibitor of papain-like proteinase

family members that is highly expressed in T cells, monocytes, and DCs, may control activity of cathepsin W (Halfon *et al.,* 1998; Ni *et al.,* 1998).

Granzyme and caspase activity can both be inhibited by serpins. The serpin CrmA/SPI-2 inhibits caspases-8, -9, and -10 and prevents Fas-induced and TNF-induced apoptosis. Other caspases involved in cytokine maturation such as caspase-1, -4, and -5 are inhibited as well, thereby downregulating activation of proinflammatory cytokines (Ray *et al.,* 1992; Miura *et al.,* 1995; Quan *et al.,* 1995; Tewari *et al.,* 1995; Zhou *et al.,* 1997). In addition, serpin PI-9 can inhibit caspase-1 and, to a lesser extent, caspases-4 and -8, and granzyme B (Bird *et al.,* 1998; Annand *et al.,* 1999; Young *et al.,* 2000). Recently, it has been suggested that leakage of granzyme B into the cytoplasm may result in apoptosis. This unwanted cell death is prevented by high expression levels of serpin PI-9 in the cytoplasm of CTLs. This protects CTLs against apoptosis by their own granzyme B, whereas their Fas DR-pathway remains functional (Fig. 4) (Bird *et al.,* 1998). In addition, PI-9 is expressed in immune-privileged sites such as eyes, testis, placenta, and ovaries. Immune cells are not active in these tissues under normal conditions, but tissue damage can induce recognition of these tissues by the immune system as being foreign. Therefore, an immune response due to CTL or NK degranulation can be deleterious and to prevent this, protection mechanisms such as PI-9 expression are required (Bladergroen *et al.,* 2001). The murine counterpart, SPI-6, is expressed in a similar fashion (Sun *et al.,* 1997). Cancer cells can escape CTL-dependent elimination by expression of SPI-6/PI-9 (Medema *et al.,* 2001a; Bladergroen *et al.,* 2002). In conclusion, granzymes, caspases, and cathepsins are crucial proteases involved in the NK and CTL killing machinery. The killing cells protect themselves from self-induced apoptosis by intracellular expression of serpins and cystatins.

D. Regulation of Apoptosis in Dendritic Cells

DCs are the most potent subset of antigen-presenting cells in the immune system. Immature DCs reside in the periphery and they are characterized by their ability to process antigens. When antigens are captured, they migrate to the draining lymphoid organs where they activate T cells. During this process, DCs mature and undergo a shift toward their costimulatory capacity, whereas their capacity to process antigens is reduced (Banchereau and Steinman, 1998). Not much is known of the fate of DCs, but 1–2 days after they have entered a lymph node they disappear, presumably by apoptosis (Ingulli *et al.,* 1997). DCs are sensitive to different apoptotic stimuli, depending on their maturation status (Fig. 5). For instance, DC apoptosis can be induced by several mechanisms, for instance DR ligands, extensive MHC class II crosslinking, UV-B exposure, and granzyme B, as a result of an attack by a CTL. DR-induced apoptosis occurs in immature monocyte-derived DCs (iDCs) (Fig. 5A). This type of apoptosis can be induced by

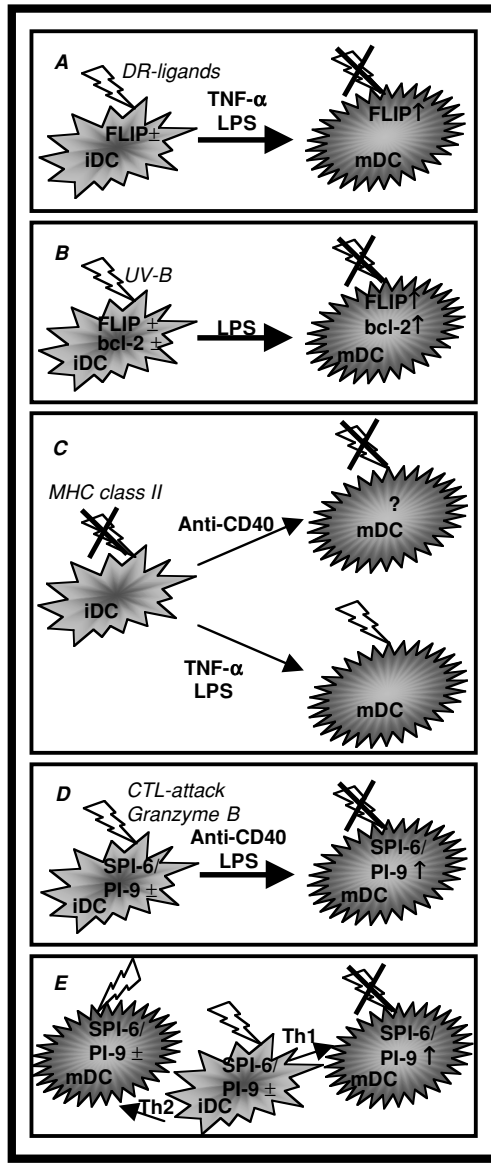


FIG. 5 Regulation of apoptosis in maturing DCs. Apoptosis of DCs can be induced via DRs, CTLs (granzyme B), UV-B, and extensive MHC class II crosslinking. Immature DCs (iDCs) are sensitive to (A) DR-, (B) UV-B-, and (D) CTL-induced apoptosis, but are not sensitive to (C) MHC class II-induced apoptosis. Mature DCs (mDCs) on the other hand are insensitive to (A) DR-, (B) UV-B-, and (D) CTL-induced apoptosis, but are sensitive to (C) MHC class II-induced apoptosis. Resistance to DR-induced apoptosis in mDCs is a result of (A) cFLIP expression. UV-B resistance is a result of expression of both (B) bcl-2 and cFLIP. Resistance to an attack by a CTL (granzyme B) is a result of (D) SPI-6/PI-9 expression. (E) Both Th1 and Th2 cells are equally well equipped to induce DC maturation. However, only Th1 cells are able to induce PI9/SPI6 expression and thus resistance to CTLs.

TNF-related apoptosis-inducing ligand (TRAIL) or FasL (for instance on $CD4^+$ T cells). However, mature DCs (mDCs) are resistant to induction of apoptosis via these DR ligands. It has been demonstrated that this resistance is not a result of downregulation of Fas or TRAIL receptors 2 and 3, respectively (Ashany *et al.,* 1999; Matsue *et al.,* 1999; Leverkus *et al.,* 2000; Willems *et al.,* 2000). Resistance to apoptosis may be developed during maturation of DCs by upregulation of the caspase-8-inhibiting protein $cFLIP_L$ (Fig. 5A; Ashany *et al.,* 1999; Leverkus *et al.,* 2000; Willems *et al.,* 2000; Nicolo *et al.,* 2001). Importantly, CD40 stimulation inhibits FasL-induced apoptosis in DCs, suggesting that $cFLIP_L$ expression is regulated by this stimulus (Bjorck *et al.,* 1997; Koppi *et al.,* 1997). DCs constitutively express Fas that, like FLIP, is upregulated by TNF-α and LPS. Remarkably, it has been shown that FasL induces FLIP expression and that it triggers both the functional and phenotypical maturation of DCs and secretion of proinflammatory cytokines such as TNF-α and IL-1β (Rescigno *et al.,* 2000).

Differences in responses between iDCs and mDCs to an apoptotic stimulus were also observed when apoptosis was induced using UV-B (Fig. 5B). UV-B-induced apoptosis of DCs results in activation of caspases-3, -8, and -9 and cleavage of PARP. iDCs are more sensitive to this form of apoptosis, whereas mDCs are resistant. This resistance of mDCs is a result of increased expression of both FLIP and Bcl-2 (Fig. 5B; Nicolo *et al.,* 2001). Alternatively, the death signal may be a result of DR signaling via other members of the TNF-R family. For instance, DCs derived from TNF-R-2-deficient mice survive only for 3–4 weeks, whereas TNF-R-1-deficient mice survive 6–9 months. Most likely, TNF-R-1-mediated signals are crucial for apoptosis and TNF-R-2-mediated signals are antiapoptotic. It is necessary to bear in mind that a functional TNF-R-1 is required for the expression of CD95 and this obviously influences CD95-dependent apoptosis (Funk *et al.,* 2000).

Interestingly, human mDCs derived from monocytes or $CD34^+$ cells show an increased susceptibility to MHC class II-induced apoptosis that acts independently of caspase activation (Fig. 5C). However, DCs stimulated via CD40 are resistant to MHC class II-induced apoptosis. In contrast, TNF-α and LPS, which are maturation factors for DCs *in vitro,* fail to rescue DCs from this form of apoptosis (Fig. 5C; Bertho *et al.,* 2000; McLellan *et al.,* 2000a). Murine iDCs are, like human iDCs, insensitive to MHC class II-induced apoptosis but they are sensitive to Fas-induced apoptosis. On the other hand, murine mDCs are sensitive to MHC class II-induced apoptosis, whereas they are insensitive to Fas-induced apoptosis (McLellan *et al.,* 2000b). Cytoplasmic PI-9/SPI-6 protects CTLs against unwanted apoptosis caused by leakage of granzyme B into the cytoplasm (Sun *et al.,* 1996; Bird *et al.,* 1998). PI-9/SPI-6 is also expressed in different subsets of DCs such as Langerhans cells in epithelial layers, interdigitating cells in the mantle zone of tonsil and spleen, and DCs in the medulla of the thymus. PI-9 is also expressed in FDCs in GCs of secondary follicles (Bladergroen *et al.,* 2001). Possibly, this intracellular PI-9/SPI-6 protects various DC subsets against a CTL attack. It has been demonstrated *in vivo* that DC can be eliminated by $CD8^+$ T cells (Hermans *et al.,* 2000).

Recently, it has become clear that induction of serpin expression protects DC subsets against CTL-induced apoptosis (Medema *et al.,* 2001b). Murine iDCs are highly susceptible to CTL-induced DNA fragmentation, whereas CD40- or LPS-induced maturation protects mDCs against CTL-induced apoptosis. This protection is a result of expression of PI-9 (human)/SPI-6 (mouse) in mDCs (Fig. 5D). It is known that Th1 cells (characterized by their interferon-γ (IFN-γ)-secreting phenotype) support CTL development, whereas Th2 cells (characterized by their IL-4- and IL-10-secreting phenotype) drive the immune system toward humoral responses (O'Garra, 1998). Both Th1 and Th2 cells are equally well equipped to induce DC maturation, however, only Th1 cells were able to induce SPI-6 expression, resulting in resistance to CTL killing (Medema *et al.,* 2001b). Most likely, secretion of IL-10 by Th2 cells is an explanation for the failure to upregulate SPI-6 expression and subsequent resistance to CTL-induced apoptosis (Fig. 5E). In conclusion, different stimuli can induce apoptosis in DCs depending on their maturation status (Fig. 5). However, it is still unknown what stimulus exactly causes apoptosis of DCs at 24–48 hr after entrance into a lymph node.

III. MHC Class II-Mediated Antigen Processing

Antigen-presenting cells (APCs), DCs, B cells, and macrophages can take up antigens such as bacterial products, which are processed into peptides that are exposed on MHC class II molecules and presented to $CD4^+$ T lymphocytes. Antigens derived from the cells themselves such as viral products are presented on MHC class I molecules to $CD8^+$ T lymphocytes. $CD4^+$ T lymphocytes that recognize the peptide-MHC class II complexes initiate an immune response (Banchereau and Steinman, 1998). MHC class II molecule expression can be induced on a variety of cells by stimulation with IFN-γ or LPS. MHC class II molecules are constitutively expressed on APCs and cortical thymic epithelial cells. The thymus is the environment in which positive selection of $CD4^+$ T cells takes place (Anderson *et al.,* 1999).

Antigens are presented on MHC class II molecules after internalization by APCs via endocytosis and degradation in the endosomal compartment (Fig. 6). Newly synthesized MHC class II $\alpha\beta$-heterodimers are assembled and associated with the invariant (Ii) chain in the ER. Ii chain trimers associate with three $\alpha\beta$-dimers forming a complex that is transported to the Golgi apparatus (Fig. 6). A specific target signal directs Ii–$\alpha\beta$ complexes to the endosomal compartment, where they encounter processed antigens. When the complexes have entered the endosomal compartment, the Ii chain is cleaved in a stepwise fashion. The first steps of Ii chain cleavage require aspartic proteinase activity and later steps require cysteine proteinase activity. Cleavage results in fragments of 21–22 kDa and 11–14 kDa,

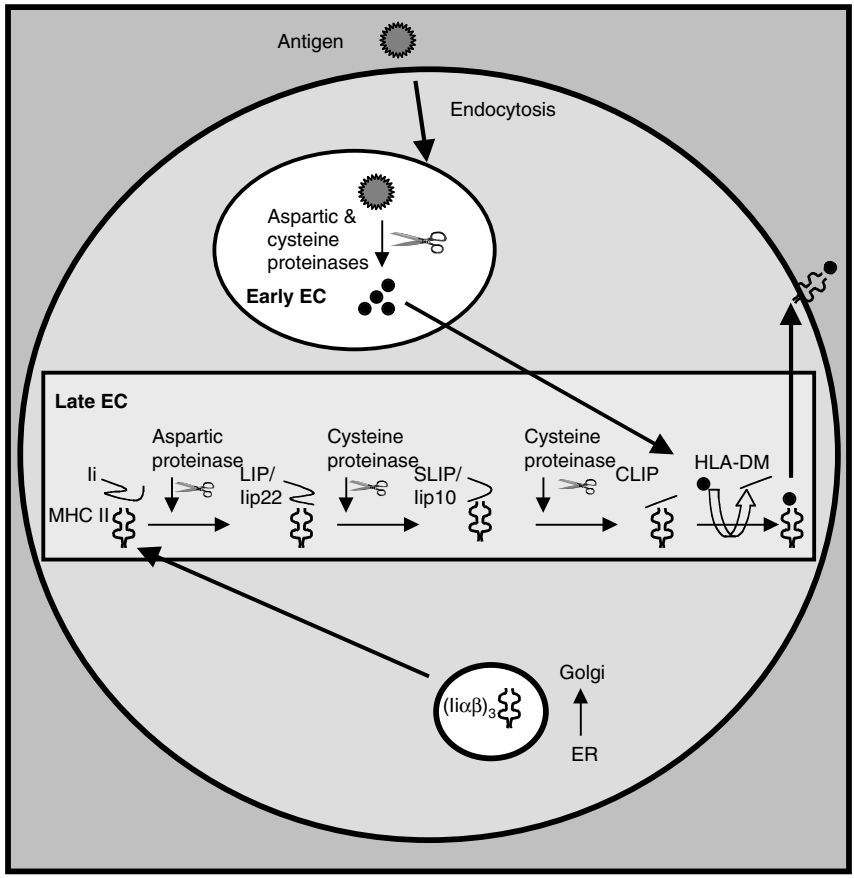


FIG. 6 Antigen uptake via endocytosis and processing by proteolytic enzymes in the endosomal compartment (EC) for antigen presentation on MHC class II molecules. Newly synthesized MHC class II heterotrimers are assembled in the ER where they complex with invariant chain ($\alpha\beta$) trimers. These complexes are transported to the EC via the Golgi apparatus. Here, the complex is first processed by an aspartic proteinase and further processed by cysteine proteinases, finally resulting in CLIP-loaded MHC class II molecules. HLA-DM exchanges the CLIP molecule for an antigen-derived peptide and the MHC class II-peptide complex is transported to the cell surface.

respectively. These fragments are named leupeptin-induced protein (LIP/Iip22) and small leupeptin-induced protein (SLIP/Iip10), respectively. Further processing of LIP and SLIP gives rise to a product called class II-associated invariant chain peptide (CLIP). Then, CLIP molecules bind to the groove of an MHC class II molecule. HLA-DM (H2-M in mice) activity is required as it catalyzes the exchange of CLIP for an antigenic peptide. These MHC class II-peptide-loaded

complexes are then transported to the cell surface (Wubbolts and Neefjes, 1999).

The proteinases that are involved in antigen processing and Ii chain degradation are cathepsins B, D, E, F, H, K, L, S, calpain, and legumain (Nakagawa and Rudensky, 1999; Villadangos *et al.,* 1999; Riese and Chapman, 2000; Villadangos and Ploegh, 2000). So far, only cathepsins F, L, and S have shown to be essential for Ii degradation in knockout mice (Nakagawa *et al.,* 1999; Shi *et al.,* 1999b, 2000). The aspartic proteinase cathepsin D is not crucial in the initial steps of Ii breakdown because mice lacking cathepsin D show normal Ii degradation. Cathepsin B is not essential either, as Ii processing in cathepsin B knockout mice is not inhibited (Deussing *et al.,* 1998). The specific proteinases that are involved in antigen processing and Ii chain degradation are discussed in the following sections. The set of proteinases in dendritic cells and B lymphocytes is different from that in macrophages and cortical epithelial cells. Therefore, the processes are described separately for the different cell types.

A. Dendritic Cells and B Lymphocytes

The process of Ii chain degradation is relatively well understood, in contrast to degradation of internalized antigens. The classic concept that peptides derived from exogenous antigens are presented on MHC class II molecules and cytoplasmic antigens are presented on MHC class I molecules is not entirely correct. It has been demonstrated that endogenous antigens can be presented on MHC class II molecules as well. For instance, epitopes derived from endogenous glutamate decarboxylase (GAD) are presented on MHC class II molecules of the B lymphoblastoid cell line Priess. GAD is processed in the cytoplasm by calpain and the proteasome complex before presentation of epitopes on MHC class II molecules (Lich *et al.,* 2000) and nonlysosomal proteinases such as cathepsin E are involved in processing of exogenous ovalbumin into antigenic peptides for presentation on MHC class II molecules in the murine B cell lymphoma A20. Until recently, it was assumed that cathepsins D and E cleave the tetanus toxin C fragment (TTCF) for antigen presentation on MHC class II molecules in humans (Bennett *et al.,* 1992; Hewitt *et al.,* 1997). However, recently it became clear that the lysosomal asparagin-specific cysteine endopeptidase (AEP), also called legumain, is involved in degradation of TTCF. Inhibitors of AEP block TTCF processing *in vitro*. Application of such inhibitors *in vivo* reduces presentation of TTCF peptides, whereas pretreatment *in vitro* of TTCF with AEP accelerates presentation. AEP is efficiently inhibited by the cystatins C and E/M, and partly by cystatin F. In contrast, activity is not blocked by cystatins A and B or low MW kininogen (Chen *et al.,* 1997; Manoury *et al.,* 1998; Alvarez-Fernandez *et al.,* 1999). TTCF contains three major AEP cleavage sites, but only one cleavage step is already sufficient for effective antigen presentation (Antoniou *et al.,* 2000).

Cathepsin S is the best characterized cathepsin involved in Ii chain degradation in DCs and B lymphocytes. These cells express cathepsin L as well, but only in an inactive form, suggesting the presence of a specific cathepsin L inhibitor (Honey *et al.,* 2001). Purified cathepsin S can specifically digest Ii chain in $\alpha\beta$-Ii trimers generating $\alpha\beta$-CLIP, whereas cathepsins B, D, and H cannot (Riese *et al.,* 1996). *In vivo,* inhibition of cathepsin S in B lymphoblastoid cells by a selective vinylsulfone inhibitor (LHVS) results in accumulation of a 13-kDa Ii fragment, indicating that complete degradation of the Ii chain is prevented (Riese *et al.,* 1998). Additional evidence for the crucial role of cathepsin S in antigen presentation was found in cathepsin S knockout mice. Their APCs fail to process Ii chain into fragments smaller than 10 kDa, resulting in delayed peptide loading and accumulation of MHC class II/10-kDa Ii fragments at the surface of the cells. Furthermore, these mice virtually lack germinal centers. Most likely, B cells are not able to degrade their Ii chains and therefore they do not express MHC class II-peptide complexes required for interactions with T cells and subsequent signaling events. In addition, B cells of cathepsin S knockout mice induce proliferation of T cells poorly. These mice show reduced isotype switching toward IgG, especially IgG_1 and IgG_3, which is in line with the absence of germinal centers (Nakagawa *et al.,* 1999; Shi *et al.,* 1999b).

Defective Ii degradation is found in DCs of cathepsin S knockout mice as well. iDCs are poor stimulators of T cells as they do not express activation markers such as CD40, CD54, and CD86. However, iDCs are very efficient in taking up antigens by endocytosis, phagocytosis, or macropinocytosis. Maturation induced by, for instance, intact bacteria, bacterial cell wall products such as LPS, or cytokines blocks their capacity to take up antigens. In contrast, mDCs express high amounts of peptide-loaded MHC class II complexes and are well equipped to activate T cells in lymphoid organs (Banchereau and Steinman, 1998). Recently, it has been demonstrated that proinflammatory stimuli evoke rapid formation of peptide-loaded class II dimers by upregulating cathepsin activity. Antiinflammatory stimuli such as IL-10 counteract this (Fiebiger *et al.,* 2001).

In summary, a shift from antigen uptake to antigen presentation at the surface of DCs takes place during maturation. This concept is supported by the finding that developmental regulation of MHC class II transport occurs in maturing mouse DCs. iDCs contain MHC class II molecules in lysosomes only. Upon maturation, MHC class II molecules are found in nonlysosomal vesicles at the periphery of DCs, whereas late DCs mainly express MHC class II molecules at their surface (Pierre *et al.,* 1997). In addition, inflammatory stimuli such as LPS, TNF-α, and CD40L initiate intracellular formation of MHC class II-peptide complexes (Inaba *et al.,* 2000). This implies that MHC class II trafficking and degradation are tightly controlled during DC development. Increased cathepsin S activity with subsequent Ii degradation has been observed in mDCs in combination with efficient delivery of MHC class II molecules to the plasma membrane. In contrast, iDCs show low cathepsin S activity and inefficient Ii degradation due to the presence of cystatin C.

Thus, the balance between cathepsin S and its inhibitor cystatin C determines the fate of newly synthesized MHC class II molecules (Pierre and Mellman, 1998). The recently described cystatin F/leukocystatin is expressed in DCs and may control Ii degradation as well (Halfon *et al.,* 1998; Ni *et al.,* 1998). MHC class II trafficking is regulated by control of Ii degradation, because in cathepsin S knockout mice and mice treated with LHVS the majority of MHC class II molecules are retained in late endocytic compartments in mDCs in a similar way as in iDCs. Moreover, traffic of MHC class II molecules to the plasma membrane is impaired (Driessen *et al.,* 1999). Thus, both B cells and DCs require active cathepsin S for proper trafficking of matured MHC class II molecules.

B. Macrophages

Macrophages are also important in MHC class II antigen presentation and Ii degradation. During inflammation, macrophages may cause tissue damage as a result of secretion of proteolytic cysteine proteinases. Despite the important role of cathepsin S in antigen presentation and Ii degradation, cathepsin S-deficient mice do not show defects in IgE responses and develop normal pulmonary eosinophilia upon challenges with ovalbumin (Shi *et al.,* 1999b). Even in the absence of both cathepsin S and L (essential in cortical thymic epithelial cells), the only essential cysteine proteinases known so far in the generation of CLIP, lung macrophages show normal peptide loading (Nakagawa *et al.,* 1998, 1999; Shi *et al.,* 1999b). However, a normal IgE response and infiltration of eosinophils into the lung are not observed when either wild-type or cathepsin S knockout mice are treated with the cathepsin S inhibitor LHVS. This implies that a cysteine proteinase different from the cathepsins S and L is required for the observed IgE responses and lung eosinophilia. Interestingly, macrophages express two other cathepsins, in addition to the cathepsins S and L that are not expressed by B cells and DCs, namely cathepsins F and Z (Shi *et al.,* 2000). Cathepsin F but not cathepsin Z degrades Iip (SLIP) as efficiently as cathepsin S, thereby generating CLIP fragments. Cathepsin F and S are both inhibited by LHVS, cystatin C, and to a lesser extent cystatin A and B.

Macrophages accumulate during inflammation and are held responsible for the destruction of elastin-rich tissues such as the elastic lamina by the use of extracellular cysteine proteinases. Human macrophages express the elastinolytic cathepsins L, S, and K and are able to secrete cathepsins B, L, K, and S into the extracellular space. A pericellular acidic environment for optimal cathepsin activity is created by upregulation of the vacuolar-type H^+-ATPase on the plasma membrane (Reddy *et al.,* 1995; Punturieri *et al.,* 2000). Macrophages of an individual lacking cathepsin K who suffers from pycnodysostosis showed normal elastinolytic activity because they were still able to express, process, and secrete active cathepsins L and S (Punturieri *et al.,* 2000). In conclusion, lung macrophages contain

a unique cathepsin, cathepsin F, that can substitute for cathepsins L and S in Ii degradation and matrix-destructive MDMs exteriorize a mixture of cathepsins, namely cathepsin K, L, and S, that all have elastinolytic activity.

C. Thymic Epithelial Cells

In contrast to DCs, B lymphocytes, and macrophages, cortical thymic epithelial cells (cTECs) do not express cathepsin S (Nakagawa *et al.,* 1998). cTECs are involved in positive selection of $CD4^+$ T cells in the thymus. These cells have self-peptides complexed with MHC class II molecules expressed on the plasma membrane (Anderson *et al.,* 1999). Studies using cathepsin L knockout mice revealed that this proteinase is essential for the degradation of Ii in cTECs, but not in bone marrow-derived APCs. Decreased Ii chain degradation and increased amounts of MHC class II-CLIP/IiP10 complexes at the surface of cTECs correlate with impaired selection of $CD4^+$ T cells (Nakagawa *et al.,* 1998). Cathepsin L activity can be inhibited by cystatins or members of the recently described thyropin family (Lenarcic and Bevec, 1998). Two isoforms of Ii chain exist, a p31 and p41 isoform. The p41 isoform contains an additional domain that shows a strong homology with members of the thyropin family. Interestingly, the thyropin domain of p41 inhibits cathepsin L, but not cathepsin S, implying a specific regulatory role during selection of $CD4^+$ T cells in the thymus (Guncar *et al.,* 1999). Human cathepsin L can be inhibited by cystatin A, C, and F/leukocystatin (Halfon *et al.,* 1998; Ni *et al.,* 1998; Cimerman *et al.,* 1999). Furthermore, the amino-terminal parts of p31 and p41 show homology with cystatins, suggesting an additional role in proteinase inhibition (Katunuma *et al.,* 1994). Recently, it has become clear that p41 is a chaperone for cathepsin L rather than an inhibitor of cathepsin L activity. p41 prevents premature destruction of cathepsin L by other lysosomal or endosomal proteases and hence contributes to high levels of intracellular cathepsin L (Lennon-Dumenil *et al.,* 2001).

Recently, human cathepsin V (or L2) has been described (Adachi *et al.,* 1998; Santamaria *et al.,* 1998a; Bromme *et al.,* 1999). Its gene is located in the chromosomal region adjacent to the cathepsin L locus. Analysis of its electrostatic surface potential indicated that human cathepsin V resembles murine cathepsin L rather than human cathepsin L. Cathepsin V is expressed specifically in human testis and thymus. Its role in testis is unknown. In the thymus it may function as the proteinase involved in selection of human $CD4^+$ T cells.

Another thymus-specific proteinase that may be involved in selection of $CD4^+$ T cells by cTECs shows sequence homology with lysosomal prolylcarboxypeptidase and is most likely a serine proteinase (Bowlus *et al.,* 1999). It has been suggested that this thymus-specific serine proteinase (TSSP) is involved in removal of the endosomal targeting signal from the Ii chain, thereby enabling MHC class II-peptide complexes to reach the cell surface. Mice lacking cathepsin L

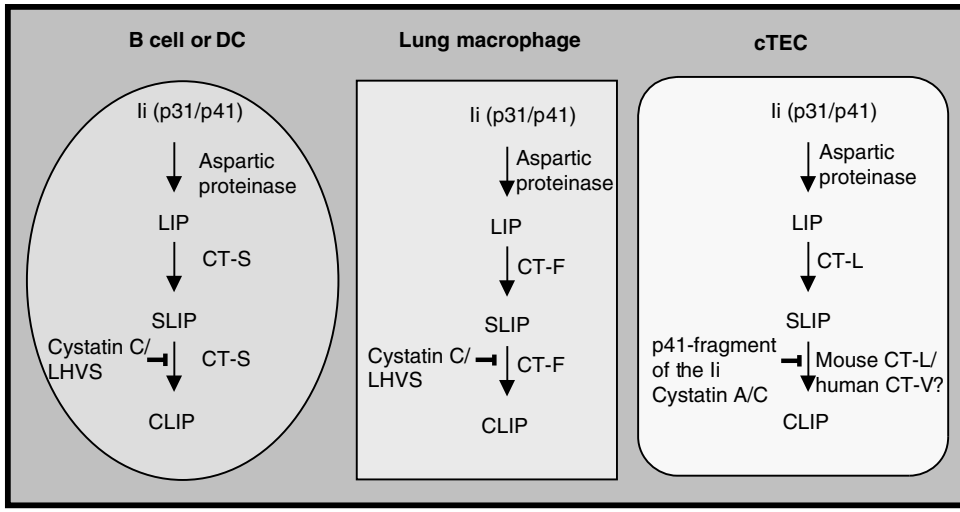


FIG. 7 Proteinases involved in proteolytic processing of antigens for antigen presentation on MHC class II molecules in B cells, DCs, macrophages, and cortical thymic epithelial cells (cTECs). B cells, DCs, macrophages, and cTECS require aspartic proteinase activity for the initial degradation of invariant chains (Ii). Further processing into CLIP fragments in the different cell types is carried out by cysteine proteinases. Cathepsin S is the essential cysteine proteinase required for Ii chain degradation in both B cells and DCs. Cathepsin F is the essential cysteine proteinase in macrophages, whereas cathepsin L (mouse) and probably cathepsin V/L2 (human) fulfill this task in cTECs.

show normal expression of MHC class II molecules and the presence of TSSP may explain why MHC class II expression is normal.

The involvement of the various proteinases in antigen presentation in the various cell types of the immune system is summarized in Fig. 7. In B lymphocytes and DCs, Ii chain degradation depends on activity of cathepsin S. In macrophages, these processes are dependent on cathepsin F activity and in cTEC on cathepsin L (mouse) and probably cathepsin V (human) activity. As far as we know, cathepsins F, L, and S are the only essential proteinases for Ii chain degradation.

IV. Interference with Proteinase Activity as a Therapeutic Strategy

A number of immune-related human diseases are associated with defects in or changed expression of proteinases. Hence, inhibition or supplementation of these enzymes may contribute to amelioration of the disease and the strategies involved are discussed in the following sections.

A. Caspases

A number of diseases are caused by defects in caspase activity. For instance, the gene for caspase-8 is frequently found inactivated in neuroblastoma, a tumor of the nervous system (Teitz *et al.,* 2000). In Alzheimer's disease, amyloid-β (Aβ) protein is deposited in senile plaques and cerebral blood vessels. Caspases are involved in the proteolytic processing of amyloid-β precursor protein and caspase activity results in elevated levels of Aβ peptide that are observed in apoptotic cells (Gervais *et al.,* 1999). Moreover, autoimmune lymphoproliferative syndrome (ALPS) or Canale Smith syndrome patients suffer from heterozygous Fas mutations, resulting in increased numbers of cells of particular lymphocyte subsets and subsequent autoimmune phenomena (Nagata, 1998). Similar phenomena have been observed in ALPS II patients, where defective Fas signaling is not a result of Fas/FasL mutations. In these patients, the caspase-10 gene is mutated and caspase-10 is an initiator caspase in DR signaling as well that can functionally substitute for caspase-8. This caspase is, like caspase-8, recruited to the DISC and may have a dominant negative effect on apoptotic signaling in ALPS II patients (Fischer *et al.,* 1999; Wang *et al.,* 1999, 2001).

Caspase inhibitors that have been used in animal models of human diseases are mainly small molecular active site mimetic peptide ketons. Such compounds have been shown to be effective in models of ischemia–reperfusion injury. Not only was decreased apoptosis observed, but also improved organ function resulting in higher survival rates. Promising results of therapeutic inhibition of caspase activity have also been reported in other animal models of diseases that are characterized by decreased apoptosis, including amyotrophic lateral sclerosis, traumatic brain injury, status epilepticus, and Parkinson's disease. In these models, neuronal survival is improved upon treatment (Nicholson, 2000). Moreover, caspase inhibition results in increased survival in models of sepsis. The protective effect is most likely enhanced immunity, which is caused by the prevention of apoptosis of lymphocytes (Hotchkiss *et al.,* 1999, 2000). Female IFN-γ transgenic mice show features of the autoimmune disease systemic lupus erythematosis (SLE) that is similar to the human disease. A major cause of death in this autoimmune disorder is immune complex-mediated kidney disease (lupus nephritis). Treatment of these IFN-γ transgenic mice with a pan-caspase inhibitor results in the amelioration of kidney disease (Seery *et al.,* 2001).

B. Cathepsins

A number of human diseases are caused by mutations in genes encoding for cathepsins or cystatins. For example, loss of function mutations in the cathepsin K gene is found to be responsible for pycnodysostosis (Gelb *et al.,* 1996), an autosomal recessive osteochondrodysplasia characterized by osteosclerosis and short stature.

Mutations in the cathepsin C gene result in periodontal diseases and palmoplantar keratosis (Toomes *et al.,* 1999). Mutations in the cystatin B gene lead to progressive myoclonus epilepsy and cystatin C gene mutations cause hereditary brain hemorrhage (Pennacchio *et al.,* 1996; Palsdottir *et al.,* 1988). In addition, a variety of diseases such as arthritis, allergy, invasion and metastasis of cancer, osteoporosis, human atherosclerosis, aortic aneurysms, and parasitic infections are characterized by an unbalanced cathepsin/inhibitor ratio.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation and destruction of bone. Increased levels of cathepsin B, K, and L are associated with RA (Hummel *et al.,* 1998; Cunnane *et al.,* 1999). Patients with RA and animals with experimentally induced arthritis have elevated levels of cathepsins in the synovial tissues and fluids. When rats, suffering from antigen-induced arthritis, are treated with a synthetic cathepsin B inhibitor, the cathepsin B activity in chondrocytes, inflammation of knee joints, and cartilage damage is reduced (Van Noorden *et al.,* 1988; Esser *et al.,* 1994). Furthermore, cathepsin S knockout mice exhibit diminished susceptibility to collagen-induced arthritis (CIA). Therefore, cathepsin S is an interesting candidate for drug targeting to manipulate the peptide repertoire without interfering with the overall class II repertoire (Nakagawa *et al.,* 1999).

Recently, a different strategy has been described to interfere with antigen presentation in RA. MHC class II peptides specific for RA were modified and used as antagonists. These peptidomimetic compounds potently inhibited T cell responses and resistance of the antagonistic peptides against cleavage by cathepsins B, D, and H was used as a selection criterium to develop these antagonists (Falcioni *et al.,* 1999).

An alternative approach in the treatment of RA may be pushing the immune system from the pathological Th1 phenotype toward the Th2 phenotype. In this way, protection against autoimmunity may be induced. Such a shift in Th subsets can be achieved by the application of exogenous CLIP. Antigen-specific responses can be downregulated as a result of competition between internalized CLIP and peptide epitopes. As long as the peptide is not presented by MHC class II molecules, a pathological Th1 response may be prevented. In this way, a favorable Th2-type response could be induced as has been demonstrated by the shift in cytokine profiles and antibody isotypes (Chaturvedi *et al.,* 2000).

In a mouse model of human multiple sclerosis, experimental allergic encephalomyelitis (EAE), it has been demonstrated that introduction of recombinant Ii chains, in which the CLIP core region has been replaced by antigenic self-epitopes, suppresses disease symptoms. In this model, recombinant Ii proteins are more potent for antigen delivery when compared to peptides and also for modulation of $CD4^+$ T cell-mediated autoimmunity (Bischof *et al.,* 2001). Modulation of Th subsets can also be induced by the application of selective proteinase inhibitors. For instance, inhibition of cathepsin B with CA074 in experimental leishmaniasis resulted in a switch from a Th2 to a Th1 response, most likely caused by modulation

of antigen processing rather than a direct effect on the parasites themselves. Others have found that treatment of *Leishmania*-infected mice with other selective cathepsin B and cathepsin L inhibitors killed the parasites without affecting the host (Maekawa *et al.,* 1998; Selzer *et al.,* 1999).

In malaria, degradation of hemoglobin by the cysteine proteinase falcipain and the aspartic proteinases plasmepsin I and II is essential for survival of the parasite. Simultaneous inhibition of these proteinases inhibited development of the parasite and hemoglobin degradation synergistically, both in cultured parasites and in a murine model of malaria (Semenov *et al.,* 1998).

In allergic lung disease, interference with antigen presentation by selective inhibitors of cysteine proteinases was also observed in an animal model of Th2-driven allergic inflammatory responses in the lung. In this mouse model, cathepsin S and/or F activity was inhibited by LHVS resulting in attenuation of IgE levels and decreased infiltration of inflammatory cells into the lung. This effect is most likely due to inhibition of cathepsin F and S in lung macrophages as cathepsin S knockout mice showed normal IgE levels and showed a defective IgE response only in the presence of LHVS (Riese *et al.,* 1998; Shi *et al.,* 1999b, 2000). In human vascular diseases such as atherosclerosis and aortic aneurysms, pathogenesis is characterized by breakdown of the elastic lamina. During arterial wall remodeling, the balance between cathepsins K and S and their inhibitor cystatin C is essential. Vascular wall smooth muscle cells produce cystatin C under normal conditions, but cystatin C levels are strongly reduced in both atherosclerotic and aneurysmal aortic lesions. This reduction results in disturbance of the balance of proteinases and proteinase inhibitors and thus to an increased breakdown of the elastic lamina. In addition, it was found that cystatin C levels correlated inversely with the diameter of the aorta in patients, indicating that proteolytic activity is highest in a bulging aortic wall. *In vitro,* proinflammatory cytokines such as IFN-γ induce secretion of cathepsins with elastolytic activity from smooth muscle cells. Interestingly, treatment with TGFβ1 inhibited this elastolytic activity by induction of cystatin C production (Sukhova *et al.,* 1998; Shi *et al.,* 1999a).

Gingivitis and periodontitis are infectious diseases of the periodontium caused by bacteria. Both oral pathogens and cells in the connective tissue can produce proteolytic enzymes such as cathepsins B, C, D, G, and L that contribute to tissue destruction (Trabandt *et al.,* 1995; Tervahartiala *et al.,* 1996). During inflammation, increased cystatin levels compensate for elevated proteolytic enzyme activity. For example, levels of the cystatins A, C, and S are increased in periodontal diseases (Henskens *et al.,* 1994; Blankenvoorde *et al.,* 1997). Of therapeutic interest is the recent finding that cystatins and cystatin-derived peptides show direct antibacterial activity against the pathogen *Porphyromas gingivalis,* independent of their function as a proteinase inhibitor (Blankenvoorde *et al.,* 1998).

In metastasis of cancer, cathepsins and cystatins play important roles. For instance, cathepsin B has been found on the plasma membrane of cancer cells where it is active and may facilitate invasion by breakdown of surrounding connective tissue

(Van Noorden *et al.,* 1998). Cathepsin B can be secreted by cancer cells and become active on the plasma membrane by complexing with annexin II (Mai *et al.,* 2000). Moreover, extracellular cathepsin B activity may result in activation of other proteolytic enzymes as it can activate, for example, urokinase-type plasminogen activator that can cleave plasminogen into plasmin that subsequently degrades elements of tumor stroma. Plasmin can activate MMPs as well, thereby stressing once more the pivotal role of cathepsin B in this cascade (Moin *et al.,* 1998; Yan *et al.,* 1998; Koblinski *et al.,* 2000). However, *in vivo* inhibition of cathepsin B delays rather than blocks tumor development, indicating that it is involved but not essential in this process (Van Noorden *et al.,* 1998, 2000). Cathepsin B may also activate trypsinogen, which is involved in the onset of acute pancreatitis (Halangk *et al.,* 2000).

V. Concluding Remarks

Therapeutical interference with proteolytic activity may be beneficial as it inhibits tissue damage or cancer progression. Also, interference with peptide processing and presentation and subsequent activation of T cells may inhibit the immune system. Furthermore, beneficial shifts in Th subsets can be induced that may result in decreased autoimmunity or parasitic infections. However, the possible side effects of such strategies need to be considered as well. For example, cathepsin L secreted by a hemangioendothelioma can generate endostatin from collagen XVIII and inhibition of cathepsin L will block formation of antiangiogenic endostatin, thereby increasing neovascularization and growth of tumors (Felbor *et al.,* 2000). In conclusion, proteinases are important players in the immune system and their activity is tightly regulated. Therapeutic interference in proteinase/inhibitor ratios looks promising, but unwanted side effects have to be taken into consideration.

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Comparative Analysis of Spore Coat Formation, Structure, and Function in *Dictyostelium*

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Dictyostelium produces spores at the end of its developmental cycle to propagate the lineage. The spore coat is an essential feature of spore biology contributing a semipermeable chemical and physical barrier to protect the enclosed amoeba. The coat is assembled from secreted proteins and a polysaccharide, and from cellulose produced at the cell surface. They are organized into a polarized molecular sandwich with proteins forming layers surrounding the microfibrillar cellulose core. Genetic and biochemical studies are beginning to provide insight into how the deliveries of protein and cellulose to the cell surface are coordinated and how cysteine-rich domains of the proteins interact to form the layers. A multidomain inner layer protein, SP85/PsB, seems to have a central role in regulating coat assembly and contributing to a core structural module that bridges proteins to cellulose. Coat formation and structure have many parallels in walls from plant, algal, yeast, protist, and animal cells.

KEY WORDS: Spore coat, *Dictyostelium,* Cell wall, Cysteine-rich domains, mucin domains.

I. Introduction

In response to starvation, amoebae of *Dictyostelium discoideum* aggregate to form a multicellular organism that reorganizes, over the course of 24 hr, to form a terminal fruiting body. The majority of cells differentiate into dormant, mononucleated spores perched atop a cellular stalk (Fig. 1A and B). Each spore acquires its own 210-nm-thick coat that allows the cell to live with minimal energy expenditure,

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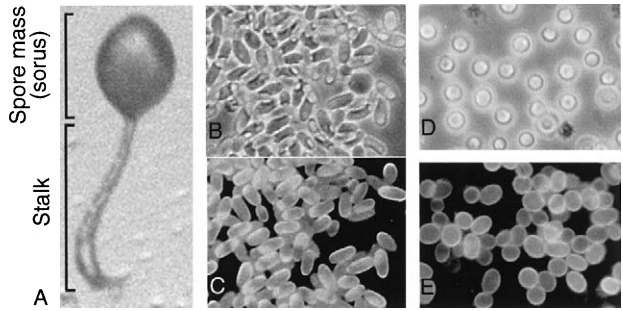

FIG. 1 Light microscopy of a *Dictyostelium* fruiting body and spores. (A) Fruiting body (normal strain Ax3), 1.5 mm tall, showing location of stalk cells and spore cells in sorus. (B) Spores (strain Ax3), about 8 μm long, removed from sorus in (A), phase contrast. (C) Same field of spores, Calcofluor White ST-induced fluorescence to localize cellulose. (D) Pseudospores formed by *dcsA* (cellulose synthase)-null cells (strain DG1099), phase contrast. (E) Round spores produced by SP85-null cells, Calcofluor.

but still allows access to nutrients and other signals that can induce germination. The spore coat is a modified cell wall with an especially well-developed barrier function. Like many cell walls, the coat contains abundant polysaccharides and structural proteins. The main polysaccharide is cellulose, also the primary component of the cell walls of vascular plants, most algae, and certain protozoans. A related polysaccharide, chitin, is an important constituent of fungal and invertebrate cell walls. There are nearly a dozen abundant proteins in the *Dictyostelium* coat and they contain an assortment of sulfhydryl-rich and mucin-like domains. The high level of protein of the spore coat, about 50%, is reminiscent of the vitelline envelopes and basement membranes of animal cells and cell walls of *Chlamydomonas* and *Volvox*. These components are organized as a three-layered "sandwich" with inner and outer protein-rich layers surrounding the central cellulose-rich layer, which at least superficially resembles the ultrastructure of many cell walls. Genetic findings document the importance of cellulose and proteins for coat formation and function, and biochemical studies support the importance of protein–protein and protein–cellulose interactions in coat organization.

The proteins of the coat are synthesized prior to sporulation and stored in the specialized prespore vesicle together with a branched galactose-rich polysaccharide, the Gal/GalNAc-PS. Coat assembly is initiated by secretion of these components, and subsequently cellulose is synthesized and deposited by a plasma membrane-associated enzyme complex. The appearance of cellulose results in the reorganization of the proteins into electron-dense outer and inner layers, each of which contains distinct proteins. The protein interactions are stabilized by extensive intermolecular disulfide bonds, which contribute to the major permeability barrier at the outer layer.

Dictyostelium diverged evolutionarily from the base of the crown group radiation and is best known as a eukaryotic model for cell biological and developmental

mechanisms (Kessin, 2001). This organism contains about 10,000 genes about 25% of which are up- or down-regulated during development (van Driessche *et al.,* 2002). Genes that encode spore coat proteins have been cloned from investigations of cell-type specific gene expression and by sequencing the coat proteins. The gene for cellulose synthase was cloned in a genetic screen for developmental genes based on a restriction-enzyme-mediated gene insertion (REMI) strategy (Blanton *et al.,* 2000). It is straightforward to inactivate genes and to express genes ectopically; the great majority of the genome has been sequenced, and an extensive collection of expressed sequence tags (ESTs) has been sequenced (Kuspa *et al.,* 2001). Thus *Dictyostelium* can be readily exploited using molecular biology, genetics, biochemistry, and microscopy to investigate cell wall assembly. Recent studies have produced a new model for the ordered sequence of events for wall assembly, identified a novel checkpoint that regulates an intermediate step in assembly, and provided new evidence for the critical roles of cellulose and specific proteins in the morphogenesis of individual layers of the coat.

II. Background on Cell Walls

Many free-living, single-cell eukaryotes use cell walls for protection from environmental variation. Walls are typically 100–1000 nm thick, closely associated with the plasma membrane, inherited from cell to cell, and designed to accommodate cell growth and multiplication. They have properties in common with corresponding structures from vascular plants and fungi, and the vitelline envelopes, exoskeletons, and basement membranes of animals, suggesting that a comparison of their molecular architectures might be mutually informative.

A. Plant and Algal Cell Walls

Most microbial and plant cell walls are predominantly polysaccharide in composition. The plant wall has been modeled as a pectic gel impregnated with cellulose fibrils that are cross-bridged to the gel and to each other by hemicelluloses (Carpita and Gibeaut, 1993). The pectins, highly branched polysaccharides rich in Gal, Ara, and GalUA, are produced in the Golgi and secreted via conventional exocytosis. They gel spontaneously in a Ca^{2+} - and boron-dependent fashion (O'Neill *et al.,* 2001). Cellulose consists of individual linear β1,4-linked glucans that emerge from the cellulose synthase complex embedded in the plasma membrane (Delmer, 1999; Brett, 2000). Nascent β1,4-glucans appear to spontaneously coalesce into parallel fibrils based on hydrogen bonding and hydrophobic interactions. The number of glucans per fibril depends in part on the organization of cellulose synthase into terminal complexes. Cellulose is required for normal wall form, as mutational blockade of cellulose biosynthesis causes altered tissue and cell morphology.

The surfaces of cellulose fibrils are thought to be coated with hemicelluloses, which consist of a β1,4-glucan backbone modified at the 6-position by branches consisting of Xyl sometimes extended by Gal and Fuc. The side chains prevent complete incorporation into the microfibril and are thought to directly associate with the pectin gel. Some cell walls lack hemicellulose suggesting alternative mechanisms for linking cellulose fibrils. The relative proportions of cellulose and pectin are highly regulated. For example, pharmacological or mutational interference of cellulose synthesis leads to increased levels of pectins and hemicelluloses. The discovery of transmembrane wall-associated kinases provides a mechanism to link wall polysaccharides to intracellular signaling to regulate wall formation or remodeling (Kohorn, 2001). The composite of cellulose, hemicelluloses, and pectins generates a highly hydrated, porous wall with tremendous tensile strength but little impediment to the diffusion of water, enzymes, other macromolecules, and other solutes.

Many proteins have been detected in the wall during assembly (Robertson *et al.,* 1997) although in aggregate they comprise only 10% of wall mass. Most proteins are classified based on sequence motifs that are rich in the amino acids HyPro, Pro, or Gly (Cassab, 1998). Many of these motifs are probably mucin-like interdomain spacers separating other functional domains. These domains are usually extensively *O*-glycosylated, which probably stabilizes rod-shaped, sometimes polyproline type II conformation, and protects from proteolysis. A large HyPro-rich group includes the extensins, which traverse and strengthen the wall by covalent cross-links resulting from transglutamination. Pharmacological and genetic studies are beginning to suggest important functions for members of the arabinogalactan, Gly-rich, and other groups of proteins (Cooper *et al.,* 1994; Fowler *et al.,* 1999; Gaspar *et al.,* 2001; Ringli *et al.,* 2001; Rizk *et al.,* 2000; van Hengel *et al.,* 2001). Other important protein groups enzymatically cross-link and remodel the wall.

Most plant wall assembly is initiated in a confined space within vesicles of the cell plate and expanded at the interface between the daughter cells, which might be important for defining the outer boundary. At the free surface of the tissue, i.e., on epidermal cells, a unique outer layer is observed, consisting of pectins and possibly protein (Vaughn and Turley, 1999), which might help delimit the outer wall surface during assembly. High M_r lipids in the outer layer of an algal cell wall might serve a similar role (Allard and Templier, 2001).

The unicellular green alga *Chlamydomonas reinhardtii* and the related colonial *Volvox* produce cell walls that appear to be composed purely of proteins and no polysaccharides. Most of the proteins contain hydroxyproline-rich amino acid repeats related to those seen in vascular plant wall proteins, usually in association with one or more globular domains that in some cases are Cys rich. One of the outer wall layers (W4–W6) can be solubilized and reconstituted from a single glycoprotein species composed of globular and rod-shaped HyPro-rich mucin-like domains (Woessner and Goodenough, 1994). Biophysical evidence suggests that assembly depends on discrete contacts between the globular heads and a kink

in the polyproline II-type helix of the rod (Ferris *et al.,* 2001). Related proteins mediate cell–cell recognition during mating (Woessner and Goodenough, 1994). Deeper layers appear to consist of related modular proteins some of which are cross-bridged by divalent cations or are covalently cross-linked by sugar–PO_4–sugar bridges and disulfide bonds (Sumper and Hallmann, 1998).

Electron microscopy of regenerating protoplasts suggests that wall formation is initiated by the formation of radial fibers of unknown composition (Woessner and Goodenough, 1994). These are subsequently decorated by proteins specific to the outer layer (W4–W6), and then the inner layer, which is subsequently further cross-linked by peroxidases and other enzymes as indicated above. The entire process appears to be delicately sensitive to the action of transglutaminase inhibitors (Waffenschmidt *et al.,* 1999) and Ellman's reagent, which implicates an important sulfhydryl moiety (Sumper *et al.,* 2000). Plant cell walls also contain proteins that contribute important functions, and the *Chlamydomonas* model provides examples for how they may contribute to structural organization in polysaccharide-rich cell walls.

B. Fungal and Protozoan Cell Walls

Another polysaccharide-rich wall is the 150- to 200-nm-thick fungal/yeast cell wall, whose "ground substance" is thought to be gel like as in plants. In *Saccharomyces cerevisiae,* this gel is comprised of long β1,3-linked glucans and equimolar but shorter β1,6-linked branched glucans (Kapteyn *et al.,* 1999). Evidence is emerging for a plasma membrane origin for the β1,3-glucans (like cellulose) and a Golgi origin for the β1,6-glucans. Other fungi express related polysaccharides such as α-glucans. Tensile strength is achieved by impregnation with chitin, a β1,4-linked *N*-acetyl-D-galactosamine (GlcNAc) polymer that is synthesized and organized in a crystalline fashion similar to that of cellulose. After secretion, these polysaccharides become covalently cross-linked to form extended, hydrated arrays (Lipke and Ovalle, 1998; Cabib *et al.,* 2001).

Proteins comprise up to 50% of the dry wall mass. Members of the major class, mannoproteins, are initially synthesized with glycosylphosphatidyl inositol (GPI) anchors linking them to the plasma membrane. At the cell surface, these are exchanged with attachments to polysaccharides allowing their movement to the outer layer where they become extensively disulfide cross-linked with their long, *N*-linked mannan chains oriented toward the environment. A major theme emerging from studies on *S. cerevisiae* is the existence of an intermediate core "module" assembled from the glucans, chitin, and mannoproteins by enzyme-catalyzed cross-links that is in turn incorporated into the entire wall (Lipke and Ovalle, 1998; Kapteyn *et al.,* 1999; Cabib *et al.,* 2001). Proteins of a second major class, the PIR proteins, have mucin-like domains extensively modified by shorter *O*-linked mannose chains. PIR proteins contain distinct sequence motifs

and are covalently cross-linked by an unknown mechanism involving the peptide backbone. An HSP70-like protein might function as a chaperone or in translocation (Lopez-Ribot *et al.,* 1996). It has been speculated that the thickness of the wall is controlled by the length of a single membrane-anchored protein. An example of how wall proteins might interact with one another comes from the interaction of a- and α-mating factors, which involves immunoglobulin (Ig)-like domains (Zhao *et al.,* 2001).

Examination of the growing tips of the water mold *Achlya* shows that β1, 3-glucan is deposited first at the apical end, followed by lateral deposition of cellulose (Shapiro and Mullins, 2002). Thus discrete components are incorporated in temporal order. This is reminiscent of the delayed insertion of cellulose into the walls of growing pollen tubes in plants (Ferguson *et al.,* 1988). Coordination between the pathways that deliver separate components is also evident in yeast. For example, β1,3-glucan synthase mutants show strongly increased levels of chitin and linkages between chitin and cell wall glycoproteins (Kapteyn *et al.,* 1999). The so-called cell wall integrity response can be induced by a variety of treatments including osmotic stress or treatment with a chitin/cellulose intercalating dye such as Calcofluor. A family of single-pass type I transmembrane proteins has been identified by genetic studies that appears to mediate an early step in this signaling pathway (Philip and Levin, 2001).

The yeast cell wall acquires additional outer layers in the ascospore. A thin electron-dense layer consisting of a novel DL-dityrosine polymer forms the outer electron-dense boundary and is subtended by an ionically-associated layer of de-*N*-acetylated chitin (chitosan) (Briza *et al.,* 1996). These layers are resistant to the action of proteases and chitinases, and may protect the spore from ultraviolet radiation.

Encystment is a vital phase of the life cycle of many protists and other unicellular eukaryotic microbes. The primitive amitochondriate *Giardia lamblia* cyst wall is composed of an unusual β1,3-linked GalNAc polymer that appears to be organized into fibrils, and protein derived from the secretory pathway (Eichinger, 2001). Cysts of *Physarum polycephalum* contain a predominant GalNAc-rich polysaccharide reminiscent of *Giardia* and a high level of a phenylalanine polymer reminiscent of the tyrosine polymer of yeast ascospores (Zaar *et al.,* 1979). *Entamoeba* species utilize chitin as their major fibrillar component and the most abundant protein contains multiple Cys-rich chitin-binding domains (Frisardi *et al.,* 2000). In contrast, *Acanthamoeba* uses cellulose as its major fibrillar wall component (Turner *et al.,* 2000). Cellulose fibrils are also major components of the cyst walls of the water mold *Achlya ambisexualis* (Shapiro and Mullins, 2002), the soil amoeba *Hartmannella glebae* (Upadhyay *et al.,* 1984), the green alga *Acetabularia* (Herth *et al.,* 1975), and the amoeba-flagellates *Naegleria gruberi* (Werth and Kahn, 1967) and *Schizopyrenus russelli* (Rastogi *et al.,* 1971) and walls of the algal plant pathogen *Phytophthera parasiticans* (Mateos *et al.,* 1997). Thus cellulose fibrils are widely distributed phylogenetically in the walls of many organisms, and is even found in the extracellular matrix (ECM) of a tunicate (Kimura and Itoh, 1996).

C. Animal Walls

Animals typically use cell walls to protect their eggs. In the mouse, the zona pellucida is a relatively simple, 6-μm-thick porous structure. Like the *Chlamydomonas* wall it consists entirely of protein, in this case the three polypeptides ZP1–3 (Wassarman, 1999). ZP2/ZP3 dimers form filaments that are noncovalently cross-bridged by ZP1. ZP3 is required *in vivo* as shown by genetic analysis. These proteins also present carbohydrate-based recognition determinants for sperm cell surface receptors (Dell *et al.,* 1999). The ZP glycoproteins are secreted by the egg in the mouse, but evidence exists that in *Xenopus,* the envelope can be assembled from precursors arriving from the bloodstream (LaFleur, 1995). The sea urchin vitelline envelope is more elaborate consisting of five layers formed sequentially from separate populations of secretory vesicles (Matese *et al.,* 1997). The primary protein of one of these layers, hyalin, is a 75-nm-long filamentous molecule with a 12-nm-diameter globular head, and microscopic data suggest that it is capable of head-to-head self-assembly (Adelson *et al.,* 1992). At fertilization, the vitelline envelope is irreversibly cross-linked by the tandem action of transglutaminases and peroxidases as in *Chlamydomonas,* and later polarizes the surface blastomeres and influences their motility. The vitelline envelope and chorion of *Drosophila* eggs are sequentially deposited and contributed by both the egg and surrounding somatic follicle cells (Waring, 2000). It consists largely of a family of Cys-rich proteins that become cross-linked by disulfide bonds and subsequently other covalent linkages for stabilization (Cernilogar *et al.,* 2001). There is evidence that structural protein precursors are segregated into separate vesicles or regions of the same vesicles prior to secretion (Trougakos *et al.,* 2001), and that proteins are dynamically localized after secretion based on specific domains (Andrenacci *et al.,* 2001). Certain proteins are proteolytically cleaved after secretion, which seems to influence their fates and mediates positional signaling to the egg (Nogueron *et al.,* 2000).

Animal mucins form a multimicrometer-thick mucus sheet at epithelial surfaces that is a less rigid kind of cell wall. They contain extensive tandem arrays of repeat sequences 5–80 amino acids in length, rich in the amino acids S, T, A, V, P, and G. S and T residues are typically modified by a Galβ1,3GlcNAcα1-core 1 moiety that is further modified by sialic acid or SO_4, conferring a high negative charge density supporting extensive hydration and an extended conformation. Many mucins also contain Cys-rich domains at their N- and C-termini. In MUC1, the C-terminal Cys-rich domain mediates dimer formation, and the N-terminal Cys-rich domain assembles dimers into extended networks that are subsequently disulfide cross-linked (Perez-Vilar and Hill, 1999). In some mucins the oligosaccharide chains contain diverse glycan structures that might act as decoys to compete attachment of parasites to the underlying cell surface.

Good examples for how wall proteins might interact with each another come from animal basement membranes (Timpl and Brown, 1996). Three of the major components, laminin, collagen type IV, and perlecan (heparan sulfate proteglycan),

can separately form self-associating gels. The intermolecular contacts that support these gel-forming networks depend upon protein–protein interactions involving epidermal growth factor (EGF) and fibronectin type I–III modules, triple-helical interactions between collagen chains (Yurchenco *et al,* 1992), and protein–carbohydrate interactions involving carbohydrate recognition domains in laminin, perlecan, and link proteins (Talts *et al.,* 1999). For example, a laminin G domain binds to a novel *O*-mannosyl modification on mucin-like repeats in the transmembrane protein α-dystroglycan to maintain essential contact with the cell (Grewal *et al.,* 2001). These protein networks are cross-bridged by a unique multidomain protein nidogen (entactin) with discrete domains, separated by EGF-like spacers, that independently bind each of the other basement membrane components (Hopf *et al.,* 2001). The protein networks are interdependent as, e.g., genetic ablation of laminin interferes with the orderly cell surface deposition of the other components, and the associated epithelial cells fail to differentiate, demonstrating signaling back to cells (Smyth *et al.,* 1999). Basement membrane precursors are usually secreted by neighboring cells, but they can diffuse great distances as for collagen type IV in *C. elegans* (Graham *et al.* 1997). Although biochemical studies suggest that the basement membrane is capable of a considerable degree of self-assembly (Kleinman *et al.,* 1983), genetic manipulations show that cell surface receptors including $\alpha_7\beta_1$ integrin (Colognato and Yurchenco, 2000) and α-dystroglycan (Henry and Campbell, 1998) are required to initiate basement membrane assembly *in vivo*. Biochemical studies show that these cell surface receptors interact with specific domains of laminin and nidogen. This is reminiscent of the role of integrins in promoting fibronectin fibrillogenesis at the cell surface (Schwarzbauer and Sechler, 1999). In addition, biochemical and genetic studies show that proteoglycans of the basement membrane binds growth factors that modulate cell behavior (Baeg-Hun and Perrimon, 2000). The basement membrane provides important examples of how specific domains of components are involved in self-assembly, cross-bridging, linkage to the cell surface, and how these components are interdependent and provide information back to the cell demonstrating the existence of both structural and regulatory functions.

III. Composition and Structure of the *Dictyostelium* Spore Coat

The purpose of development in *Dictyostelium* is to produce aerial spores for survival (Loomis, 1975; Kessin, 2001). An essential feature of the spore is its cell wall or coat. Development is a specific response to starvation and involves chemotactic aggregation of the normally solitary amoebae into a multicellular organism. Cells subsequently differentiate molecularly into prespore cells or one of several types

of prestalk cells, which then sort out from one another. During culmination, the prespore cells rise up around an extending core of stalk cells, resulting in a fruiting body in which 80% of the cells become aerial spores (Fig. 1A). The spores (Fig. 1B and C) are maintained by surface tension in a droplet of secretory fluid known as the interspore matrix (ISM). The spore coat is assembled from premade and newly synthesized precursors during the final hour of the 24-hr developmental cycle.

A. Ultrastructure

In spores that have been aldehyde fixed, osmicated, and conventionally embedded, thin sections decorated with heavy metal salts reveal a three-layered wall at the cell surface (Fig. 2B and C). The central electron-lucent core is bounded at the outside by an electron-dense layer and on the inside, adjacent to the plasma membrane, by a less electron-dense layer. Similar images are seen after fixation in permanganate (Hemmes *et al.,* 1972) or by freeze-substitution (Erdos and West, 1989). In freeze-fracture replicas, the coat is about 210 nm thick with the central layer occupying about 160 nm. The coat retains its ultrastructural appearance after purification from lysed spores and remains firmly attached to the plasma membrane (Fig. 2B). The ultrastructure is unchanged by germination except that the plasma membrane becomes detached, and the coat retains its shell-like shape (George *et al.,* 1972; Loomis, 1975).

Freeze-fracture replicas show that the central region is fibrillar with two sublayers (Hemmes *et al.,* 1972). Outer sublayer fibrils are oriented primarily circumferentially around the long axis in contrast to those of the inner sublayer, which are randomly oriented. The replicas suggest that microfibrils have multinanometer diameters. Negatively stained images suggest that the spore coat fibrils have a uniform diameter of 4.8 ± 0.3 nm, compared to the broad range of diameters seen for cellulose elsewhere in the organisms (Section VII). The wall material is resistant to boiling in 5% H_2SO_4 followed by 5% NaOH, and the similar structures in the stalk tube diffract with reflections consistent with cellulose (Muhlethaler, 1956). Microfibrils are removed by treatment of disrupted spores with cellulase (Hemmes *et al.,* 1972). In thin sections, this region of the coat is heavily labeled with a probe consisting of a cellulose-binding domain or cellulase adsorbed to colloidal gold (Berg *et al.,* 1988), and this region is absent from a cellulose-null mutant (see below). Coats are intensely labeled with Calcofluor White ST and other fluorescent dyes (Fig. 1C), which have a high affinity for cellulose and related polysaccharides (Harrington and Raper, 1968). Together these results indicate that the middle layer of the coat is highly enriched in and probably consists predominantly of 4.8-nm-thick cellulose microfibrils.

The outer layer is strongly contrasted with uranyl acetate and lead citrate suggestive of protein. The localization of glycoprotein-associated epitopes in this region and the sensitivity of several coat proteins to protease applied to intact spores

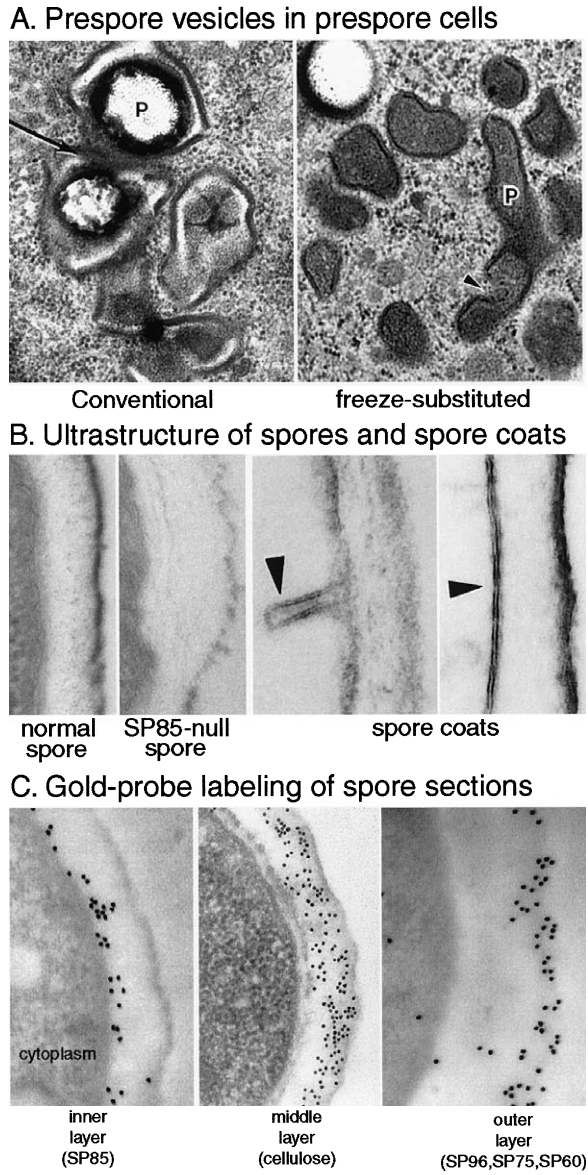

FIG. 2 Electron microscopy of *Dictyostelium* prespore and spore cells. (A) Thin sections were prepared from slugs prepared by conventional fixation (Zhang *et al.,* 2001) or freeze-substitution (Erdos and West, 1989), embedded in Epon, and stained with lead citrate and uranyl acetate. p, prespore vesicle; arrow denotes possible continuity between vesicles. (B) Thin sections (Epon) were prepared from spores or purified coats (Zhang *et al.,* 1998), and stained with lead citrate and uranyl acetate. Normal (strain Ax3) and SP85-null (HW70) spores were processed in parallel. Cytoplasm is to the left and the outer layer is to the right. Arrowheads denote plasma membrane, which in the third panel has formed a pleat. (C) Thin sections were prepared from conventionally fixed spores embedded in LR White or Epon, and probed with mAbs and gold-conjugated secondary antimouse IgG or cellulase-gold (Erdos and West, 1989) as indicated. Images were generously provided by G. W. Erdos.

(see West and Erdos, 1990) support this interpretation. Extraction of spores or purified postgermination coats by hot sodium dodecyl sulfate/2-mercaptoethanol (SDS/MSH), which removes >90% of total coat protein based on amino acid analysis (West *et al.,* 1996), leaves residual electron-dense material (Yoder *et al.,* 1994) that can still be labeled with antiglycoprotein antibodies. The outer layer is also resistant to digestion by Pronase (Hemmes *et al.,* 1972). It is, however, susceptible to boiling in 10% KOH for 45 min (Maeda, 1984). The appearance of this material is reminiscent of the DL-dityrosine polymer of the yeast spore wall (Section II.B), but the coat residue is not especially enriched in any amino acids. Early conclusions that the outer layer contains the Gal/GalNAc-PS (see below), based on sugar analysis of enzymatically prepared outer layer material and antibody probing (Hemmes *et al.,* 1972), were not confirmed by probing with the lectins RCA-I and soybean agglutinin (Erdos and West, 1989), which unlike the antibodies do not cross-react with coat glycoprotein epitopes. The outer layer seems to be organized symmetrically because immunogold labeling with monoclonal antibody (mAb) 83.5 shows a double-track pattern in freeze-substituted material (Erdos and West, 1989) that is also seen in conventional preparations of purified coats (Fig. 2B). Thus proteins may be anchored on each side of a planar core that seems to be irreversibly cross-linked as seen in other cell walls and vitelline envelopes (Section II). In mutant spores missing selected outer layer proteins or an inner layer protein (Fig. 2B), the outer layer consists of a planar lattice of electron densities with a spacing of about 35 nm (Metcalf *et al.,* 2002). This lattice might represent a core structure on which other outer layer proteins are assembled.

The inner layer is less electron dense than the outer layer. It contains distinct proteins based on probing with mAb 16.2 against SP85 (West and Erdos, 1988) and antibodies against certain lysosomal enzymes (Free *et al.,* 1989). The inner layer localization of SP85 has been confirmed by immunofluorescence (Watson *et al.,* 1994). The inner layer also contains the Gal/GalNAc-PS (Section III.B.2) based on probing sections with the lectins RCA-I or soybean agglutinin (Erdos and West, 1989; Lenhard *et al.,* 1989b). Labeling diminishes in the middle cellulosic region, suggesting that the Gal/GalNAc-PS does not fulfill a hemicellulose- or pectin-like role. A remnant of the inner layer remains visible in the electron microscope after extraction with hot SDS/MSH, suggesting the existence of cross-linked material as for the outer layer.

When coats are purified from mechanically disrupted spores, they remain associated with fragments of the plasma membrane (Cotter *et al.,* 1969) (Fig. 2B). However, the membrane is often pleated, suggesting that the coat contacts can diffuse within the plane of the membrane when the coat curls. The plasma membrane remains visible when coats are extracted with 0.1% Triton X-100 before preparation for electron microscopic (EM) analysis, suggesting an unusual stability of the lipid components. Coats are membrane free when examined after germination.

The coat may thus be summarized as a polarized protein–cellulose–protein/polysaccharide sandwich that is tightly associated with the plasma membrane in a

reversible fashion. The core is predominantly cellulose as for plant cell walls. The inability to completely dissociate the coat in hot SDS/MSH suggests the existence of irreversibly cross-linked material as observed in many cell walls, but chemical analysis shows $<10\%$ of total protein is bound up in this form.

B. Molecular Components of the Coat

Spore coats can be highly purified from either germinated or mechanically lysed spores by isopycnic centrifugation in renografin gradients (Akalehiywot and Siu, 1983; Gonzalez-Yanes *et al.,* 1989; Zhang *et al.,* 1998). Wild-type coats have a buoyant density in this medium of about 1.25 g/ml, which is altered in some coat mutants (see below). Each postgermination coat contains about 450 fg of protein, about 1% of total spore protein, and 330 fg of cellulose, based on amino acid analysis after acid hydrolysis and sugar analysis after trifluoroacetolysis or cellulase digestion (Zhang *et al.,* 1998; West *et al.,* 1996, 2002b). The other known polysaccharide, the Gal/GalNAc-PS, is present at only about 10 fg/coat based on monosaccharide analysis after hydrolysis in 4 *M* trifluoroacetic acid (TFA). The coat appears to lack the hemicelluloses thought to cross-bridge the cellulose fibrils of most but not all plant cell walls (Section II.A).

1. Cellulose

Cellulose appears to be mostly crystalline based on resistance of the glucan chains to hydrolysis by 4 *N* TFA at 100° C for 4 hr (Zhang *et al.,* 1998; West *et al.,* 2002b) and the acetic-nitric reagent of Updegraff (1969) (unpublished data). In this respect it is similar to cellulose of stalk (Blanton *et al.,* 2000) and slime sheath (Freeze and Loomis, 1977). A crystalline organization of the cellulose in other *Dictyostelium* ECMs has been confirmed by certain reflections seen in X-ray diffraction analysis (Muhlethaler, 1956). However, further study is required to determine whether the β1,4-glucan chains are in antiparallel (type II) or parallel (type I) alignments and, if the latter, whether type Ia or Ib packing is employed.

Dictyostelium contains a single gene for the catalytic subunit of cellulose synthase (*dcsA*), which is required for synthesis of cellulose in all of its ECMs (Blanton *et al.,* 2000). *dcsA* is homologous to cellulose synthase catalytic subunits of bacteria and plants, which all belong to the family 2, sugar nucleotide-dependent inverting glycosyltransferases, which usually transfer sugar to the nonreducing terminus of an acceptor sugar chain. Uridine dephosphoglucose (UDPGlc) is an efficient Glc donor in extracts of differentiating stalk cells (Blanton, 1993), consistent with the dramatic rise in UDPGlc pyrophosphorylase (Fishel *et al.,* 1982) concomitant with the disappearance of glycogen at the end of development when most cellulose is made. Based on recent studies on cotton cellulose synthase (Peng *et al.,* 2002), cellulose synthesis appears to be initiated by the action of the catalytic subunit on a sterol glucoside primer oriented toward the cytoplasm that is the product of a

sterol glucosyltransferase (Warnecke *et al.,* 1999). The initial sterol oligoglucose moiety is thought to be hydrolyzed by the action of the *korrigan* cellulase allowing the free reducing terminus to be cosynthetically translocated across the membrane.

Cellulose synthase enzyme complexes can be detected in the plasma membrane by freeze-fracture analysis as an array of intramembranous particles (IMPs). In higher plants, these terminal complexes are organized as rosettes, which consist of a hexagonal group of IMPs (Delmer, 1999; Brett, 2000). As a result, the β1,4-glucan chains emerge in close proximity to one another allowing spontaneous association to form crystalline microfibrils. A terminal complex of intramembranous particles is also seen in *Dictyostelium* prestalk cells as they produce stalk tube cellulose (Section VII.B). The particles initially have a linear arrangement, which converts to 2-dimensional clusters possibly by lateral aggregation, as cells shift from synthesizing stalk tube cellulose to stalk cell wall cellulose (Grimson *et al.,* 1996). This might explain the different dimensions of cellulose microfibrils in these ECMs. The terminal complex of maturing spores, whose cellulose microfibrils have a distinct diameter (Section III.A), has not been studied.

2. Gal/GalNAc-Polysaccharide

A spore-associated galactose-rich polysaccharide was first discovered in the water-soluble fraction of the sorus, i.e., the interspore matrix fluid in which the spores are suspended (White and Sussman, 1963). The acid- and alcohol-precipitable fraction consisted predominantly of Gal, GalNAc, and GalUA, with no evidence for amino acids, and was referred to as an acid mucopolysaccharide. An antiserum raised against spores of the related species *D. mucoroides* reacted with the *D. discoideum* polysaccharide in Ouchterlony tests (Takeuchi, 1963). Based on serological cross-reactivity the polysaccharide was detected at the surface of *D. discoideum* spores and, at an earlier stage, in the prespore vesicles (PSVs) (Ikeda and Takeuchi, 1971).

The polysaccharide is synthesized in the Golgi apparatus based on EM autoradiography (Takemoto *et al.,* 1985) and biochemical studies reveal a UDPGal:polysaccharide galactosyltransferase in this compartment (Ikeda, 1981). The finding that the polysaccharide:protein ratio in the coat (1:45) is less than that (1:15) of purified PSVs (Ikeda and Takeuchi, 1971) is consistent with delivery of some of the polysaccharide to the interspore matrix.

A possibly related polysaccharide was characterized in a search for potential ligands of an abundant lectin expressed by prespore cells, discoidin II, which has specificity for β-linked Gal and GalNAc (Cooper *et al.,* 1983). The discoidins accumulate to high levels in the cytoplasm but there is evidence that they are externalized via an unconventional secretory pathway (Barondes *et al.,* 1987) that does not appear to involve PSVs. The discoidin II-binding polysaccharide has water, acid, and alcohol solubility characteristics, and a size, which are consistent with the previously characterized polysaccharide. However, this material lacks GalUA but because GalUA is also not detected in acid hydrolysates of spore coats (West *et al.,* unpublished data), the presence of this sugar in the original

polysaccharide remains to be confirmed. These results raise the possibility that these molecules interact in the spore coat, but thus far there is no evidence for discoidin II in the coat.

To characterize the polysaccharide further, an ethanol (EtOH)-insoluble, trichloroacetic acid (TCA)-soluble extract from a sporeless mutant of *D. mucoroides* has been fractionated by gel filtration, ion-exchange, and affinity chromatography (Sakurai *et al.,* 2002). Most of the Gal content coeluted with neutral fractions that were active acceptors for the above-mentioned UDPGal:polysaccharide GalTase. Acceptor activity bound to an RCA-60 column, but was heterogeneous with respect to size and binding affinity to RCA-120. Composition analysis, methylation analysis, nuclear magnetic resonance (NMR), controlled Smith degradation, and exoglycosidase digestions of these fractions suggest that the polysaccharide consists of the repeating branched trisaccharide Galβ1,3(Galβ1,6)GalNAcα1,3-. This material appears to be a free polysaccharide because protein was not found and a free reducing terminus was detected. The absence of uronic acids in the more recent studies and the relatively low abundance of the Gal/GalNAc-PS suggest that it is not comparable to the pectins of vascular plant walls.

Two lectins that recognize Gal and GalNAc residues, RCA-I(120) and soybean agglutinin, have also been used to detect Gal- and GalNAc-containing polysaccharides. These lectins are specific for a target that migrates in the stacking gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Erdos and West, 1989) and do not react with proteins. As described above, these lectins label the PSV, inner layer of the coat, and the ISM, where the Gal/GalNAc has been previously localized, and do not label intact spores unless they are preextracted with hot urea/MSH (Gonzales-Yanes *et al.,* 1989). Under these conditions, most Gal and GalNAc are retained in the coat (Zhang *et al.,* 1999). The lectins are more specific than the original antispore polysaccharide antiserum as they do not cross-react with spore coat glycoproteins.

All of the above studies probably describe the same population of polysaccharides, which will subsequently be referred to as the Gal/GalNAc-PS. Functional analysis of the Gal/GalNAc-PS must await identification of the glycosyltransferases responsible for its biosynthesis.

3. Coat Proteins

Ten major protein species are detected in highly purified postgermination coats separated on two-dimensional O'Farrell gels (West *et al.,* 1996) (see Table I). Compared to the amino acid composition of total spore protein, coat proteins are enriched in the amino acids Ser, Thr, Gly, Pro, and Cys, and deficient in the hydrophobic amino acids Leu, Ile, and Phe. The residue remaining after extraction with SDS/MSH sample buffer contains only 5% of the original protein and is enriched in hydrophobic residues.

TABLE I

Properties of Major Spore Coat Proteins

Protein	Gene (linkage group)	GI or accession number	N-terminus[a]	Polypeptide M_r[b]	Apparent M_r (PAGE)	Predicted p*I*	Measured p*I*	Polymorphisms[c]	*N*-Glycosylated	*O*-Glycosylated[d]	Layer(s)	References[e]
SP96	*cotA*(2)	S07638	21 (P)	57,510	96,000	5.4	4.3		≤1	FGNP, FP, F	Outer	1
SP87	*pspD*/PL3(2)	P54643	22 (P)	69,354	87,000	7.1	7.6		≤1		Outer/middle	2
SP85	*pspB*/14E6(2)	AF066071	20 (D)	57,455	85,000	6.1	6.8	WS576, WS380B	≤1	GN	Inner	3
SP80					80,000		5.7		Unknown	FP, GN	Outer	4
SP75	*cotD*/DP87(4)	BAA03083	23 (D)	55,989	75,000	7.7	5.2	WS380B	1–3	FP	Outer	5
SP70	*cotB*(2)	P15269	21 (D)	54,508	70,000	4.8	5.7–5.8		1	Fuc	Outer	6
SP65	*cotE*(2)	AF279135	18 (D)	45,358	65,000	7.9	7.0–7.2		≤4		Unknown	7
SP60	*cotC*(2)	S11676	24 (D)	47,272	60,000	4.8	5. 6		0		Outer	6
SP52					52,000		5.3		Unknown		Unknown	8
SP35	*psvA*/EB4(2)	P08798	23 (D)	53,006	35,000	9.2	6.5–7.5	WS576	≤1		Unknown	9

[a] Position after start codon: (P), predicted; (D), determined.

[b] In strain Ax3, after signal peptide cleavage.

[c] Compared to strain Ax3 (see Section III.B.3.a.ii).

[d] FGNP, Fucα1,3GlcNAcβ-PO_4-; FP, Fucβ1-PO_4-; F, Fucα1-; GN, GlcNAcα1-; Fuc, Fuc in unknown linkage (see Section III.B.3.b.i–ii).

[e] (1) Fosnaugh and Loomis (1989a); Tasaka *et al.* (1990); (2) Yoder *et al.* (1994); (3) Zhang *et al.* (1998); Powell-Coffman and Firtel (1994); (4) Aparicio *et al.* (1990); (5) West *et al.* (1996); Nakao *et al.* (1994); (6) Fosnaugh and Loomis (1989b); (7) Zhang *et al.* (1999); (8) West *et al.* (1996); (9) Zhang *et al.* (1998); Hildebrandt *et al.* (1991).

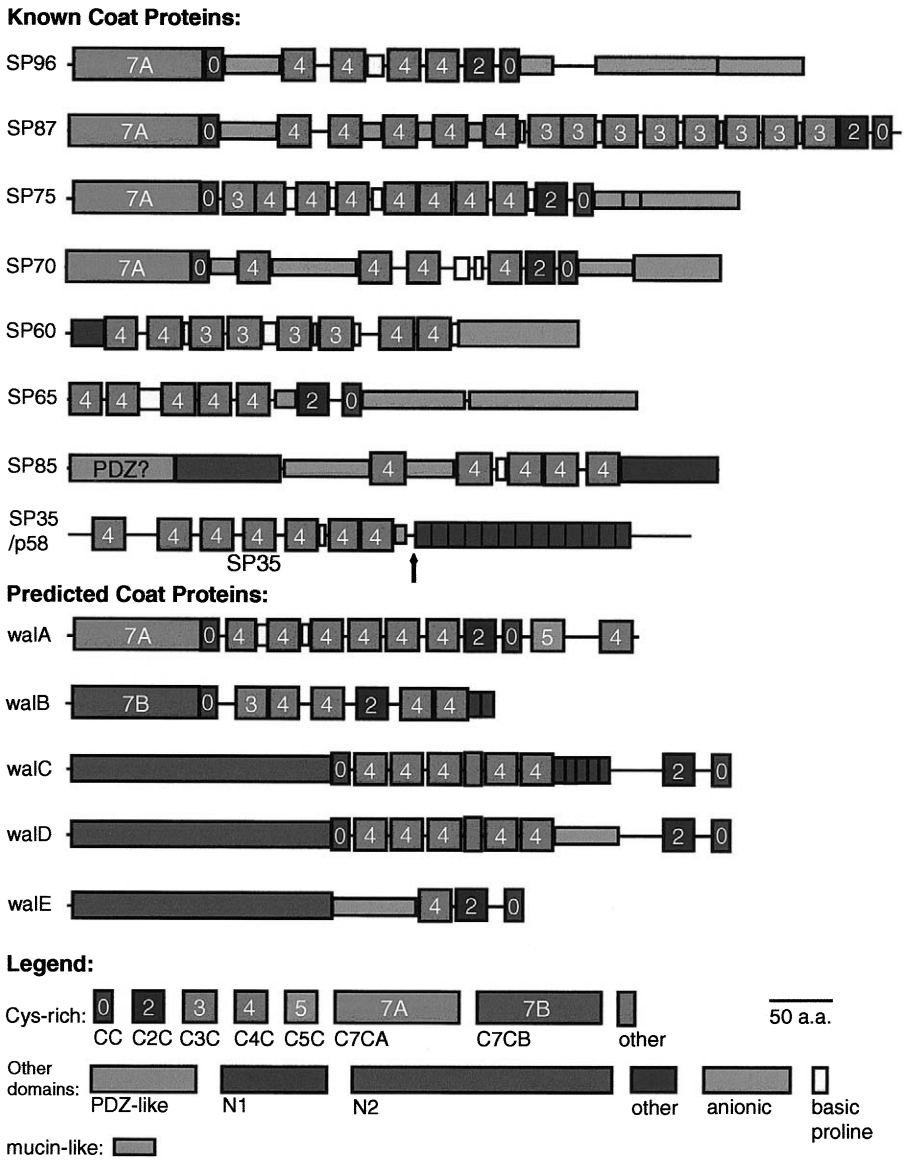

FIG. 3 Modular architecture of *Dictyostelium* spore coat proteins. Known coat proteins are shown at the top and predicted proteins with similar sequence motifs are shown below. Sequence motifs are represented by blocks. The tallest blocks, colored in shades of red, represent distinct Cys-rich motifs, which are listed in Table II. The shortest blocks represent mucin-like motifs, with repeats varying from 4 to 20 amino acids rich in the amino acids G, P, A, V, S, and T. Examples are given in Table II. The

Coat protein genes have been identified starting from both amino acid sequence data and the characterization of developmentally regulated genes with abundant transcripts. Complete gene and cDNA sequences are now known for eight of the major proteins. They are each transcriptionally active for many hours only in prespore cells, during which their protein products accumulate in PSVs (Hopper *et al.,* 1995). One coat protein gene, *psvA*, may be under antisense regulation to direct its expression in prespore cells (Hildebrant and Nellen, 1992). A search of *Dictyostelium* genomic and cDNA databases for similar sequences suggests the existence of five additional proteins related to the known coat proteins (Fig. 3). Some of these might encode minor coat proteins (West *et al.,* 1996) or be expressed in other ECMs (Section VII). The predicted polypeptide M_r values of the coat protein precursors range from 35,000 to 55,000, but they are generally extensively *O*-, *N*-, and phosphoglycosylated resulting in apparent M_r values of 58,000 to 110,000 based on SDS–PAGE. Metabolic labeling with [^{35}S]Met, $^{32}PO_4^{2-}$, and [^{3}H]Fuc shows that coat proteins are the major synthetic products of the slug and the primary recipients of Fuc and PO_4 modifications (Gregg and Karp, 1978; Watson *et al.,* 1993; Akalehiywot and Siu, 1983).

a. Sequence Motifs Sequence analyses show that the coat proteins consist predominantly of characteristic Cys-rich, mucin-like and other motifs (Fig. 3, Table II). They are distinct from related motifs seen in other *Dictyostelium* ECMs, and their order within individual proteins defines related subsets of coat genes and suggests an overall hierarchy of evolutionary descent (West *et al.,* 1996).

All known coat proteins have at their N-terminus a typical cleavable signal peptide (von Heijne, 1986) whose cleavage has been confirmed for most of the proteins (Table I). This is consistent with evidence that all coat protein precursors are processed through the rough endoplasmic reticulum (rER)-to-Golgi secretory pathway. The few introns found ($\sim \leq 1$/gene) are invariably located between individual sequence motifs consistent with the idea that motifs evolved as discrete structural and functional units.

i. Cysteine-Rich Motifs Six classes of Cys-rich motifs exist based on the spacing of Cys residues and propensities for β-turn residues at characteristic locations (Table II).

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medium-height boxes represent other motifs. All sequences have documented or predicted cleavable signal peptides at their N-termini (not shown). Predicted proteins were identified by Cys-spacing pattern searches in the Tsukuba EST sequence database (see http://www.csm.biol.tsukuba.ac.jp/). Sequences were confirmed and extended by alignment with uncurated genomic sequence reads from databases at Baylor and Jena (see http://dicty.sdsc.edu/). Predicted *walA–E* sequences are represented by the following cDNA clones (Tsukuba database): *walA,* SLA426; *walB,* SSM152; *walC,* SLC712; *walD,* SLA680, SSG386, and CFC681; *walE,* SSF605. (See also color insert.)

TABLE II
Classes of Sequence Motifs in *Dictyostelium* ECM Proteins[a]

Motif class	Sequence	Similarity
Cys rich		
Prespore/spore		
C4C	$Cx_4Cx_5Cx_{6-10}C$	N-terminal subdomain of EGF module
C3C	$Cx_3Cx_5Cx_{7,8}CCV$	
CC	$CCx_{4,5}C$	
C2C	$Cx_2Cx_{4,6}Cx_{4,6}C$	Thioredoxin
C7C-A	$Cx_{7,8}Cx_{3-6}Cx_7CCx_6Cx_{6,7}Cx_{5-7}Cx_{14,15}Cx_{15-22}Cx_{3-7}Cx_{6,9}Cx_{3-7}C$	
C7C-B	$Cx_{7,8}Cx_{3-6}Cx_7CCx_6Cx_{19}Cx_{31}Cx_4Cx_6Cx_7C$	
Prestalk/stalk		
ecmA/ecmB/ SSC290	$Cx_{5-6}Cx_4Cx_{5-9}C$	
AmpA	$CCx_2Cx_7Cx_6Cx_8CCxC$	Disintegrin
	$Cx_4Cx_6Cx_8CCxC$	Ornatin
SLA301/SLE577	$Cx_4Cx_4Cx_{12}CCx_2C$	
Mucin like		
SP96	GGQTS GGSTT, STIAG STIAS, SSSASGSSAA, SSSPSSSAA SSSPSSSA, AATT AATT IATT	
SP87/PL3	GGGSSGGTSGGSSSGG GGGTSGGSSSTGTSGG	
SP85/14E6	TQPP TYPP	
SP75/DP87	STIATTA STVATTT, GTTTG GTTTG, APSSSASS APSSASSS	
SP70	SHTTTGGSTTG SHTTTGGSTTG, STIAA, STIAS	
SP65	THSP THTP	

[a] The six families of Cys motifs in coat proteins are defined by their Cys-spacing patterns. Distinct Cys-spacing patterns are seen in proteins associated with ECMs produced by prestalk/stalk cells. Representative mucin-like sequences, defined by prevalence of A, G, P, S, T, and V and occurrence in tandem arrays, are listed in the lower part of the table. Some of these have been shown to be *O*-glycosylated (see text). Figure 3 shows the arrangement of these motifs in coat proteins.

The most common pattern, the C4C motif, contains about 25 residues and tends to occur in tandem arrays (Fig. 3). An extreme example is a coat protein from the related cellular slime mold *Polysphondylium pallidum,* which consists entirely of 12 tandem C4C motifs (Gregg and Cox, 2000). These motifs contain four Cys-residues with a spacing similar to that of the N-terminal subdomain of the EGF module (Davis, 1990). The second and third Cys residues are frequently separated

by the sequence PxGxx as often found in EGF modules. Based on these similarities, the C4C motif is predicted to have C1–C3 and C2–C4 disulfide bonds. A splice variant of an EGF-like growth factor removes the last C, which would also support only two disulfide bonds (Loukianov *et al.,* 1997). A similar repeat containing four Cys residues occurs in the finger module of tissue-type plasminogen activator and fibronectin (Patthy, 1993), which differ from EGF modules in the length of the loops between them. With few hydrophobic residues, the C4C motif probably relies on disulfide bonding to stabilize its folding. Some of these motifs contain an extra C residue between C3 and C4, which would be available for additional disulfide bonds. C4C motifs typically occur in up to 12 tandem copies, each separated by 2–15 residues, in the central region of coat proteins. Neighboring motifs tend to be related, suggesting that they evolved by endoduplication. Individual EGF modules possess sufficient structural information for highly specific receptor recognition, but tandem EGF modules are found in many animal ECM and cell surface proteins and may function as units (e.g., Handford *et al.,* 2000). There is evidence that the EGF domain can be a ligand for other receptor proteins, and it has been suggested that tandem EGF domains can form an inert rod domain. For example, in the *Drosophila* cell surface pair-rule transmembrane protein Ten-m/Odz, an octad cluster of EGF-like repeats forms a fine 16-nm-long rod that mediates homodimerization with another Ten-m/Odz protein (Oohashi *et al.,* 1999). Interestingly, the second and fourth repeat pairs are each missing their third Cys residues, leaving the odd Cys residue available for stabilizing parallel alignment of the octad repeats by interdomain disulfide bonds. A similar function may be associated with odd Cys residues that occur in the *Dictyostelium* C4C motif. A single C4C motif is found in the small *staB* protein probably secreted by stalk cells (Robinson and Williams, 1997). Thus C4C repeats can act alone, and are not unique to the spore coat ECM.

The C3C motif has a similar pattern of Cys residues except that it invariably has a fifth one (Table II), implying extensive interdomain disulfide cross-linking for this motif.

The C7C-A motif, about 100 amino acids in length, occurs only once, near the N-terminus, of four coat proteins (Fig. 3). This motif contains 13 Cys residues with characteristic spacings (Table II), and many other residues are also conserved. The C7C-A motif is invariably followed by a single CC motif (see below) with three Cys residues. The combined pairs of C7C-A and CC motifs have an even number of Cys residues therefore allowing all of them to be internally disulfide bonded. A similar, somewhat shorter C7C-B motif occurs in a similar location in a predicted protein that also shares other coat protein motifs (Fig. 3).

The CC motif contains three Cys residues in 10 amino acids (Table II). Copies occur twice in the four coat proteins that contain the N-terminal C7C-A or -B motif (Fig. 3). One follows the C7C motif and the other always follows the C2C motif (see below) near the C-terminus. Together the CC motifs tend to bracket a central

region of C4(3)C motifs. The role of the CC motif is unknown, but its odd number of Cys residues implies that one of them, i.e., from the CC pair, is available for disulfide bonding outside of the motif.

The C2C motif contains four Cys residues and is always found associated with the C-terminal CC motif (Fig. 3). The CX_2C subsequence, unique to this motif, is also found in the active sites of protein disulfide isomerases as part of the general thioredoxin motif (West *et al.,* 1996). Four residues C-terminal to the CX_2C are also loosely conserved with consensus thioredoxin sequences. There is a need for cell surface protein disulfide isomerase activity to cross-link proteins after they achieve their final positions in the coat, which might be provided by proteins with the C2C motif. There is evidence for this mechanism for animal fibronectin (Langencach and Sottile, 1999).

Distinctive Cys-rich motifs (Table II) are found in proteins likely to be associated with the stalk and slime sheath (Section VII), suggesting that the coat Cys motifs support coat-specific functions such as its defined architecture and outer permeability barrier.

ii. Mucin-like Motifs Six of the eight known coat protein sequences contain tandem arrays of short (4- to 20-residue) repeats rich in the amino acids S, T, P, G, A, and V, which comprise up to 40% of their overall length. These arrays are similar to sequences that occur in animal mucins, and in spacer regions between multidomain proteins such as cellulases (Gilkes *et al.,* 1991). The *Dictyostelium* repeats have diverse sequences (Table II). Within a given tandem array a motif may be perfectly repeated or show significant random or progressive variation, as seen in animal mucins. The number of repeats in SP85 (Zhang *et al.,* 1998) and probably SP75 (McGuire and Alexander, 1996) is polymorphic between strains as also seen in the animal mucins.

Hydroxyamino acids in mucin repeats are usually extensively modified by short, *O*-linked, anionic oligosaccharides (Perez-Vilar and Hill, 1999), and the coat protein repeats may be similarly extensively modified (see Section III.B.3.b). Related repeats are seen in the *Dictyostelium* GPI-anchored prespore cell surface protein SP29/PsA between its membrane anchor and its globular ectodomain (Zachara *et al.,* 1996), and in a *Dictyostelium* endoglucanase between its catalytic and cellulose-binding domains (Ramalingam *et al.,* 1992). Both regions are heavily *O*-glycosylated as in animal mucins.

Coat proteins have two other features that resemble traditional animal mucins. They contain terminal Cys-rich domains involved in intermolecular disulfide cross-linking (Section II.C), which contributes to the viscosity of animal mucus, and exhibit limited *N*-glycosylation.

The *Dictyostelium* mucin-like repeats are likely to assume the rod-like shapes described for animal mucins and in related regions seen in hydroxyproline-rich cell wall proteins in plants and *Volvox* (Section II.A). These might serve as interdomain spacers but, as they can also occur at the C-terminus, they may have alternative roles such as for protein–protein contact as suggested for GP-1 in *Chlamydomonas*

(Ferris *et al.,* 2001) and for laminin-α-dystroglycan contact in the muscle basement membrane (Section II.C).

iii. Other Motifs C3C and C4C repeats are often separated by short 5–12 amino acid motifs rich in His, Pro, and basic residues, and devoid of acidic residues. These may interact with anionic *O*-glycan chains or negatively charged peptide regions. SP60 and SP70 each contains 60–90 residues at its C-termini, which are $\geq$40% acidic residues and devoid of basic residues.

The Cys-rich, mucin-like, basic proline, and acidic motifs comprise the entire length of most of the coat proteins (Fig. 3). Exceptions >20 amino acids in length are found in SP85, which contains novel 95-residue C-terminal and 220-residue N-terminal domains. The beginning of the N-terminal domain does, however, show similarity to a PDZ domain found in a spore coat protein of *Bacillus subtilis* (Hoa *et al.,* 2001).

b. Posttranslational Modifications The apparent M_r values of coat proteins are generally 20,000–40,000 greater than predicted from their deduced amino acid sequences (Table I), which appears to be due to the mass increase and conformational effects of extensive *O*-glycosylation. Coat proteins are modified by at least four *O*-glycosylation pathways that differ in their linkage to the protein. Two pathways involve phosphoglycosylation in which the Fuc or GlcNAc is attached via a phosphodiester linkage to Ser or Thr, resulting in a negative charge. In the other two pathways, Fuc and GlcNAc are attached directly in a conventional glycosidic linkage. These sugars can be extended by additional glycosylation steps. Glycosylation appears to occur in the Golgi based on evidence that the coat proteins are modified a short time after synthesis (West *et al.,* 1986; Watson *et al.,* 1993).

i. O-Glycosylation Simple *O*-Fuc modification of SP96 and total prespore cell proteins was first suggested based on recovery of [^{3}H]fucitol by β-elimination of material from cells metabolically labeled with [^{3}H]Fuc (Riley *et al.,* 1993). The ability of slug cells to synthesize a Fuc1-Ser linkage was confirmed by mass spectrometry (MS) analysis of synthetic mucin-like peptides that were fucosylated by cell extracts (Srikrishna *et al.,* 1998). Analysis of a single coat protein, SP96, suggested that it contains about 28 *O*-Fuc modifications, based on gas chromatography (GC)/MS analysis after reductive β-elimination (Mreyen *et al,* 2000). Resistance of SP96 fucopeptides to Pronase digestion (Riley *et al.,* 1993) indicates that *O*-Fuc and other fucose modifications are clustered as expected for the modification of mucin-like domains. Fucα1-Ser/Thr has also been found on certain EGF repeats of mammalian secretory proteins (Harris and Spellman, 1993).

A second simple modification found in prespore cells is the linkage of α-GlcNAc to Ser or Thr. Nonreducing terminal GlcNAc linkages were originally suspected on developmentally regulated glycoproteins based on the ability of free GlcNAc to inhibit binding of certain mAbs that recognized gp80 and PsA/SP29 (Bozzaro and Merkl, 1985), and the recognition of these glycoproteins by the lectin wheat germ agglutinin (West *et al.,* 1986). mAb binding was abolished by the *modB* mutation,

resulting in a decrease in apparent M_r consistent with a defect in glycosylation (Murray *et al.,* 1984). Related mAb antibodies recognize the coat proteins SP85, SP86, and SP80 in a *modB*-dependent fashion. For structural studies, PsA/SP29 was expressed in growing cells (instead of prespore cells) without its membrane anchoring sequences, and found to still produce the *modB*-dependent epitope. Recombinant PsA/SP29 was modified by simple *O*-αGlcNAc residues at almost all of the Thr and Ser residues present in its mucin-like repeat region (Zachara *et al.,* 1996), based on MS studies, Edman degradation, and GC/MS analysis of the products of reductive β-elimination. Subsequent Edman degradation analysis of a glycopeptide isolated from normal PsA/SP29 showed that GlcNAc was in α1-linkage to Ser and Thr (Zachara, 1998). Resistance of this glycopeptide to proteolytic digestion confirmed that it was cluster glycosylated, and many of the GlcNAcα1 moieties are further modified by phosphoglycosylation (see below).

The GlcNAcα1-Thr modification appears to also be prevalent on the coat proteins SP85 and possibly SP80. These proteins carry a *modB*-dependent epitope recognized specifically by two mAbs, 16.1 and MUD102 (West *et al.,* 1986; West and Erdos, 1988; Alexander *et al.,* 1988), possibly located in a region of tandem mucin-like TXPP-tetrapeptide repeats in SP85 (Zhang *et al.,* 1999). The specificity of these mAbs for SP85 might reflect an SP85-specific glycosyl extension or an involvement of the peptide backbone in the epitope. Recent evidence suggests that SP85 and PsA/SP29 are modified by the cis4c gene (Li *et al.,* 2000), predicted to encode a glycosyltransferase that is homologous to the cytoplasmic UDP GlcNAc:Skp1 hydroxyproline GlcNAcTase and other Golgi polypeptide Ser/Thr GalNAcTases (West *et al.,* 2002a).

ii. Phosphoglycosylation Evidence exists for both GlcNAcα1-PO_4 and Fucβ1-PO_4 modifications of Ser/Thr residues on coat proteins. Phosphoglycosylation is an important modification in *Leishmania* and other protozoans (Haynes, 1998), and in fungal (Lipke and Ovalle, 1998) and *Volvox* (Sumper and Hallman, 1998) cell walls, in addition to its role as a peripheral modification of *N*-glycans in lysosomal protein targeting in animal cells.

Mucin-like repeats in a lysosomal cysteine protease are known to be cluster modified on their Ser and Thr residues by an GlcNAcα1-PO_4 moiety (Ord *et al.,* 1997; Mehta *et al.,* 1997). SP96 is heavily phosphorylated at Ser residues (Akalehiywot and Siu, 1983), contains mucin-like repeats, has an acidic p*I* suggestive of phosphodiester substituents, and is recognized by an antibody specific for GlcNAcα1-PO_4 (West and Freeze, unpublished data), suggesting that SP96 might also contain GlcNAcα1-PO_4-Ser modifications. This linkage was directly detected after nonreductive β-elimination of SP96 followed by sugar analysis of the product after mild acid hydrolysis and HF treatment, or NMR (Mreyen *et al.,* 2000).

Fucβ1-PO_4 modification of Ser residues has also been detected in SP96. This moiety was first observed on a synthetic mucin-like peptide in an extract of prespore cells provided with GDP-[^{3}H]Fuc (Srikrishna *et al.,* 1998). Its identity was confirmed by MS analysis of the peptide and radiochemical characterization after

β-elimination and acid hydrolysis. Fucophosphorylation of the peptide conferred upon it reactivity with mAb 83.5 (Srikrishna *et al.,* 1998), suggesting that SP96, recognized by mAb 83.5, also has the Fucβ1-PO_4 modification. In another study, Fuc-PO_4 was detected on SP96 as a minor species as fucitol after β-elimination and alkaline phosphatase treatment (Mreyen *et al.,* 2000).

In addition, GlcNAcα1-PO_4-Ser can be modified by Fucα1,3, as shown by MS–MS and NMR studies (Mreyen *et al.,* 2000). Sugar and phosphate measurements show that there are approximately 56 Fucα1,3GlcNAcα1-PO_4 modifications on SP96 (Mreyen *et al.,* 2000), which might explain the ca. M_r 40,000 mass discrepancy between SDS–PAGE-based estimates and amino acid sequence predictions. Formation of the Fucα1,3GlcNAc linkage is specifically blocked in a *modD* mutant. This mutation also prevents recognition of coat proteins by mAbs MUD62 and 83.5 (Champion *et al.,* 1995), which suggests that the recognition determinant for these two mAbs involves the Fuca1,3GlcNAcα1-PO_4 moiety (but see below). This structure may also reside on SP80 and SP75, other proteins recognized by mAb 83.5. Because mAb 83.5, whose binding can be competed by free Fuc (West *et al.,* 1986), appears to bind both Fucβ1-PO_4-Ser/Thr and Fucα1,3GlcNAc, the *modD*352 mutation is implied to affect both structures, possibly by interfering with Fuc utilization or a compartment-specific function. The GlcNAcα1-Thr(Ser) linkage also appears to be extended by phosphoglycosylation on the GPI-anchored prespore cell surface protein PsA/SP29. Normal PsA contains both phosphate and fucose modifications in its mucin-like region (Haynes *et al.,* 1993). MS analysis of material released from the mucin-like glycopeptide by HF provided evidence for extension of *O*-GlcNAc residues by GlcNAc-PO_4, Fuc-GlcNAc-PO_4, and PO_4-Fuc-GlcNAc-PO_4 moieties (Zachara, 1998). The presence of phosphodiester linkages in PsA is consistent with the very acidic apparent p*I* (4.0–4.5) of this protein (West and Loomis, 1985). These outer anionic modifications of core *O*-αGlcNAc are not likely to be prominent on the major known coat structural proteins except for SP96, the only major coat protein that has a p*I* as acidic as SP29/PsA (West *et al.,* 1996).

iii. N-Glycosylation Most coat proteins are *N*-glycosylated based on slightly increased apparent M_r values by SDS–PAGE in a *modA* mutant (Table I), which targets an early trimming step in the *N*-glycosylation pathway mediated by α-glucosidase-II (Freeze *et al.,* 1997). The *N*-glycans may be α1,3-core fucosylated by an enzyme known to be expressed in prespore cells (Srikrishna *et al.,* 1998), which would explain why their M_r values were not affected by PNGase F (Watson *et al.,* 1993). Coat proteins are minimally *N*-glycosylated, based on the small number of consensus NxS/T *N*-glycosylation sequons, at most 1–3 per protein (see Table I). *N*-Glycans of prespore cells are heterogeneous including a neutral class with possibly intact Man chains ($Man_9GlcNAc_2$, and smaller, neutral $Man_{3-5}GlcNAc_2$ (Riley *et al.,* 1993) forms that are variably α1,3-core fucosylated, consistent with the developmental expression of trimming mannosidases-1 and -2 (Sharkey and Kornfeld, 1991). The minimal *N*-glycosylation of the coat proteins

implies that their folding in the rER is not subject to extensive quality control by the calnexin/calreticulin-dependent pathways (Helenius and Aebi, 2001).

iv. Proteolytic processing The overall M_r profiles of coat proteins seen in Western blots of prespore cells and spores are similar indicating that proteolytic processing is not a major event in coat assembly. However, the product of the *psvA* gene, p58, was originally detected in PSVs as an M_r 58,000 polypeptide but not in the coat or the interspore matrix (Hildebrandt *et al.,* 1991). Subsequently, the coat protein SP35 (M_r 35,000) was found to derive from the *psvA* gene (Zhang *et al.,* 1998). Based on amino acid sequence and amino acid composition data, SP35 corresponds to the first ca. 292 amino acids of p58. Because Northern blotting indicates only a single transcript from *psvA*, it is likely that SP35 arises from p58 by proteolytic processing (West *et al.,* 1996).

Cysteine proteases have been detected in the PSV (Srinivasan *et al.,* 2001) and interspore matrix, and coat proteins may be exposed to them during coat formation (Cavallo *et al.,* 1999). In a strain expressing the NC2 fragment of SP85, SP96 is expressed as an M_r 80,000 protein that is no longer retained in the coat (West *et al.,* 2002b), which might result from aberrant proteolytic cleavage. Proteolytic processing occurs during formation of the *Drosophila* vitelline envelope/chorion (Nogueron *et al.,* 2000), and might be a general form of assembly regulation.

v. Disulfide Bond Formation Reducing agents are required for denaturing extraction of most proteins from the coat (Wilkinson and Hames, 1983), suggesting that they are disulfide cross-linked to one another. Nevertheless, denaturing treatment in the absence of reducing agent is able to release about half of each protein type (Aparicio *et al.,* 1990), raising the possibility that not all proteins are disulfide cross-linked. However, when coats were prealkylated with iodoacetimide, denaturing agents alone were unable to extract any protein with the exception of SP96 (West *et al.,* 1996). This suggests that free sulfhydryl groups are available in the coat to mediate disulfide exchange reactions under denaturing conditions that can artifactually release normally cross-linked proteins. Potential donor sulfhydryls might preexist in coat proteins as observed for animal ECM proteins (Misenheimer *et al.,* 2001; Yan and Smith, 2001) and possibly *Chlamydomonas* (Section II.A), or derive from intracellular sources. Thus all proteins except SP96 appear to be quantitatively disulfide cross-linked together in the coat.

IV. Intermolecular Interactions

A. Binding Interactions in Extracts

The mechanism by which coat proteins interact with one another has been investigated using biochemical and genetic approaches. Initial studies examined coat protein precursors solubilized from prespore cells, which are packaged in PSVs and

do not contain cellulose. Using antibodies specific for SP96 or SP70, immunoprecipitation of prespore cells lysed in the presence of nonionic detergent pulled down the three most abundant coat proteins, SP96, SP70, and SP60 (Devine *et al.* 1983). Similar results were subsequently obtained using an mAb (MUD102) against SP85 (also referred to as PsB) and another mAb (MUD3) against SP96 (Watson *et al.,* 1993, 1994), using metabolic labeling with [^{35}S]Met to detect the proteins. This complex contained SP96, SP85, and probably SP70 and SP60, and clearly lacked other coat proteins such as SP80 and SP75. After partitioning into the aqueous phase after Triton X-114 extraction, the protein complex chromatographed with an apparent M_r of >670,000 (Watson *et al.,* 1993). The complex was dissociated by treatment with 8 *M* urea and MSH. Treatment with reducing agent alone did not affect the complex, but urea alone selectively extracted SP96, indicating that it was associated in a distinct fashion. A third way in which this complex was pulled down was by mixing the PSV extract with cotton cellulose particles (McGuire and Alexander, 1996). Although only a small fraction of the coat proteins bound cellulose, binding was reportedly specific for cellulose compared to other polysaccharides. The same four proteins and, in addition, SP75 were selectively bound to the cellulose. These results support the existence of a preformed complex of coat proteins, which might be important for packaging in the PSV or later assembly of the coat.

To test the role of individual proteins in the complex, the existence of the complex was examined in strains genetically lacking SP96, SP70, or SP60 (McGuire and Alexander, 1996). The findings suggested that the multiprotein complex consists of a pairwise series of molecular contacts in the order SP60–SP85–SP70–SP96, with SP96 mediating contact with cellulose but only when the complex is intact. Further studies are required to determine whether these contacts are direct and occur *in vivo*.

A fraction of proteins normally found in the coat is present in the ISM surrounding the spores, and probably represents a pool of unincorporated precursors. Immunoprecipitation with mAb MUD102 pulled down a complex that was distinct from the complex obtained from prespore cell extracts (Watson *et al.,* 1994). It contained SP96, SP85, and apparently SP60, was deficient in SP70, and also contained SP80, SP75, and possibly another protein, SP65. Extraction of SP96 from the complex by urea could not be accomplished without MSH, suggesting that it had become disulfide cross-linked. Thus the original complex appears to be significantly modified after secretion. The immunoprecipitation approach was also applied to a fraction of coat proteins that could be extracted from washed coats by SDS in the absence of reducing agent, which yielded a complex similar to that described in the ISM.

In another approach, proteins were obtained directly from coats by denaturing extraction with hot 8 *M* urea/MSH, which releases 85% of total coat protein. When urea and MSH were removed by gel filtration, about half of the total protein could be pulled down after incubation with Avicel cellulose (Zhang *et al.,* 1998).

Pull-down was specific for cellulose. Two coat proteins, SP85 and SP35, bound to a greater extent, and SP96 bound less well, suggesting that SP85 and SP35 might bind cellulose directly with other proteins piggybacking on them. Poor binding of SP96 correlated with its relatively poor incorporation into the coat *in vivo* (West *et al.,* 1996). Evidence for direct binding of SP85 and SP35 to cellulose, and for simultaneous binding of SP85 to the coat protein SP65, is discussed in the next section.

Coats purified from spores remain attached to their associated plasma membrane (Section III.A). Several proteins that are unique to the prespore/spore plasma membrane are candidates to mediate these contacts. SP29/PsA is a small M_r 29,000 glycoprotein anchored to the membrane by a C-terminal GPI anchor (Haynes *et al.,* 1993). Its N-terminal ectodomain with unknown function projects from the lipid bilayer by a 100-amino-acid long mucin-like spacer. WGA80B is a larger M_r 80,000 prespore/spore-specific glycoprotein also predicted to contain a mucin-like region and a GPI anchor (West and Loomis, 1985). The SpiA glycoprotein, with an apparent M_r of 31,000, is the latest known prespore cell gene product to be expressed. SpiA appears to be localized at the plasma membrane consistent with its four predicted transmembrane domains (Richardson and Loomis, 1992). *spiA*-null spores produce coats but die under stress, showing that SpiA contributes an as yet unidentified function. The *lagC* protein (gp150), an M_r 150,000 glycoprotein thought to be membrane attached by a C-terminal hydrophobic region, is essential for sporulation by a cell-autonomous mechanism (see Wang *et al.,* 2000). Additional studies are required to evaluate the potential roles of these proteins in coat formation.

B. Binding Interactions of Purified Proteins

In a directed search for individual coat proteins that could bind cellulose *in vitro,* proteins extracted with 8 *M* urea/MSH were separated by anion-exchange chromatography in the presence of urea to inhibit intermolecular associations, and individual fractions were assayed for Avicel cellulose-binding activity in pull-down assays (Zhang *et al.,* 1998). This study identified two coat proteins, SP85 and SP35, that bound Avicel specifically after purification. Other proteins, including SP96, failed to bind Avicel. Though this might simply reflect failure to renature or the presence of inhibitors, before separation the proteins of the original extract bound cellulose robustly after renaturation (see above).

To eliminate the possibility that cellulose binding depended on an unknown copurifying factor, SP85 cDNAs were expressed recombinantly in *Escherichia coli* (Zhang *et al.,* 1998). An SP85 construct that lacked the central 110 amino acids that encode the middle (M)-domain and flanking TXPP-tetrapeptide repeats bound cellulose efficiently, as did a more truncated version consisting of the C-terminal C1 and C2 domains (see domains in Fig. 3). In contrast, the expressed N-terminal region did not bind. Binding of the C1C2 region renatured

from inclusion bodies depended on the presence of glutathione, suggesting a need for proper disulfide bond formation by the C1 region, which contains 20 Cys residues in four tandem C4C motifs. In contrast to these results, SP85 in extracts of prespore cells failed to bind cellulose in strains deleted in either SP96 or SP70 (McGuire and Alexander, 1996), which suggests either the existence of an inhibitor in prespore cells or that cellulose-binding activity is activated during sporulation in a way that can be mimicked by expression in *E. coli*.

To distinguish the contributions of the C1 and C2 regions to cellulose binding, they were separately fused to the N-terminal domain and expressed under control of the prespore cell-specific *cotB* promoter in the cellulose-null strain DG1099 (West *et al.,* 2002b). The N domain was included based on its ability to be targeted to the PSV (Zhang *et al.,* 1999) and as a potential aid in folding the short C1 and C2 domains. When sporulation was induced by suspending dissociated slug cells in the presence of 8-Br-cAMP, a significant fraction of total coat protein can be recovered in the soluble extracellular fraction that is equivalent to a diluted interspore matrix. NC1 but not NC2 from this fraction bound cellulose efficiently *in vitro,* indicating that cellulose binding maps to the C4C motifs found in the C1 domain (Metcalf *et al.,* 2002). The other cellulose-binding protein, SP35, is composed almost entirely of C4C repeats that, however, share no obvious sequence identity with the SP85 repeats.

During studies to define the cellulose-binding activity of SP85, SP85 tended to copurify with the coat protein SP65. Subsequent studies showed that the recombinant C-terminal (but not N-terminal) region of SP85 binds SP65 directly in a pull-down assay, and that the C region can bind both cellulose and SP65 simultaneously (Zhang *et al.,* 1999). Furthermore, the NC1 fusion but not the NC2 fusion described above binds a band at the SP65 position of a Far Western blot containing purified spore coats (Metcalf *et al.,* 2002). These results show that C4C motifs can bind either cellulose or other proteins, but it remains to be determined whether the C4C motifs act autonomously or synergistically.

A search for cellulose-binding proteins in the interspore matrix fraction yielded an M_r 95,000 protein band that was identified as β-glucosidase-2 based on its M_r value, amino acid sequence data, and reactivity with anti-β-glucosidase-2 antibodies (Zhang, 1999). β-Glucosidase-2 could be eluted from Avicel cellulose by water like other known cellulose-binding proteins (Tomme *et al.,* 1998), but its sequence appears unrelated to known cellulose-binding domains. β-Glucosidase-2 is synthesized in prespore cells (Bush *et al.,* 1994), and probably cooperates with endoglucanases and cellobiohydrolases to digest cellulose during germination (Section VI.B). Although cellulose-binding domains are often found in endoglucanases and cellobiohydrolases (Gilkes *et al.,* 1991), this is a novel feature for a β-glucosidase and might ensure retention of the enzyme with the coat until it is needed during germination.

Two cellulose-binding proteins, CelA and CelB, are expressed during germination and are probably associated with cellulose breakdown (see Section VI.B). SP85 does not appear to have cellulase activity (Zhang *et al.,* 1998) nor does it

interact with the *celA* endoglucanase *in vitro* (Zhang and West, unpublished data). Thus there is no evidence that SP85 associates in a cellulosome-like multiprotein cellulase complex found in certain anaerobic bacteria and fungi (Bayer *et al.,* 1998).

Polysaccharide-binding proteins are likely to be a general feature of cell walls. Two new cellulose-binding proteins have been described recently in the stalk and slime sheath (Section VII). The cellulose-containing wall of the plant pathogen *Phytophthora* contains an abundant modular glycoprotein with two Cys-rich cellulose-binding domains (Mateos *et al.,* 1997), the chitin-rich cyst wall of *Entamoeba invadens* contains a major protein with five Cys-rich chitin-binding domains (Frisardi *et al.,* 2000), and a fungal cell wall contains a galectin-like protein (Bouliamme *et al.,* 2000). SP85 and these other lectin-like proteins may cross-bridge proteins to polysaccharides via multiple binding activities mediated by separate domains, as observed also for animal basement membrane proteins (Section II.C).

C. Protein–Protein and Protein–Cellulose Interactions *in Vivo*

Studies on mutant coats have yielded several examples supporting the concept that coat proteins interact by specific and discrete contacts. In SP85-null spores, the coat protein SP65 fails to incorporate significantly into the coat and is instead found in the interspore matrix surrounding the spores (Zhang *et al.,* 1999). Because SP85 simulataneously binds SP65 and cellulose *in vitro,* this result strongly supports the relevance of these interactions *in vivo*. Incorporation of SP65 can be partially restored by reexpression of fragments of SP85 containing its C domain, consistent with *in vitro* domain studies (see above). Thus the SP65–SP85–cellulose triad may constitute a core structural module in the coat. SP96 may also belong to this core module as it too is deficient in SP85-null coats (Metcalf *et al.,* 2002). The major coat proteins SP70 and SP60 are still efficiently incorporated, suggesting that they are anchored by another mechanism.

The mechanism by which SP85 is incorporated into the coat has been examined by observing the localization of fragments of SP85 expressed in prespore cells under the *cotB* promoter (Zhang *et al.,* 1999). The 180-amino acid N domain is targeted correctly to the PSV but is not retained in the coat and is recovered quantitatively in the interspore matrix. In contrast, the 200-amino acid C-terminal domain is not properly targeted to PSVs but is efficiently incorporated into the coat proper, possibly arriving by a different pathway. Retention of the C-terminal domain in the coat is consistent with its *in vitro* cellulose-binding activity. The contribution of the C1 and C2 subregions to coat retention was examined by expressing the NC1 and NC2 domain fusions described above in normal and mutant backgrounds. NC1 bound the coat very efficiently, even when expressed in the cellulose-negative or SP85-null strains (West *et al.,* 2002b). The majority of NC2 also bound, though not as completely as NC1 but to a much greater extent

than N alone. Thus the C1 and C2 fragments appear to represent separate folding domains each expressing distinct binding activities *in vivo* to components of the coat in addition to cellulose, as binding did not depend on its presence.

A triple-mutant strain lacking SP96, SP70, and SP60 contains a normal complement of other coat proteins except for SP35, which is absent (West *et al.,* 1996). Because SP35 has cellulose-binding activity *in vitro* and would be expected to bind the coat in the absence of these proteins, SP35 may not be properly proteolytically processed from its p58 precursor. In a strain lacking SP75, all other proteins seem to be incorporated at normal levels into the coat. Together these results are consistent with evidence that SP96, SP70, SP60, and SP75 are in the outer layer and depend on other proteins for their incorporation.

Evidence that SP96 and SP80 are bound to the coat via discrete domains comes from the analysis of glycosylation mutants. In *modB* mutants, SP80 is not properly *O*-glycosylated and is quantitatively modified during germination to yield an M_r 68,000 fragment that no longer binds the coat (Aparacio *et al.,* 1990). Similarly, in a general fucosylation mutant SP96 is released from the coat during germination as an M_r 45,000 fragment (Gonzales-Yanes *et al.,* 1989). In a strain expressing the NC2 fusion of SP85, SP96 is expressed as an M_r 80,000 fragment that fails to incorporate during assembly (West *et al.,* 2002b). These results confirm the existence of protease activities during coat formation and germination, and demonstrate that binding of these two proteins to the coat depends on terminal regions that probably correspond to specific domains predicted by the sequence analyses (Fig. 3).

V. Formation and Functions of the *Dictyostelium* Spore Coat

A. Assembly

The protein and Gal/GalNAc-PS precursors of the coat are synthesized in advance and stored in PSVs. Their exocytosis signals the initiation of coat assembly (stage I). Cellulose is deposited separately via the plasma membrane-associated cellulose synthase complex. Coat assembly depends on the appropriate coordination of these two pathways over a time interval of about a half-hour once sporulation is triggered. The three stages of coat assembly are summarized in Fig. 4A.

1. Prespore Vesicle Formation

The prespore vesicle (PSV) accumulates in prespore cells and is a developmental marker for this cell type (Hohl and Hamamoto, 1969; Gregg and Karp, 1978). All coat protein precursors whose localization has been examined are stored in the PSV together with the Gal/GalNAc-PS, based on antibody colocalization and cell

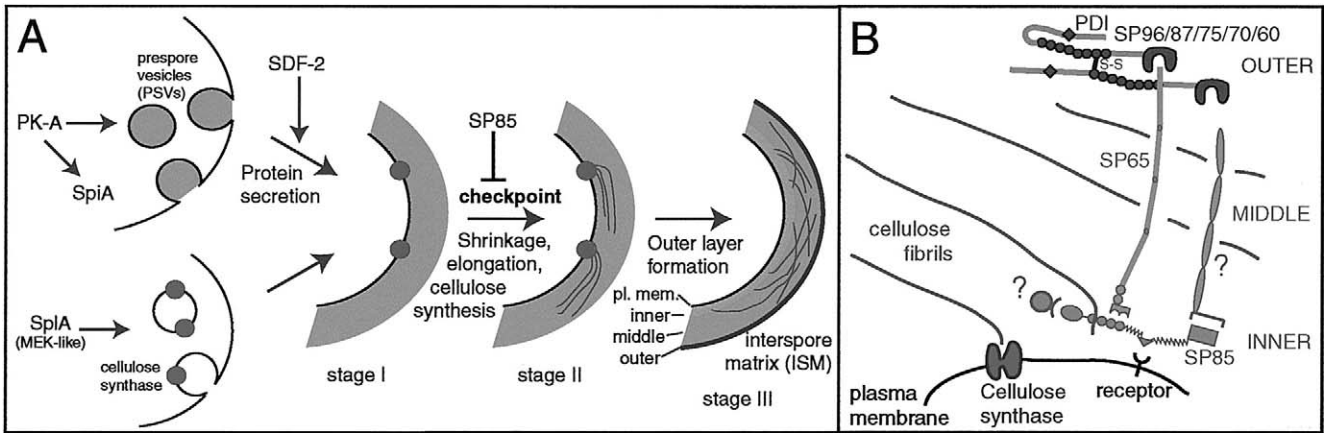

FIG. 4 Schematic model of spore coat assembly and structure. (A) Proposed assembly pathway. Delivery of the two major components of the coat, protein and cellulose, is proposed to be under the control of separate signaling pathways that depend upon PK-A (Loomis, 1998) and SplA (Nuckolls *et al.,* 1996), respectively. SDF-2 (Wang *et al.,* 1999) is suggested to initiate coat formation by stimulating exocytosis of future coat proteins from PSVs (upper arm), taking them to stage I. The pathway and timing of arrival of cellulose synthase (lower arm) to the plasma membrane are not known. Stage II, during which cells shrink and elongate, and cellulose is synthesized, depends on a checkpoint that is negatively regulated by SP85 (Adapted from West *et al.,* 2002b. Outside-in signaling of cellulose synthesis by a spore coat protein in *Dictyostelium. Euk. Cell* **1,** 281–293, with permission from American Society for Microbiology.). Deposition of cellulose results in condensation of proteins into the outer and inner layers (stage III). Locations of the inner, middle, and outer layers are shown. (B) Proposed organization of proteins and cellulose. As described in the text, SP96, SP87, SP75, SP70, and SP60 are associated with the outer layer where they comprise an electron-dense permeability barrier. The outer layer including SP96 depends on SP85, which resides in the inner layer and binds cellulose. This invokes the need for a bridging adaptor protein(s) that connects the outer and inner layers, which might be fulfilled by SP65, which also binds SP85. This molecular quartet SP96–SP65–SP85–cellulose is proposed to represent a core structural unit that helps to cross-bridge the three layers to each other at the plasma membrane. This might be related to an SP96–SP70–SP85–SP60 complex described in prespore cells (Section IV.A). Additional predicted binding partners for SP85 are denoted with a question mark. (See also color insert.)

fractionation studies (Devine *et al.,* 1983; West and Erdos, 1990). PSVs can be purified by density gradient purification and a proteomics study has confirmed the accumulation of coat protein precursors (Srinivasan *et al.,* 1999, 2001). PSVs are devoid of cellulose. Coat protein precursors are specifically and coordinately synthesized in prespore cells (Devine *et al.,* 1982; Wilkinson and Hames, 1983) and comprise up to 9% of the protein output of the cell at their peak of production (Wilkinson *et al.,* 1983). PSVs derive from the Golgi apparatus, which presents as a conventional cisternal stack in *Dictyostelium* during coat protein synthesis (Takemoto *et al.,* 1985). Their apparent number, protein cargo content, and density increase steadily until culmination (Srinivasan *et al.,* 1999).

PSVs also appear to be enriched in lysosomal enzyme proteins based on biochemical and microscopic studies (Lenhard *et al.,* 1989a,b). Pulse-chase experiments suggest that at least some of these proteins derive from preformed lysosomes, but PSVs do not seem to be functional lysosomes because they are not acidic relative to the cytoplasm (West and Erdos, 1990). The presence of lysosomal enzymes in the inner layer of the spore coat (Lenhard *et al.,* 1989b) and in the ISM (Seshadri *et al.,* 1986) is consistent with derivation from PSVs, though the significance of this localization is not known.

PSVs appear as discrete vesicles when conventionally prepared thin sections are examined in the electron microscope (Hohl and Hamamoto, 1969), and when prespore cells are visualized by immunofluorescence using antibodies or lectins that recognize coat precursors (Gomer *et al.,* 1986; Gonzales-Yanes *et al.,* 1989). In the EM, their contents appear heterogeneous with a layer of electron-dense material often contracted away from the inner surface of the vesicle membrane (Fig. 2A), and immunogold localization studies suggest that individual epitopes are clustered in separate domains of the same vesicle (Erdos and West, 1989), as also observed in vesicles containing multiple vitelline envelope/chorion precursor in *Drosophila* follicle cells (Trougakos *et al.,* 2001). However, when cells are prepared by freeze-substitution, which is generally thought to yield superior preservation (Wesley-Smith, 2001), PSVs appear to have a homogeneous composition with only a lightly contrasted layer closely associated with the vesicle membrane (Fig. 2A). This is more consistent with evidence that at least half of the coat protein is soluble after prespore cell lysis (West *et al.,* 1996) and associated with a soluble precursor complex (Watson *et al.,* 1993). Considering that PSV contents are likely to be highly concentrated and dehydrated following emergence form the Golgi, the irregular rim of material and other electron densities seen in conventional but not freeze-substituted material may represent artifactual rearrangement and precipitation.

Examination of serial sections of freeze-substituted preparations suggests that PSVs are not independent, but interconnected by tubular extensions to form a network (West and Erdos, 1990). A similar organization was also visualized by fluorescein isothiocyanate (FITC)-RCA-I labeling of the Gal/GalNAc-PS using confocal microscopy. This PSV network is not unlike the membrane systems

associated with the contractile vacuole, endosomes, and peroxisomes in this and other organisms. Conventional fixation might lead to artifactual fragmentation of the membrane system into round vesicles, and the PSV system might be most accurately described as a tubular membrane system with vesicular swellings.

2. Exocytosis

The contents of PSVs are quantitatively secreted at sporulation resulting in their disappearance from spores (Hohl and Hamamoto, 1969), which constitutes stage I of coat formation. Thus the PSV is comparable to the regulated secretory compartments of animal endocrine and exocrine cells (Burgess and Kelly, 1987; Srinivasan *et al.,* 2000a). Secretion involves a fusion event between the membrane of the PSV and the plasma membrane, although the tubulovesicular morphology of the PSV described in Section V.A.1. suggests that exocytosis may not necessarily involve transfer of a large area of membrane. An M_r 22,000 G-protein found in the PSV membrane fraction may be involved in membrane recognition during exocytosis (Srinivasan *et al.,* 1999, 2001).

PSV exocytosis appears to initiate coat formation based on four lines of evidence. Examination of developing spores in the transmission electron microscope (TEM) reveals individual cells that have exocytosed their PSVs but not yet acquired the smooth contours of fully dehydrated spores. In these cells, the future coat proteins localize amorphously at the cell surface but cellulose and the electron-dense layers are absent (West and Erdos, 1990). In another study, flow cytometry identified a subpopulation of small prespore cells that expresses both plasma membrane antigens and a coat protein epitope (Browne *et al.,* 1989). This population may represent the same intermediate seen by electron microscopy. Third, analysis of an SP45–GFP fusion protein in *P. pallidum* indicates that this coat protein is secreted before cells shrink, elongate, and produce cellulose (Gregg and Cox, 2000). Finally, coat proteins accumulate amorphously at the cell surface in a cellulose-null mutant (see next section), showing that secretion does not require cellulose deposition.

As prespore cells prepare for sporulation they rise up the forming stalk in a process that appears to require locomotion based on a cell-autonomous requirement for myosin regulatory light chain (Chen *et al.,* 1998). However, coat formation appears to be initiated before cells reach the stalk apex and there is evidence that anterior-like prestalk cells that have sorted out on either end of the prespore cell group generate force through their movements to complete the elevation of the spores (Sternfeld, 1998). The last known gene to be induced in prespore cells, *spiA,* is induced in a temporal–spatial gradient initiated at the apical end of the rising prespore cell mass prior to coat formation (Richardson *et al.,* 1995). SDF-2, a peptide secreted from prestalk cells that might activate a sensor histidine kinase receptor on prespore cells (Wang *et al.,* 1999), is the latest signal known. Cellulose is synthesized in dissociated cells within 30 min of the application of SDF-2 without a requirement for protein synthesis. SDF-2 appears to inhibit cAMP

phosphodiesterase thereby activating protein kinase A, which is known to regulate spore differentiation in a cell-autonomous fashion (Loomis, 1998).

PSV exocytosis is greatly delayed in *dcsA*-null strains (Zhang *et al.,* 2001), which might be due to the inability of prespore cells to reach the apex of the mechanically compromised stalk (Blanton *et al.,* 2000) to receive a signal. The delay can be overcome by mixing cellulose-negative with normal cells or treating mutant cell suspensions with 8-Br-cAMP, indicating that mutant cells fail to signal sporulation upstream of protein kinase A activation. Although the role of cellulose might be physical, it may also influence SDF-2 localization like animal ECMs control presentation of growth factors (Section II.C).

3. Protein Precoat

PSV exocytosis is proposed to result in the formation of a protein precoat as an intermediate stage in coat assembly. *dcsA*-null cells that are induced to sporulate in timely fashion form cellulose-negative pseudospores (Fig. 1D) that, based on immunofluorescence localization, have exocytosed their PSVs (Zhang *et al.,* 2001). These pseudospores retain at least half of their secreted coat proteins at their cell surface, even in shaken suspension, but this material does not organize into anything but amorphous electron-dense material. This association seems to depend on an active, postsecretion mechanism because in normal development, several secreted proteins, including SP96, SP80, and SP75, appear to exchange between neighboring spores based on flow cytometry studies on interstrain chimeras (West and Erdos, 1992). This non-cell-autonomous mechanism of protein incorporation is reminiscent of examples from animal cell walls (Section II.C), and suggests the involvement of postsecretion binding events among soluble precursors or complexes. These probably occur early prior to cellulose deposition, as coats form normally on solitary cells sporulating in suspension, and the subsequent incorporation of cellulose is cell autonomous (Zhang *et al.,* 2001). In contrast, SP86 appears to be retained on the surface of its cell of origin, and might play a special role in initiating coat assembly (West and Erdos, 1992).

The residual coat of pseudospores may be an intermediate in normal coat assembly. As a precoat, it may help to deadhere prespore cells from one another, and may subsequently provide a favorable microenvironment for the crystallization of nascent cellulose. The appearance of a precoat prior to later deposition of cellulose is similar to what is seen in growing tips of the mold *Achlya* and plant pollen tubes (Section II.B).

An early EM study identified electron-dense structures at the early spore surface prior to the deposition of cellulose (Hohl and Hamamoto, 1969), which was suggested to be similar to material associated with the inner membrane leaflet of the PSV. This led to a model in which the outer coat layer is derived from preassembled vesicle-derived coat precursor fragments, as is believed to occur in siliceous walls of sarcodinian protozoans (Anderson, 1994). However, the proposed precursor structures were not seen in PSVs from freeze-substituted

preparations (Section V.A), and are difficult to reconcile with more recent evidence that incorporation of coat proteins is not cell autonomous (West and Erdos, 1992). Further study is required to determine the significance of the originally reported cell surface structures, which are not commonly seen.

Comparisons of proteins from prespore vesicles and spore coats by two-dimensional gel electrophoresis indicate that the apparent M_r and p*I* values of most of the major coat proteins do not change during secretion. However, one protein, p58, appears to be cleaved into an M_r 35,000 form referred to as SP35 (Section III.B.3.b.iv). Cleavage may expose SP35's intrinsic cellulose-binding activity, as p58 was not detected as a cellulose-binding protein in an assay of prespore cell extracts (McGuire and Alexander, 1996). Subsequent reorganization of the precoat into electron-dense layers appears to depend on the appearance of cellulose.

4. Intermediate events

Spores appear to achieve their final form before the cellulose is deposited. Microscopic observations on sporulating *dcsA*-null cells show that they transiently acquire an elongate, smooth-surfaced, refractile appearance typical of mature spores (Zhang *et al.,* 2001). However, within less than a half hour these pseudospores become spherical (Fig. 1D), suggesting that subsequent cellulose deposition is necessary to maintain their elongate morphology. Because certain microtubule mutants also have a round spore shape phenotype (Welker and Williams, 1983), the shape of spores might be generated by the cytoskeleton and then stabilized by cellulose. Pseudospores are sensitive to lysis in hypoosmotic medium, unlike prespore cells, indicating that they also down-regulate their osmoregulatory pathways during sporulation.

5. Deposition of Cellulose

Cellulose is synthesized after the appearance of the protein precoat by the cellulose synthase complex embedded in the plasma membrane (Section III.B.1). Application of the fluorescent brightener Calcofluor to sporulating cells suggests that cellulose deposition is initiated at one pole, and therefore advances as a wave across the cell (Zhang *et al.,* 2001). Cellulose synthesis appears to be very rapid and synchronous throughout the sorus, as it is unusual to find cells with partial cellulose walls or to see heterogeneity in the walls of different cells.

Sporulation in a strain that is disrupted in *splA,* a gene encoding an MEK-like kinase, proceeds up to the point of cellulose deposition, which occurs to only a partial extent in a fraction of the cells (Nuckolls *et al.,* 1996; West, unpublished data). Thus SplA appears to regulate the cellulose synthesis arm of coat assembly (see Fig. 4A) but, since mutant spore plasma membranes also become leaky to propidium iodide, it is not known if the effect on cellulose synthesis is direct or indirect.

Cellulose synthesis appears to be regulated by a checkpoint mechanism. The existence of a checkpoint was initially suggested by the observation that when

the NC1 domain fusion of SP85 is expressed, cells exocytose their PSVs but do not synthesize cellulose (West *et al.,* 2002b), as seen in *dcsA*- and *splA*-null cells. The effect of the NC1 fusion is non-cell autonomous in interstrain chimeras, suggesting it acts at the cell surface. SP85-null spores present an opposite phenotype in which spores differentiate prematurely before reaching the top of the stalk, leaving them half-mast, and cellulose is slightly overproduced in a disorganized fashion based on sensitivity to acid hydrolysis. Examination of individual sori suggests that in the absence of SP85, cellulose synthesis is desynchronized among neighboring cells, and is not polarized within the individual cell (West, unpublished data). The round shape of SP85-null spores (Fig. 1E) suggests that cellulose deposition is not properly coordinated with other events of sporulation including cell elongation. Expression of the NC2 domain fusion has an effect similar to SP85 deletion. In a double mutant, absence of SP85 is epistatic to NC1 expression suggesting that NC1 inhibits SP85 instead of expressing a novel function.

Together, these results suggest the existence of an outside-in signaling pathway that constitutes a checkpoint to ensure that cellulose synthesis does not occur until coat proteins are properly organized at the cell surface and early sporulation steps are complete. Checkpoint execution is proposed to be negatively regulated by SP85, which is in turn under the negative and positive influence of contacts with other coat proteins that are mediated via SP85's C1 and C2 domains and competed, respectively, by the presence of NC1 and NC2. This model predicts the existence of a checkpoint sensor in the plasma membrane, which remains to be identified. Site-specific mutatgenesis of the C1 domain suggests that each of the C4C motifs contributes to the function of NC1 in interfering with checkpoint execution (Metcalf *et al.,* 2002). Recent evidence suggests that the sensor may control turnover of a critical regulatory protein, because overexpression of two components of an $E3^{SCF}$-Ubiquitin ligase, Skp1 or cullin-A, yield phenotypes that resemble SP85-null cells with respect to stranding spores half-mast up the stalk and round spore shape. $E3^{SCF}$-Ubiquitin ligases mediate the degradation of many important regulators of the cell cycle, development, and nutrient responses in cells (West, 2002; Mohanty *et al.,* 2001).

The checkpoint is probably involved in the regulation of all stage II events including, in addition to cellulose synthesis, conversion of the amoeboid stage I cell to a small, elongated, refractile cell (Fig. 4A). This is based on the effect of expressing NC1 in submerged *dcsA*-null cells induced to sporulate with 8-Br-cAMP (Zhang *et al.,* 2001). Not only is pseudospore formation blocked but the cells also remain amoeboid, suggesting that they are also unable to expel water and acquire smooth surface contours.

6. Formation of the Inner and Outer Layers

The electron-dense inner and outer layers do not appear until after the initiation of cellulose deposition (West and Erdos, 1990; Zhang *et al.,* 2001), which is

therefore defined as stage III. During stage III, the protein and Gal/GalNAc-PS components thought to be intermixed in the precoat appear to segregate yielding the polarized distribution observed in the finished coat. This may be mediated via specific protein domains as suggested by studies of the *Drosophila* vitelline envelope/chorion, and the *Caenorhabditis* basement membrane (Section II.C). The role of cellulose is presumably mediated by cellulose-binding domains in certain coat proteins (Section IV).

The outer layer contains SP96, SP87, SP75, SP70, and SP60 (Table I). These appear to be the major components as the electron density of the outer layer is greatly diminished in a mutant lacking SP96, SP70, and SP60 (Metcalf *et al.,* 2002). In this mutant, the normally continuous outer layer is replaced by a lattice of electron densities with a spacing of about 35 nm, which might represent an uncompleted assembly intermediate. A speculative model is that these electron densities represent nucleation centers contributed by trans-coat adaptors from the inner layer, related to radial fibers in the *Chlamydomonas* cell wall (Section II.B). The colocation of outer layer proteins at the coat surface suggests that they interact both with one another and the underlying cellulose layer. Interaction with cellulose may be mediated by SP85, as SP85-null coats also have incomplete outer layers. However, a contribution by the other known cellulose-binding protein, SP35, is possible. If outer layer proteins contact cellulose via SP85 then it is likely that adaptor proteins are involved, as SP85 is located in the inner layer and is not long enough to traverse the middle layer (see Fig. 4B). A candidate adaptor protein is SP65, which is part of the SP96–SP65–SP85–cellulose structural core model proposed above (Section IV.C). If SP85 is associated with the cell surface as suggested by its inner layer localization and anticheckpoint activity, then the proposed adaptor proteins may define the thickness of the coat under tension from the thickening mat of intervening cellulose fibrils.

Proteins of the outer layer are disulfide cross-linked (Section III.B.3.b.v). Considering the non-cell-autonomous mechanism of protein incorporation, cross-linkage is likely to occur after secretion as occurs for minicollagen during assembly of the *Hydra* nematocyst capsule wall (Engel *et al.,* 2001). This activity might be contributed by some of the proteins themselves (Section III.B.3.b.v), or by thioredoxins detected recently in PSVs (Srinivasan *et al.,* 2001).

SP85 and the Gal/GalNAc-PS are associated with the inner layer. SP85 might mediate adhesion of the coat to the plasma membrane via an integral plasma membrane protein such as WGA80B, SpiA, SP29, or LagC/gp150 (Section IV.A). As described above, SP85 appears to contribute a cross-bridging function to anchor outer layer proteins. Consistent with this model, expression of the NC2 domain fusion and other SP85 fragments also interferes with formation of the outer layer both functionally (Zhang *et al.,* 1999) and structurally (Metcalf *et al.,* 2002), suggesting that other SP85 domains contribute critical binding activities to this cross-bridge that are competed by the fragments. In addition, expression of the NC1 domain fusion causes replacement of the smooth outer layer by a dense

"thicket" of outward-radiating, noodle-like projections that contain outer layer proteins based on immunoelectron microscopy (Metcalf *et al.,* 2002). This effect is separable from NC1's inhibitory effect on checkpoint execution based on site-specific mutagenesis of NC1. Although the composition of the "noodles" is not known, their induction by NC1 attests to a major organizing role for the C1 domain of SP85 in coat morphogenesis.

7. Formation of the Interspore Matrix (ISM)

The ISM occupies the space between mature spores (West and Erdos, 1990), where it provides a desiccation-resistant fluid in which spores are dispersed as single cells, and a solvent for inhibitors and activators of germination (Section VI).

Most coat proteins and the Gal/GalNAc-PS are detected in the ISM based on Western blot analysis and immunoelectron microscopy of sori fixed in the presence of cetylpyridinium chloride to precipitate polyanions (West and Erdos, 1988). Although coat proteins are the most abundant proteins of the ISM fraction from wild-type cells, they are a small fraction of the amount in the coat, suggesting that the ISM is probably the default destination for unincorporated proteins (see Section IV.C). In the ISM they may contribute to the fitness of the sorus as receptor decoys for potential pathogens to compete attachment to spores or germinating amoebae. In addition, the Gal/GalNAc-PS may provide viscosity and hydration to the ISM.

Other vesicles also appear to be exocytosed at sporulation. This includes contents of the Golgi, as exemplified by the UDPgalactose polysaccharide transferase (Sussman and Lovgren, 1965), lysosomes (Seshadri *et al.,* 1986), and vesicles marked by a protease (North *et al.,* 1990) and glycoantigens XXX and XXXII (West and Erdos, 1988), also possibly lysosomal (West and Erdos, 1990). The protein concentration of the ISM fraction from axenic mutants is higher than that of wild-type cells (West *et al.,* 1996). There is evidence that lysosomal enzymes are secreted via intermediate fusion with the PSV in axenic cells (Lenhard *et al.,* 1989a,b), though a lysosomal enzyme was not detected in maturing PSVs of wild-type cells (Srinivasan *et al.,* 1999). Global exocytosis may explain how the C-terminal domain of SP85 could incorporate into the coat despite not being targeted to the PSV (Zhang *et al.,* 1999). Global exocytosis may relieve the future germinated amoeba of developmental proteins not required for future growth.

B. Functional Properties

The coat contributes uniquely to spore functionality by protecting the dehydrated protoplast from environmental extremes including hypo- and hyperosmotic stress, desiccation, enzymatic digestion, detergents, toxins, and heat. For example, spores

are resistant to passage through the digestive tracts of migratory birds (Suthers, 1985) and nematodes (Kessin *et al.,* 1996). The coat is, however, permeable to water and small regulators of germination (Section VI). As reviewed above, genetic analysis shows that cellulose, proteins, and carbohydrate modifications on the proteins each contributes to spore fitness.

The mat of cellulose fibrils in the middle layer contributes the tensile strength that physically constrains the spore and probably preserves the elongate spore shape. As a result, the spore is able to down-regulate its osmoregulatory apparatus (Zhang *et al.,* 2001). However, the cellulose layer is unlikely to contribute significantly to the permeability barrier of the coat based on information from plant walls.

The macromolecular permeability barrier is the feature that most distinguishes the spore coat from other cell walls. It appears to be mainly mediated by the electron-dense, proteinaceous outer layer. Extraction of spores with urea/MSH increases labeling of spores with FITC-RCA-120, a lectin that binds the Gal/GalNAc-PS localized to the inner layer (Gonzalez-Yanes *et al.,* 1989). The increased labeling, which can be quantitated by flow cytometry (Zhang *et al.,* 1999), suggests that protein contributes to the permeability barrier, as cellulose and the Gal/GalNAc-PS are not extracted by this treatment (Zhang *et al.,* 1998). Each of the outer layer proteins SP96, SP75, SP70, and SP60 contributes to the permeability barrier, as mutant spores lacking these proteins exhibit greater accessibility of probes to both the Gal/GalNAc-PS (Fosnaugh *et al.,* 1995; West *et al.,* 1996) and SP85 (Srinivasan *et al.,* 2000b). The increased permeability of some pairwise combinations of gene deletions suggests that their contributions are additive. Increased permeability correlates at least in part with decreased viability during spore aging (Srinivasan *et al.,* 2000b), suggesting that macromolecular impermeability is important for coat function. Glycosylation of coat proteins is also important for the permeability barrier. Mutants that are deficient in fucosylation or *modB*-dependent *O*-glycosylation (Section III.B.3.b.i) exhibit both increased permeability to FITC-RCA-120 and aging defects (Gonzalez-Yanes *et al.,* 1989; Aparicio *et al.,* 1990). Since the underglycosylated proteins still accumulate at normal levels, glycosylation might stabilize the conformation of the mucin domains or neighboring globular domains, as described for an animal ECM protein (Loomes *et al.,* 1999). Alternatively, the missing glycan might provide an essential ligand for an unknown receptor similar to the role of α-dystroglycan glycans for laminin in animal basement membranes (Section II.C).

Like the outer layer, the inner layer and plasma membrane also contribute to the outer layer permeability barrier and spore stability based on analyses of mutants lacking SP85 (Zhang *et al.,* 1999) or the transmembrane protein SpiA (Richardson and Loomis, 1992), respectively. As described in Sections IV.A and V.A.6, this can be explained by proposed roles for these proteins in outer layer assembly (Fig. 4B). In addition, SP85 appears to contribute to the existence of an internal barrier. This is suggested by the more rapid germination of SP85-null spores both *in vitro* (Zhang *et al.,* 1999) and in the presence of bacteria. In the absence of SP85,

the coat may be more readily attacked by secreted cellulases and proteases that break down the coat from within (see Section VI.B). SP85 may function by cross-bridging as expression of specific fragments of SP85 also shorten germination time.

VI. Germination of *Dictyostelium* Spores

An important aspect of coat biology is its breakdown to allow emergence of the enclosed amoeba during germination. Three stages of germination can be resolved: (1) initial activation involving new gene expression, (2) swelling, in which the cell and its coat enlarge and assume an irregular shape but maintain a smooth but less refractile profile, and (3) emergence from the coat (Cotter and Raper, 1968).

A. Activation and Swelling

Activation is regulated by the opposing influences of activators and inhibitors. For 3 weeks spores are unable to germinate without activation whereas older spores may spontaneously germinate in a simple salts buffer (Cotter *et al.,* 2000). Resistance of young spores to germination is mutable but the genes have not yet been identified. High NH_3 concentration and hyperosmotic media, conditions found in the sorus (Cotter *et al.,* 1999), inhibit germination. A small diffusible nucleoside, discadenine (Abe *et al.,* 1981), may be a natural inhibitor of germination (or inducer of dormancy). The DhkB histidine kinase is required for dormancy and this signal transducer may sense discadenine (Zinda and Singleton, 1998). DhkB may promote high intracellular cAMP, as mutation of a spore-specific adenylate cyclase results in spontaneous germination (Virdy *et al.,* 1999). A normal spore coat also appears to be necessary for dormancy, as young SP85-null spores spontaneously germinate in simple salts buffer (West *et al.,* 2002b).

Younger spores can be activated under harsh conditions such as urea, high temperature, detergents, or organic solvents, which have been proposed to denature a hypothetical sensor protein (Cotter, 1981). There is also evidence for low-molecular-weight diffusible activators of germination. These may act via inositol 1,4,5-trisphosphate (IP_3), which can induce activation in the absence of other signals (Van Dijken and Van Haastert, 2002).

During the activation phase, which spans several hours, many new genes are expressed and some of these, including cellulases (see below), are transient (see Cotter *et al.,* 2000). Gene expression during this interval is essential based on the use of inhibitors (Kelly *et al.,* 1987). Germination appears to be related to dedifferentiation of prespore cells when they are exposed to a food source based on similar patterns of gene expression (Chandrasekhar *et al.,* 1992).

The first morphological event of germination is swelling, which is a localized expansion of the coat and enclosed cell as a lobe-like structure. Calmodulin appears to be involved based on the effects of calmodulin inhibitors (Lydan and Cotter, 1994). Swelling is reversible, which is not easy to reconcile with the expected tensile strength of cellulose fibrils. Possibly catalytic remodeling, such as that mediated by expansins in plant cell wall growth (Cosgrove, 1999), is involved in reversible shape change.

B. Coat Breakdown and Emergence

The activated amoeba exits the coat along a longitudinal fracture. No landmarks anticipating the location of the fracture zone have been found. Thus fracture is likely to occur cataclysmically as turgor pressure generated by the amoeba exceeds the burst threshold of the coat after it has been generally weakened by the combined actions of cellulases and proteases. The outer two layers, which include cellulose, provide the greatest resistance as they break first followed later by the more compliant inner layer (Cotter *et al.,* 1969).

Biochemical assays have detected cellulolytic activity in germinating spores and their associated interspore matrix fraction. Less activity was detected in association with dormant spores (Roseness, 1968; Jones *et al.,* 1979), which might be explained by an inhibitor in sori that disappears during germination (Jones and Gupta, 1981). However, a gene (*celA*) has been cloned that encodes a protein with homology to a plant endoglucanase, and its expression is germination specific. Expression of CelA in vegetative (growing) amoebae yielded an endoglucanase activity against noncrystalline (amorphous) but not crystalline cellulose *in vitro* (Blume and Ennis, 1991; Ramalingam *et al.* 1992). As found for other endoglucanases, CelA possesses a classic type 1 bacteria-like cellulose-binding domain attached to the C-terminus of the catalytic domain via a 100-amino acid-long, mucin-like region composed of glycosylated tetrapeptide (TETP) repeats. A second protein with a similar architecture (CelB), including a C-terminal mucin-like and a bacterial cellulose-binding domain, is coordinately regulated with CelA, but no cellulolytic activity could be detected (Ramalingam and Ennis, 1997). Although expected to be localized with cellulose *in vivo,* CelB might degrade another polysaccharide like the Gal/GalNAc-PS. An endoglucanase capable of hydrolyzing crystalline coat cellulose remains to be described.

Cellulose breakdown requires, in addition to endoglucanases, an exocellobiohydrolase to remove glucose disaccharides from the exposed ends generated by the endoglucanase, and a β-glucosidase to cleave cellobiose into its component glucose residues. The third enzyme is likely to be encoded by the *bglA* locus. *bglA* encodes both β-glucosidase-1, expressed during growth, and β-glucosidase-2, expressed in prespore cells (Golumbeski and Dimond, 1987). Both enzymes exhibit activity against cellobiose. β-glucosidase-2 is thought to be lysosomal in late

development but its pH activity profile is more consistent with an extracellular function. Interestingly, β-glucosidase-2 binds cellulose *in vitro* (Section IV.B), which might explain why the enzyme remains associated with the spore during germination (Chan and Cotter, 1982).

Proteolytic activity is also associated with germinating spores and may contribute to coat weakening. Cysteine proteases have been detected in extracts (Cavallo *et al.,* 1999), and germination-specific cleavage of specific coat proteins has been observed in glycosylation mutants (Gonzales-Yanes *et al.,* 1989; Aparicio *et al.,* 1990). Proteolytic action must, however, be selective, as major changes in coat protein profiles are not seen by SDS–PAGE comparisons of pre- and postgermination spore coats (Zhang *et al.,* 1998). Proteolysis is essential for germination in other organisms, such as excystation of the primitive eukaryote *Giardia* (Ward *et al.,* 1997). Reduction of disulfide cross-links may also occur but has not been investigated.

VII. Comparison with Other *Dictyostelium* ECMs

When *Dictyostelium* cells aggregate to form tipped mounds, the surface cells begin to secrete a cellulosic slime sheath that drapes over the aggregate. The sheath probably helps keep cells organized as a single unit and protects them from desiccation and predation (Kessin *et al.,* 1996), and may provide a barrier to the diffusion of morphogens (Loomis, 1972). As the aggregate elongates and then bends over and adsorbs to a surface, migration occurs within the continuously formed slime sheath that trails behind the slug (Wilkins and Williams, 1995). Cell outlines can be visualized on the sheath using mAbs against cell surface and ECM glycoproteins, Calcofluor (a cellulose dye), and Nessler's reagent (Feit, 1994). These outlines have been interpreted as "footprint" modifications of the sheath resulting from cell traction (Vardy *et al.,* 1986), although they may actually represent cell remnants (McRobbie *et al.,* 1988). At culmination, migration ceases and the slug becomes vertical and "squats" down. The central, primary prestalk cells begin to elaborate a nascent stalk tube around themselves, and continue this process as they migrate basally to contact the basal group of prestalk cells. The stalk tube is extended apically by the remaining, secondary prestalk cells that contribute to its outer layer, and then enter its open end to contribute to its inner surface and subsequently differentiate into vacuolated stalk cells. Differentiating stalk cells surround themselves with a cell wall that is shared between neighbors or with the inner layer of the stalk tube. Isolated prestalk cells can still form walls showing that the process is cell autonomous as for spores (Grimson *et al.,* 1996).

As an alternative to fruiting bodies, macrocysts are formed from mixtures of opposite mating types under certain conditions. A dominant cell within the aggregate phagocytoses the others followed by recombination of the various DNAs.

The macrocyst cell wall is derived from the original slime sheath-like layer that surrounded the aggregate (primary wall) and subsequent layers (secondary and tertiary walls) that are added from within (Erdos *et al.,* 1973). Finally, amoebae from some cellular slime mold species can bypass cell aggregation to directly form spore-like microcysts, which are surrounded by cell walls (Hohl *et al.,* 1970). Thus walls are formed around all differentiating cells or groups of cells in this organism. Although all the ECMs contain cellulose (Harrington and Raper, 1968), each is distinctive in its ultrastructural, physical, and functional properties, and contains distinctive proteins.

A. Slime Sheath

The slime sheath exhibits variable thickness in the electron microscope but is on average thinner than the spore coat (Loomis, 1975). At its outer surface lies an electron-dense layer that appears similar to the outer layer of the spore coat (Section III.A) based on its trilaminar, unit-membrane-like structure and thickness (George *et al.,* 1972). After boiling in urea and SDS, the slime sheath residue consists of 60% cellulose, 15% protein, 3% heteropolysaccharide, 5% lipid, and no detectable PO_4 (Freeze and Loomis, 1977). Cellulose is estimated to be 84% crystalline, consistent with the presence of fibrils seen in the EM (Hohl and Jehli, 1973). Fibril diameter is heterogeneous, with diameters clustering around 2.7, 4.0, and 5.9 nm (Grimson and Blanton, unpublished data), based on examination of negatively stained material. Cellulose crystallinity increases and heteropolysaccharide composition varies as the sheath matures. Cellulose is not essential for sheath formation (Blanton *et al.,* 2000) and the presence of residual metachromatic Calcofluor White ST–positive material in *dcsA*-null sheaths (West, unpublished data) is consistent with the importance of other polysaccharides.

Slime sheath recovered from slug trails is at least 50% protein as determined by extraction with protein denaturants in the absence of reducing agents. Several proteins are found in the sheath and some of these are unique to this ECM. A group of proteins is released by digestion of the trails with cellulase, which led to the identification of the sheathins, cellulose-binding proteins with apparent M_r values of 30,000–40,000 that are also found in stalk tubes (Grant and Williams, 1983; Zhou-Chou *et al.,* 1995; Wang *et al.,* 2001). Additional proteins include EcmA, EcmB, AmpA (D11) protein, and the lagC protein (gp150). EcmA and EcmB are high M_r proteins (apparent values of 430,000 and 310,000) that are also found in the stalk tube and stalk cell wall (McRobbie *et al.,* 1988), with EcmA enriched in the sheath and EcmB enriched in the stalk cell wall. EcmA and EcmB are related structural proteins that consist exclusively of reiterated tandem repeats of a single Cys–rich motif about 26 residues in length, and are extensively *N*-glycosylated in contrast to the *modB*-dependent *O*-glycosylation of the sheathins. The Cys-spacing pattern, $CX_5CX_4CX_5CX_{6}$, is similar but not identical to that of the C4C

repeat of the coat proteins. AmpA has distinct Cys-rich motifs (Table II) (Varney *et al.,* 2002). Immunoelectron microscopy shows that the slime sheath, but not the stalk tube, is highly enriched in carbohydrate epitopes known as glycoantigens (GAs)-XXX, -XXX1, and -XXXII (West and Erdos, 1988). GA-XXXI, defined by mAb 40.1, is associated with complex, possibly glycolipid-like material (West *et al.,* 1986). This prestalk-specific product migrates as a cloud punctuated with a ladder of sharp bands in standard SDS–PAGE gels, and contains Fuc and PO_4 (based on metabolic labeling), and GlcNAc (based on recognition by wheat germ agglutinin), but not protein (based on insensitivity to Pronase digestion). It is highly acidic suggestive of phosphodiester-linked substituents. Immunoelectron microscopy also shows that the glycoantigen recognized by mAb 83.5, which is thought to be specific for coat proteins, can be detected in the posterior region of the slime sheath and stalk tube associated with prespore cells (West and Erdos, 1988), which might explain an early observation that the posterior slime sheath is enriched in GlcNAc. The possible importance of disulfide cross-linking of sheath proteins has not been investigated. Thus the general composition of the slime sheath is highly divergent from that of the spore coat.

These ECM proteins contribute distinct functions to the organism. Genetic analysis shows that EcmA is required for normal slug shape (Morrison *et al.,* 1994). *modB*-glycosylation mutant slugs have inhibited motility consistent with difficulty in forming the cellular footprints on the sheath (Zhou-Chou *et al.,* 1995). The *in vitro* cellulose-binding activity of sheathin D suggests that it and related sheathins might be important for organizing and interacting with cellulose (Wang *et al.,* 2001). Two plasma membrane proteins are up-regulated at the time of sheath formation and accumulate potentially soluble derivatives that immunofluorescence localization studies suggest might associate with the sheath. gp150(lagC) is a heterophilic cell–cell adhesion protein for slug cells (Wang *et al.,* 2000) and thus might be involved in slug–sheath contacts. AmpA/D11 is proposed to have anti-cell adhesion activity consistent with the Cys-rich disintegrin- and ornatin-like sequence motifs of which it is composed (Varney *et al.,* 2002). Both LagC and AmpA are candidates for influencing cell–sheath interactions.

B. Stalk Tube and Stalk Cell Walls

The stalk tube is several times thicker than the slime sheath or spore coat, and lacks the electron-dense lamina that characterizes the outer layers of the latter ECMs. Cellulose is an essential component of the stalk tube as it is unable to support culmination in a cellulose-null strain (Blanton *et al.,* 2000). The outer layer of the stalk tube contains longitudinally oriented cellulose fibrils, corresponding to the direction of movement of upwardly migrating external prestalk cells that deposit them, and an inner layer containing randomly oriented fibrils deposited by stationary differentiating stalk cells within the tube (George *et al.,* 1972). The

SDS/urea-insoluble fraction of stalks (including both tubes and walls) and slime sheath are very similar in their relative content of cellulose, residual protein, heteropolysaccharide, and fatty acids (Freeze and Loomis, 1978). EcmA and EcmB, discussed above, are also shared between the stalk and slime sheath. They appear to be secreted as they are synthesized by prestalk cells (George *et al.,* 1972). EcmA-null and EcmB-null cells form normal appearing stalks (Morrison *et al.,* 1994), but the double-null strain remains to be described. A small, 115-amino acid protein (St15) containing a single cellulose-binding domain is also found in the stalk tube (Wang *et al.,* 2001).

Monolayer cultures of prestalk cells can be induced to deposit cellulose in a fashion that appears to recapitulate stalk tube and stalk cell wall formation *in vivo* (Grimson *et al.,* 1996). Stalk tube cellulose microfibrils average 5.6 × 2.6 nm, with a subpopulation of 1.6-nm-wide microfibrils. The main population is large enough to exhibit crystalline packing, consistent with the resistance of stalk tube cellulose to acetic acid/nitric acid reagent. They are estimated to contain 36–48 glucan chains, and are thought to derive from linear arrangements of intramembranous particles within the plasma membrane, with each particle able to synthesize three or four glucan chains. These terminal complexes deposit the cellulose microfibrils as the prestalk cells migrate apically, and microfibrils can associate into bundles up to an average of 13 nm wide.

Several hours after induction, cells begin to form stalk cell wall. Cellulose microfibrils become wider, averaging 11 nm in width, and form larger bundles, up to an average of 29 nm (Grimson *et al.,* 1996). These changes correlate with the rearrangement of terminal complexes into patches containing larger numbers of particles. These terminal complexes appear to move in the plane of the membrane to accommodate the growing microfibrils as the cells are now stationary. Nothing is known about the terminal complexes of the developing spore but, because *Dictyostelium* contains only a single cellulose synthase gene (Blanton *et al.,* 2000), spore coat cellulose is expected to be synthesized by a similar complex.

C. Macrocyst and Microcyst Walls

The primary, secondary, and tertiary macrocyst walls are each as thick or thicker than the spore coat, and have cellulose distributed throughout (West and Erdos, 1990). Each wall is characterized by a distinct combination of glycoprotein-associated carbohydrate epitopes that are also found in the spore coat, and the Gal/GalNAc-PS appears to reside in the inner, tertiary wall. The localization of the epitopes within the layers is distinct from their arrangements in the spore coat. Further studies are required to address whether the antibodies recognize the same glycoprotein targets between the two ECMs.

Microcysts of the related cellular slime mold *Polysphonydylium pallidum* have walls that are slightly less thick than that of spores (Hohl *et al.,* 1970). However,

they lack the electron-dense inner and outer layers characteristic of spore coats, and may also have a capsule-like outer layer. Chemical analysis suggests that they are about 60% glucose polymer, about half of which is crystalline cellulose II, 25% protein, and 15% lipid, which might represent membrane (Toama and Raper, 1967). Synthesis of cellulose was correlated with the appearance of new plasma membrane proteins (Philippi and Parish, 1981) and additional intramembranous particles that were not clustered (Erdos and Hohl, 1980) as in the terminal complexes of prestalk cells (see above). Microcyst wall proteins have not been studied.

VIII. Implications for Future Studies

The multidimensional interactions of cell wall components make them fascinating but challenging to analyze. However, as illustrated here, the spore coat of *Dictyostelium* and other ECMs are yielding to reverse genetic analyses of both their polysaccharide and protein components. Although the proteins and polysaccharides of cell walls are remarkably diverse, overarching generalizations are emerging from a comparison of walls of plants, algae, protists, fungi, and animals. Proteins are mostly delivered via Golgi-dependent secretory pathways. Proteins are modular with specific binding functions mediated by discrete domains ranging in size from 25 to 200 amino acids. Domains are often Cys rich falling into numerous families based on their Cys spacings. These Cys residues form characteristic intramolecular and intermolecular disulfide bonds. Domains are linked together by mucin-like spacer domains such as those that link the catalytic domains of cellulases with cellulose-binding domains. Lectin-like receptors for polysaccharides and mucin oligosaccharides are found in walls with evidence that they not only cross-bridge but can also signal the associated cell. These activities can be uncovered by dominant negative domain expression approaches that add new information in addition to knockout studies.

Small branched-chain polysaccharides tend also to be produced by the secretory pathway, but the long carbohydrate polymers must be formed by plasma membrane-associated GT2 glycosyltransferases from sugar nucleotide precursors in the cytoplasm. After translocation to the cell surface they can assemble with proteins and the shorter polysaccharides, often resulting in reorganization into multiple layers. There is evidence for outside-in signaling to ensure proper coordination of secretion and polysaccharide synthesis. These assembly pathways are designed to work in the absence of a prior template or an opposing surface. Terminal covalent cross-links may stabilize walls but are probably not required for initial assembly.

Genetic strategies must usually be complemented by biochemical approaches to elucidate function. Remarkably, many wall proteins can be efficiently renatured following the denaturation steps that are required to extract them from wall and for

purification. Isolation of protein precursors prior to coat assembly offers promise that is tempered by evidence for protein processing concomitant with assembly.

Ultrastructural analyses provide a third approach to understand mechanisms. Although cell walls are remarkably stable structures, caution must still be applied to interpretation of images and localization data owing to problems of accessibility, retention of epitopes, and molecular rearrangements. Molecular rearrangements during dehydration can be countered by freeze-substitution or other cryoapproaches.

Recent work has revealed new mutant coat phenotypes that will stimulate new forward genetic searches to complement the earlier reverse genetic studies. Mutant gene identification will be greatly facilitated by REMI mutagenesis strategies, and the comprehensive data set of genomic and cDNA sequences now available for *Dictyostelium*. With the knowledge and tools now available in this model system, it is likely that the functions of coat protein domains can be deciphered in the forseeable future and ultimately provide invaluable insights about related domains in the walls of other organisms.

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