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Integration of Viral DNA into the Host Genome

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With 10 Figures

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But I am constant as the northern star,
Of whose true-fix'd and resting quality
There is no fellow in the firmament.

W. Shakespeare
Julius Caesar III, 1.

They have their exists and their entrances:
And one gene in its time plays many parts,

W. Shakespeare
As you like it II, 7,
(modified).

I. Introduction

Animal viruses can interact with their host cells in different ways: (1) The virus can actively replicate and finally destroy the cell in a lytic or productive infection. (2) The infection can become abortive or non-productive and lead to the apparent or inapparent persistence or the eventual loss of the viral genome. As a consequence of viral infection, the properties of the cell can be fundamentally altered and a malignantly transformed cell can arise. In such cells the viral genome persists and continues to be expressed. (3) Lastly, viral infections can remain latent for an extended time period and kill the host after many years. This latter type of virus-host interaction can best be followed in animals and has been termed "slow virus" infection (HOTCHIN, 1971; TER MEULEN et al., 1972; FUCCILLO et al., 1974). Only in recent years, has it been recognized that in several species viral genetic information can be transmitted from one generation to the next. These so called endogenous viral genomes have been extensively studied in avian and murine systems. Their role in the causation of neoplasia may be a crucial one, this role is, however, not understood.

This review will be restricted to viruses which have been shown to insert their DNA into the genomes of the host cells. This linear insertion by covalent phosphodiester bonds between the viral and host DNAs is called integration. The term integration in "sensu strictiori" should be used only according to this definition and should be differentiated from terms like association or persistence of viral DNA.

The best studied and most definitely established examples of integrated viral genomes are those of the temperate bacteriophages, e.g. lambda, P2,

P22, and Mu. Among animal viruses, integration of viral DNA was shown to occur in DNA and RNA tumor viruses. To account for the state of the viral genome in virus-transformed cells, the model of lysogeny was first proposed by LWOFF (1953). However, integration of animal virus DNA is not restricted to transformed cells, but is observed as well in productively and abortively infected cells. Perhaps integration is a general phenomenon in some of the animal viruses which contain DNA or replicate via a DNA intermediate. Recently, evidence has been presented (BURGER and DOERFLER, 1974; HIRAI and DEFENDI, 1974; HÖLZEL and SOKOL, 1974) that in cells productively infected with adenovirus or simian virus 40 (SV40) a number of viral genome equivalents are integrated into the host DNA. The significance of this recombination event between virus and host genes and its role in viral replication and in the expression of host genes are as yet unknown.

Since integrated viral genes become "truly fixed" to the genome of the host, the control of viral gene expression might become subject to the regulation by host genes or, vice versa, viral genes might influence the transcription of host functions. Even in the most extensively analyzed systems, viz. in bacteria lysogenic for temperate bacteriophages, very little is known about the effect of integrated viral genes on host functions. TAYLOR discovered in 1963 that infection by bacteriophage Mu caused mutations in Escherichia coli in those genes into which viral DNA was inserted. It will be interesting to investigate whether the integration of viral DNA can elicit mutations in eukaryotic cells as well. The model of integrated viral genes acting as mutators in eukaryotic cells might explain some of the genetically stable alterations in virus-transformed cells. However, a very detailed genetic analysis of both the host genes at the site(s) of integration and the integrated viral genes will be required to test the validity of the proposed analogy with phage Mu.

In this survey, the emphasis will be on the integration of the DNA of animal viruses. For obvious reasons, current research in this field has been most prolific with the oncogenic viruses. This review will also present selected topics dealing with lysogeny and the temperate bacteriophages λ and Mu. This juxtaposition of data on integration gathered in the prokaryotic and the less completely analyzed eukaryotic systems will perhaps help to improve our understanding of the basic mechanisms involved in integration. The most important questions which remain for future research both in the prokaryotic and eukaryotic systems concern the consequences of viral integration for the host cell. Is malignant transformation of the virus-infected eukaryotic cell one of these crucial consequences?

The early work on integration of animal virus DNA has been reviewed by TEMIN (1971); WINOCOUR (1971), and SAMBROOK (1972). These authors emphasized work on integration of the DNA of oncogenic viruses. More up-to-date discussions of the subject are found in The Molecular Biology of Tumour Viruses (edited by TOOZE, 1973) and in the 1974 Cold Spring Harbor Symposium on Tumor Viruses.

The survey of the literature which is summarized in the present paper was completed in September/October 1974.

II. Methods used to Demonstrate Integration of Viral Genomes

The main part of this paper will be prefaced by a brief summary of the methods currently employed in research on integration. In the work with bacteriophages, genetic methods were used very effectively to establish the integrated state of the viral genome. A genetic analysis of comparable sophistication has not yet been achieved for viral genomes integrated into the genomes of eurkaryotic cells. Therefore, it was necessary to develop biochemical and biophysical methods to demonstrate directly the covalent linkage between viral and host DNAs. Improved cytogenetic techniques using cell hybrids (RUDDLE, 1974) and chromosome banding techniques (CASPERSSON et al., 1970) are rapidly becoming available. These methods permit the localization of presumably virus-specific functions (e.g. the T-antigen) or the entire SV40 genome on specific chromosomes of SV40-transformed or SV40-infected cells (CROCE et al., 1973). Other investigators have succeeded in demonstrating chromosomal uncoiling at specific sites on one particular chromosome in human cells infected with adenoviruses (MCDOUGALL et al., 1973). The latter approach has not yet yielded conclusive results on the actual state of the viral genome.

In the following, the methods most frequently used to study integrated viral DNA are listed. Experimental details will be discussed in section III.

A. Work with Temperate Bacteriophages and Lysogenic Cells

1. Genetic Techniques

Determination of the permutation of the gene order in vegetative phage and prophage (CALEF and LICCIARDELLO, 1960). Analysis of cotransduction of bacterial and prophage markers (ROTHMAN, 1965; FRANKLIN et al., 1965). Genetic analysis of deletion mutants of phage which originated from faulty excision; genetic analysis of transducing phage genomes (ARBER, 1958; CAMPBELL, 1959; HOGNESS and SIMMONS, 1964).

2. Direct Electron Microscopic Measurement

Direct electron microscopic measurement of phage genes inserted into bacterial episomes (MARTUSCELLI et al., 1971).

3. Heteroduplex Mapping

Heteroduplex mapping of phage genes integrated into bacterial episomes and heteroduplex mapping of defective phage genomes (SIMON et al., 1971; FIANDT et al., 1971; HSU and DAVIDSON, 1972) (Fig. 5).

B. Experimental Approaches to the Analysis of Viral Genomes in Chromosomes of Eukaryotic Cells

1. Separation of Cellular and Viral DNA Under Conditions that Denature DNA

a) Zone sedimentation in alkaline sucrose density gradients (SAMBROOK et al., 1968; DOERFLER, 1968; 1969; BURLINGHAM and DOERFLER, 1971; HIRAI and DEFENDI, 1971; BURGER and DOERFLER, 1974).

b) Equilibrium sedimentation in alkaline CsCl density gradients (DOERFLER, 1968; 1970; BURGER and DOERFLER, 1974).

2. "Network Technique"

"Network technique" (BRITTEN and KOHNE, 1968): Cellular DNA is denatured and subsequently reannealed without prior fragmentation. Reannealing of the highly repetitive sequences leads to complex formation. The complexes contain cellular and integrated viral DNA, but not appreciable amounts of free viral DNA and can be isolated by low speed centrifugation (VARMUS et al., 1973b).

3. The Integrated Viral Sequences in Cellular DNA

The integrated viral sequences in cellular DNA can be determined by DNA-DNA or DNA-RNA filter hybridization using viral DNA or RNA (c-RNA) synthesized in vitro on a viral DNA template. For the exact quantitation of the number of integrated viral genome equivalents per cell, the measurement of reassociation kinetics (BRITTEN and KOHNE, 1968; WETMUR and DAVIDSON, 1968) has proved useful.

4. Cotranscription of Integrated Viral Genomes

Integrated viral genomes can be cotranscribed with cellular genes. Such "mixed transcripts" are isolated on polyacrylamide gels which contain formamide to avoid the formation of unspecific RNA aggregates. High molecular weight RNA molecules whose size exceeds that of unit length viral RNA can be shown to contain viral and cellular sequences. This RNA is hybridized first to viral DNA on filters (LINDBERG and DARNELL, 1970; ACHESON et al., 1971), and subsequently is eluted from the filters and hybridized to cellular DNA in the second step of the reaction (WALL and DARNELL, 1971; ROZENBLATT and WINOCOUR, 1972). It has also been shown that a large proportion of the high molecular weight RNA hybridized to SV40 DNA remains sensitive to RNase. This result has been interpreted to indicate that the SV40-specific, high molecular weight RNA contains cellular sequences (JAENISCH, 1972).

5. Excision of Integrated Viral Genomes

Excision of integrated viral genomes from cellular DNA with the aid of restriction enzymes (BOTCHAN and MCKENNA, 1973). Analysis of substituted viral genomes (see 6.) by cleavage with restriction enzymes and separation of

the fragments on agarose or polyacrylamide gels (BROCKMAN et.al., 1973; ROZENBLATT et al., 1973).

6. Demonstration of "Substituted Viral Genomes"

Demonstration of "substituted viral genomes" in SV40 and polyoma virions which presumably originate from faulty excision of integrated viral DNA (compare defective phage genomes). Substituted SV40 genomes are usually shorter than normal viral DNA and contain increasing lengths of cellular DNA depending on the number of high multiplicity passages of the virus (LAVI and WINOCOUR, 1972; BROCKMAN et al., 1973; LAVI et al., 1973; ROZENBLATT et al., 1973; LAVI and WINOCOUR, 1974).

7. Heteroduplex Mapping

Heteroduplex mapping has been used mainly in the analysis of substituted viral genomes of SV40 and the SV40-adenovirus hybrids.

8. In situ Hybridization

In situ hybridization (GALL and PARDUE, 1969). Metaphase preparations of chromosomes from virus-transformed or virus-infected cells are treated with alkali to "denature" the DNA, and virus-specific sequences are visualized by hybridization with c-RNA of high specific radioactivity and subsequent autoradiography. The c-RNA is synthesized in vitro on a viral DNA template with DNA-dependent RNA polymerase from *E. coli*.

This method does not permit the unequivocal differentiation between true integration and association of viral DNA with chromosomes.

9. Fusion of Virus Transformed Cells

Fusion of virus transformed cells with permissive cells allowing viral replication and rescue of the viral genome (GERBER, 1966; KOPROWSKI et al., 1967; TOURNIER et al., 1967; WATKINS and DULBECCO, 1967). Fusion of different cell types is effected by UV-inactivated Sendai virus (HARRIS and WATKINS, 1965).

This technique represents one of the most powerful tools to prove that the entire viral genome persists in some strains of virus transformed cells. However, successful rescue of the viral genome is not necessarily relevant for the physical state of the viral genome in transformed cells.

10. Chemical Induction of Viruses

Similar reservations apply to results of experiments in which virus was chemically induced from transformed (BURNS and BLACK, 1966; ROTHSCHILD and BLACK, 1970) or apparently normal cells (TEICH et al., 1973).

11. Mendelian Genetics

Mendelian genetics has proved to be a very successful approach to the localization of the genome of murine viruses in high incidence leukemia strains of mice (ROWE, 1973; LILLY and PINCUS, 1973, CHATTOPADHYAY et al., 1975).

III. Virus Systems

In this section, the available evidence for integration of viral DNA into eukaryotic genomes will be presented for a number of virus systems. This discussion will be preceded by a survey of some of the most important features of temperate bacteriophage and lysogenic bacteria. Obviously, it will be impossible and unnecessary to include a complete discussion of lysogeny. In combining discussions of lysogeny and integration of animal virus DNA, it should be kept in mind that it might be unrealistic to emphasize too much any analogies between lysogeny and the integrated state of viral genomes in eukaryotic cells. However, there are undoubtedly parallels and it may be useful to consider in context the entire gamut of known interactions between viral and host genomes in prokaryotic and eukaryotic cells.

A. Bacteriophage Lambda

A number of outstanding reviews on different aspects of bacteriophage lambda have been published (DOVE, 1968; SIGNER, 1968; ECHOLS, 1972; HERSKOWITZ, 1973). The most comprehensive survey on lambda is found in The Bacteriophage Lambda (A.D. HERSHEY, editor, 1971).

1. Lambda Genetics and Lambda DNA

From a functional point of view the genetic map of bacteriophage lambda (Fig. 1) can be divided into two regions:

a) The early genes, comprising genes for early controls (repressor, immunity region), for recombination, for excision and integration, and for viral DNA replication.

b) The genes for late controls, for functions involved in lysis of the host cell, and for the structural genes of the phage head and tail.

The functions of the individual genetic elements in phage lambda are described in the legend to Fig. 1. The DNA of bacteriophage lambda is a double-stranded molecule of a molecular weight of 31×10^6 (BURGI and HERSHEY, 1963) or $29.6 \pm 1.5 \times 10^6$ (FREIFELDER, 1970), depending on the methods of determination and calculation. A detailed discussion of these calculations is found in DAVIDSON and SZYBALSKI, 1971. Lambda DNA has cohesive ends (HERSHEY et al., 1963) which represent single-stranded, complementary regions on either 5′-terminus of the molecule. The single-stranded ends consist of 12 nucleotides whose sequence (see legend to Fig. 1) has been determined by WU and TAYLOR (1971). The cohesive ends of the molecule allow the construction of hydrogen-

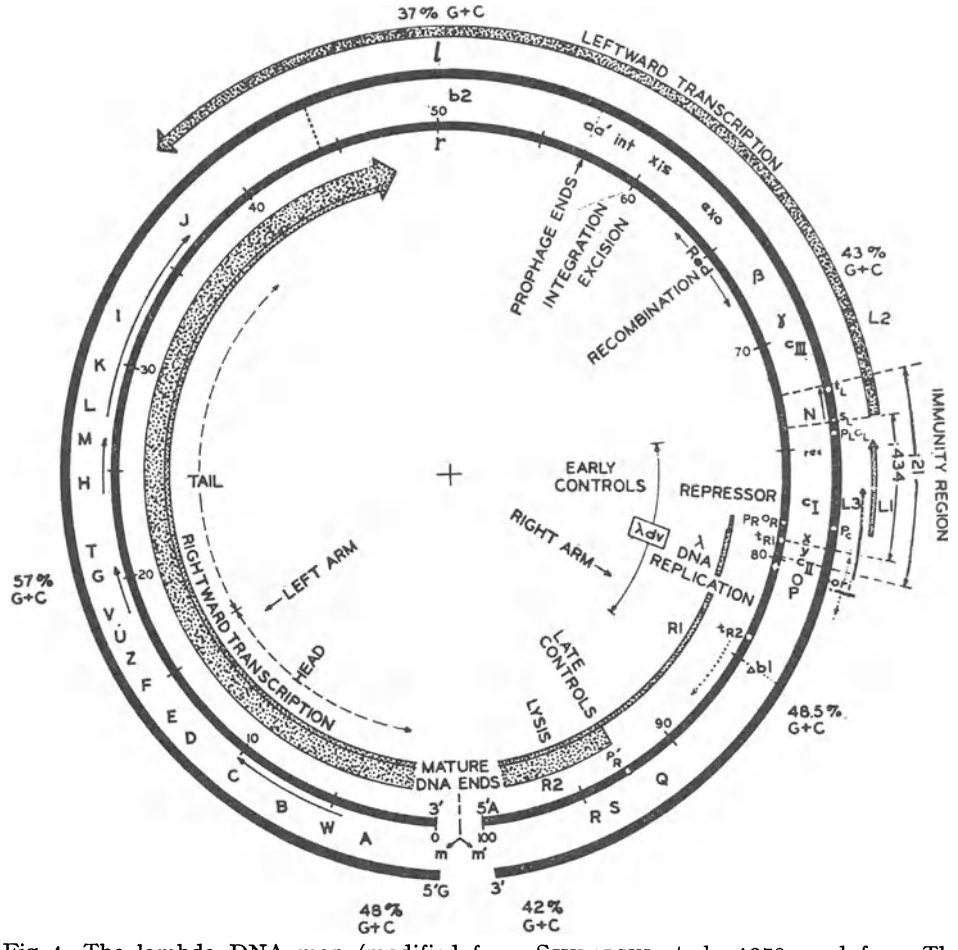


Fig. 1. The lambda DNA map (modified from SZYBALSKI et al., 1970, and from The Bacteriophage Lambda, 1971).

A, W, B, C, Nu3, D, E, F_I, F_{II}: Genes involved in formation of phage head (KAISER and MASUDA, 1973).

Gene A is responsible for the formation of the cohesion of the DNA and the cleavage of polymeric precursor DNA (WANG and KAISER, 1973).

Gene E protein makes up 75% of the mass of the head proteins (CASJENS et al., 1970; BUCHWALD et al., 1970). The phage head is a $T = 7$ structure, containing 420 subunits each of proteins E and D which are distributed over the entire surface of the phage head (CASJENS and HENDRIX, 1974).

Gene F_{II} probably codes for the attachment site on heads for tails (CASJENS, 1974).

Z, U, V, G, T, H, M, L, K, I, J: Genes involved in formation of phage tail.

Gene V: Major tail protein which is distributed along the length of the phage tail.

Gene J: determines host range.

int: Responsible for prophage insertion (and excision).

xis: Responsible for prophage excision.

exo or red: Lambda exonuclease. Promotes general recombination in rec A^- and pol A^- host cells.

β or red: β protein. Promotes general recombination.

γ: Facilitates growth in rec A^- host cells.

cIII: Establishment of immunity.

N: Positive regulator, regulates transcription of genes to the left of N and to the right of cro.

rex: Inhibits the growth of T4rII mutants.

bonded circular DNA. Upon infection of *E. coli* with phage lambda, the viral DNA is covalently closed (YOUNG and SINSHEIMER, 1964; BODE and KAISER, 1965; DOVE and WEIGLE, 1965) by the action of a DNA ligase (GELLERT, 1967) which is presumably a host specified enzyme (TOMIZAWA and OGAWA, 1968).

The strands of the DNA of bacteriophage lambda can be separated by equilibrium centrifugation in alkaline CsCl density gradients (DOERFLER and HOGNESS, 1968a, b) or by the binding of poly I, G and subsequent equilibrium centrifugation in CsCl density gradients (HRADECNA and SZYBALSKI, 1967). The heavier strand in alkaline CsCl gradients binds less poly I, G and thus is the lighter strand by the latter separation method. The heavy strand in alkaline CsCl density gradients carries the 3′-OH end on that terminus of the molecule which is adjacent to gene R (HOGNESS et al., 1966).

Transcription of lambda DNA is asymmetric (Fig. 1) and starts at the early leftward promoter (p_L) on the strand whose 3′-OH terminus is located next to gene R. Leftward transcription continues through the b2 region. The c_I gene, the gene for the lambda repressor, is transcribed from the same strand, and its transcription starts at the p_c promoter. The genes for DNA replication, late controls, lysis, and for all the structural proteins are transcribed from the opposite strand starting at the rightward promoter (p_R). The details of lambda

cI: Lambda repressor. Maintenance of immunity (KAISER, 1957).

cro, tof or fed: Negative controls of immunity. Responsible for turnoff of transcription of genes N through int.

y: Interacts with the cII/cIII oligomer.

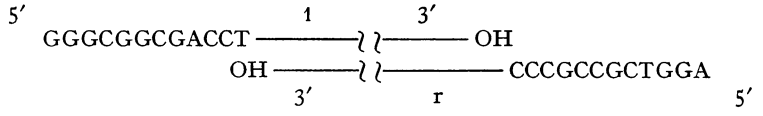
cII: Establishment of immunity.

O, P.: DNA replication

Q: Positive regulator of late gene transcription.

S, R: Cellular lysis. R is the gene for the endolysin, an endopeptidase.

m, m′: Left and right cohesive ends of lambda DNA. The nucleotide sequence of the cohesive ends was determined by WU and TAYLOR (1971):



att: Site of interaction ("attachment") of lambda DNA with host DNA during integration step. P.P′ (a.a′) designates the phage DNA site and B.B′ (b.b′) the bacterial DNA site.

P_L: Early leftward promoter (Mutants sex and t 27 define this promoter and are deficient in transcription of genes N through int).

O_L: Operator controlling transcription of genes N through int. Left binding site of the lambda repressor. Site of V2 mutation (vir L).

P_R: Early rightward promoter situated in the x region. Lambda x^- mutations are deficient in transcription of genes cro through Q.

P_R': Late rightward promoter. Required for late gene expression. Site of action of gene Q product.

O_R: Operator controlling transcription of genes cro through Q. Right binding site of the lambda repressor. Site of V3 and V1 mutations.

ori: Origin of DNA replication.

t: Termination for transcription.

i^{21}, i^{434}: Immunity regions which are altered in lambdoid phages 21 and 434.

A more detailed map of the lambda genome has been published by SZYBALSKI (1974)

transcription and the hybridization methods used in the investigation on lambda transcription have been described in detail by SZYBALSKI et al. (1970); KUMAR et al. (1970); KOURILSKY et al. (1970), and HERSKOWITZ and SIGNER (1970). In the analysis of the lambda transcription map, the availability of specific fragments of lambda DNA was very useful (SKALKA et al., 1968).

2. Lytic Development or Lysogeny

After infection of the host cells by bacteriophage lambda, the phage can either start replicating and eventually destroy the host cell or the phage DNA can become integrated into the cellular DNA with a concomitant repression of most of the viral gene functions: The cell becomes lysogenized. In the lysogenic state the viral genome replicates in the integrated form synchronously with the host chromosome. Repression of viral gene functions is effected by a phage coded protein, the repressor (PTASHNE, 1967a and b) which interacts, probably in a dimeric form (CHADWICK et al., 1970), with two sites on the viral genome (O_l and O_r, see Fig. 1), and thus prevents presumably the host RNA-polymerase from transcribing the early viral genes. Transcription of the cI gene which is responsible for repressor synthesis continues and is essential for the maintenance of lysogeny. A certain intracellular titer of repressor bestows upon the cell immunity towards superinfection with the same phage. Since the interaction between the repressor and the O_l and O_r sites on the viral genome is highly specific, the lambdoid phages, e.g. phages 434, 424, 21, which differ from lambda only in their immunity regions (Fig. 1), are not susceptible to lambda repression and can replicate in a cell lysogenic for phage lambda.

In the analysis of events leading to the lysogenization of a cell, three steps are essential: establishment of lysogeny, maintenance of lysogeny, and the capacity for induction. Establishment of lysogeny is a very complex process involving, at the same time, expression of the early viral genes required for integration, repression of the late genes responsible for lytic development, and a highly specific recombination event between specific sites on the phage (P.P') and the host (B.B') genomes (Fig. 2). The phage specific int function is necessary for the integration of the phage genome. The products of genes cII and cIII, perhaps in the form of an oligomeric complex, are essential for the establishment of lysogeny, since mutants in these genes exhibit a marked deficiency in the frequency of lysogenic conversion. Lysogeny is maintained through the action of the repressor.

For the induction of lytic development in a lysogenic host, it is necessary to release repression and to excise the viral genome. Several agents known to inhibit cellular DNA synthesis (irradiation with ultraviolet light, mitomycin C, thymine deprivation etc.) inactivate the repressor by a mechanism that is not understood. In the mutant λcI857 the repressor is temperature-sensitive and can be inactivated by high temperatures. Subsequently, viral genes can be expressed, the viral genome is excised through the action of the int and xis functions and late transcription starts leading to lytic development. Some features

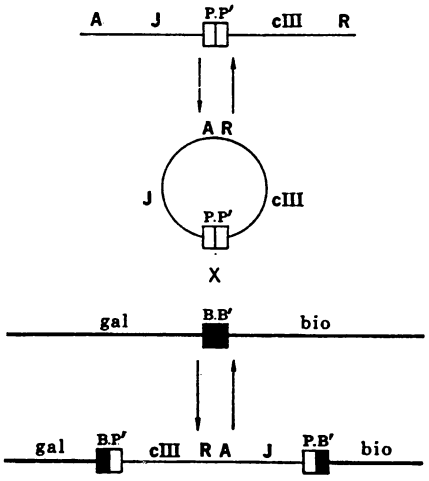


Fig. 2. Circularization of the lambda chromosome and prophage insertion and excision according to the CAMPBELL (1962) model. A, J, cIII and R are lambda genes whose sequence is permuted in the process of insertion. B.B′ and P.P′ are the bacterial and phage attachment sites, respectively. Upon insertion of the prophage, the distance between the bacterial genes gal (utilization of galactose) and bio (synthesis of biotin) is increased (ROTHMAN, 1965). This scheme was taken from GOTTESMAN and WEINBERG (1971)

of the very complicated control apparatus involved in lysogenization and induction will be dicussed in the next section.

The factors which influence the outcome of an infection with phage lambda and lead to lysis or lysogeny, are poorly understood. At high multiplicity of infection, lysogenization is favored over the lytic cycle. Cells in "poor nutritional conditions" are lysogenized at higher frequency than those actively growing. At temperatures higher than 37° C the frequency of lysogenization of the cell decreases. In cells which are deficient in the enzyme adenyl cyclase or in the catabolite activator protein (CAP), lysogenization is also reduced (GRODZICKER et al., 1972).

Phages P22 (LEVINE, 1972) and P2 (BERTANI and BERTANI, 1971) are very similar to lambda in many respects. On the other hand, phage P1 apparently does not have to integrate its genome and is able to replicate as a free episome synchronously with the host chromosome (IKEDA and TOMIZAWA, 1968).

3. Controls in Phage Lambda

The scheme in Fig. 3 summarizes the most important control functions in phage lambda. In the lytic cycle the products of genes N and Q control the transcription of the early and late genes, respectively. Immediate early transcription starts to the left (O_l, P_l) and to the right (O_r, P_r), comprising genes N, cII, O and P. Without the N product only the N and tof genes are transcribed at wild type levels. The *E. coli* protein rho terminates transcription at t_L (to the left of N) and t_R (to the right of tof) (see Fig. 1). It has been proposed that

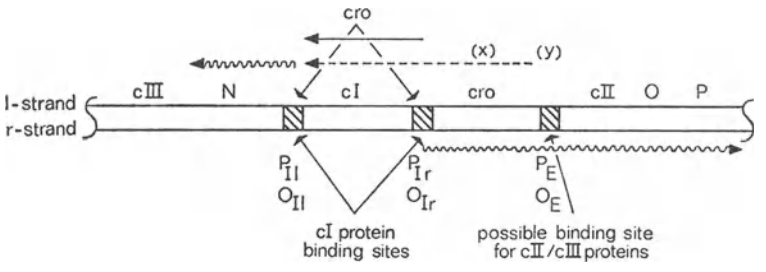


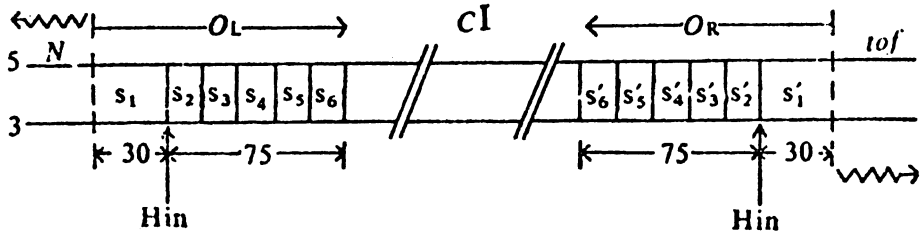
Fig. 3. The control region of phage lambda. Details are described in the text. This diagram was taken from ECHOLS (1972)

the N gene product acts as an antiterminator antagonizing rho (ROBERTS, 1970). In the delayed-early period, the N gene product activates transcription of genes cIII through int, and cII through Q. The product of gene Q then facilitates transcription of all the late genes.

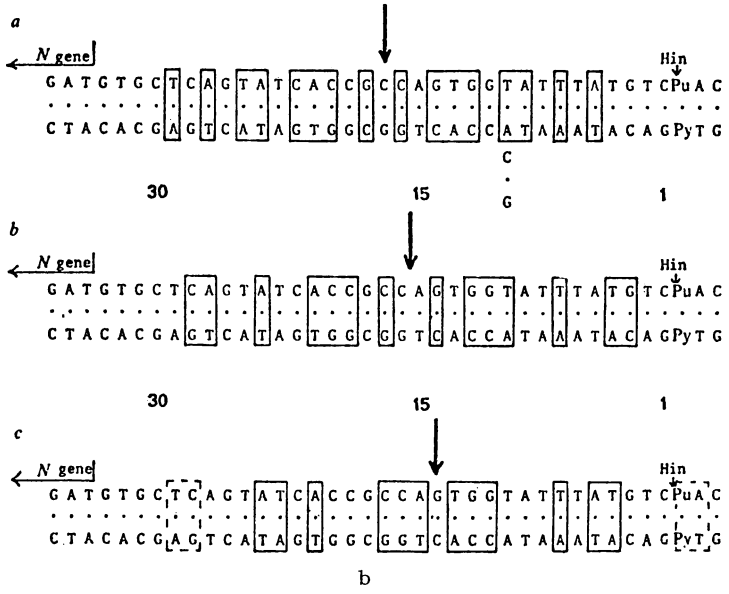
The lysogenic state is maintained by the product of gene cI, the lambda repressor. The repressor is a protein of 28000 molecular weight which binds in an oligomeric form to lambda DNA at the O_l and O_r sites (PTASHNE and HOPKINS, 1968) and thus blocks lytic development. The leftward (O_l) and rightward (O_r) operators are different in base sequence and each operator contains several different recognition sites for the lambda phage repressor (MANIATIS et al., 1973). The molecular structure of the operators has recently been elucidated by MANIATIS et al., (1974). The operators are 105 base pairs long (Fig. 4a). A sequence of 33 nucleotides in the leftward operator immediately preceding the N operon has been determined (Fig. 4b). This sequence contains three overlapping interdigitating symmetries (MANIATIS et al., 1974) which, except for the repressor, might also be recognized by other proteins, viz. the tof product, the N-product and RNA polymerase. The leftward operator is not transcribed, but rather leftward transcription starts in the N gene beyond the 33 nucleotide sequence. The sequences of the O_r operator (PIRROTTA, 1975, MANIATIS et al., 1975) and the P_r promoter (WALZ and PIRROTTA, 1975) have also been determined.

When a lambda lysogenic cell is superinfected with λ and phage λ imm 434, phage λ imm 434 can replicate but λ replication is almost completely repressed, although λ imm 434 does supply the lambda-specific N, O and P functions. These results suggest that the cI gene product also affects, in an as yet unknown way, sites on the lambda genome which are located outside the leftward and rightward operators. This block cannot be overcome by the N, O and P gene products.

For the understanding of lysogeny it is essential to investigate how the synthesis of the repressor is regulated. It is possible, albeit not proven that the cI gene product activates its own synthesis. The cI gene is transcribed from the leftward strand (KOURILSKY et al., 1970; HEINEMANN and SPIEGELMAN, 1970), hence the O_R operator (see Fig. 1) must control its expression. The molecular mechanism of the self-activation of gene cI is not understood, but it



a



b

Figs. 4a and b. The lambda operator. (a) Lambda operators and adjacent genes. The wavy lines indicate the direction of transcription of the repressor-controlled genes N and tof. The order of repressor binding from the first sites (S_1 and S'_1) to the last sites (S_6 and S'_6) in O_l and O_r, is shown by the arrows O_l and O_r. The restriction endonuclease from Hemophilus influenzae cuts the O_l and O_r operators at the sites indicated by the Hin arrows. The numbers 30 and 75 refer to the number of nucleotide pairs within the operators on either side of each endonuclease cleavage site. (b) Base sequence elucidated in leftward lambda operator. The bases in boxes are symmetric about two-fold rotational axes indicated by vertical arrows. There are three interdigitating symmetries in this region of the leftward lambda operator. From MANIATIS et al. (1974)

must be independent of any other viral gene, since repressor control functions in an N⁻x⁻lysogen (HEINEMANN and SPIEGELMAN, 1970). In the regulation of the expression of gene cI, the cII and cIII functions are involved, since mutants in genes cII or cIII are defective in the synthesis of repressor (REICHARDT and KAISER, 1971; ECHOLS and GREEN, 1971). The regulatory activities of genes cII and cIII are not direct, but rather are effected through the interaction of the presumptive cII/cIII oligomer with the y site.

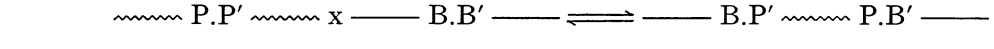
There is yet another regulatory gene in lambda whose function on a molecular level is difficult to understand: Gene cro antagonizes the establish-

ment of repression. A hypothetical way in which cro regulation works was suggested by ECHOLS (1972). The cro gene product binds to the right and left of gene cI (Fig. 3), perhaps at the same sites with which the repressor interacts. Moreover, the cro product represses transcription of gene N after inception of late transcription. How the activation of cro is delayed until the proper time point late in transcription remains enigmatic. Perhaps, cro is also able to inhibit the synthesis and activity of the cII and cIII proteins.

Although the details of many individual gene functions have been unraveled, one of the main riddles in the regulation of lambda genes is still to explain in a comprehensive way how the decision between the lytic and the lysogenic pathways is made.

4. On the Mechanism of Integration

The discussion in this chapter leans on a review by GOTTESMAN and WEISBERG (1971). In 1962, CAMPBELL suggested a model for the mechanism of integration of the genome of phage lambda (Fig. 2). The essential features of this model were proven to be correct (ROTHMAN, 1965; FRANKLIN et al., 1965; YOUNG and SINSHEIMER, 1964; BODE and KAISER, 1965). The infecting phage DNA molecules are circularized and, by reciprocal recombination between the P.P′ site on the phage DNA and a specific site (called att λ or B.B′) (SIGNER and BECKWITH, 1966) on the host chromosome, the viral genome is inserted between the gal and bio sites at 17 min on the genetic map of *E. coli*. This recombination step can be formalized in this way:



(B.P′ and P.B′ represent the junctions between the bacterial —— and phage ~~~ chromosomes).

ROTHMAN (1965) was able to show that insertion of the lambda chromosome leads to a reduction in the frequency of cotransduction of the gal and bio markers by phage P1. The insertion of the lambda chromosome into the bacterial DNA is alkali stable and is therefore presumed to be effected by a covalent bond (FREIFELDER and MESELSON, 1970). In the process of integration the gene order of the phage is permuted (CALEF and LICCIARDELLO, 1960; ROTHMAN, 1965; FRANKLIN et al., 1965).

In 1967, WEISBERG and GALLANT demonstrated that integration of the phage genome (the prophage) was dependent on protein synthesis. At the same time, mutants deficient in the integration function (int$^-$ mutants) were isolated in several laboratories. Thus, it appeared likely that a phage gene was responsible for the synthesis of the "integrase" function (ZISSLER, 1967; GINGERY and ECHOLS, 1967; GOTTESMAN and YARMOLINSKY, 1968a). The int protein has been characterized to some extent. It has a molecular weight of approximately 42000 (HENDRIX, 1971; AUSUBEL et al., 1971).

The recombination step catalyzed by the int function is highly specific for the viral and host sites. This has been clearly demonstrated in experiments crossing int$^+$ red$^-$ lambda mutants in a rec$^-$ host. In this cross the general

recombination genes of the phage (red) and the recombination system of the host (rec) are inoperative. The phage recombinants found in these experiments were restricted to the region between b2 and int (WEIL and SIGNER, 1968; ECHOLS et al., 1968). It is not understood, how the int protein (perhaps in cooperation with other functions) recognizes the P.P′ and B.B′ sites. Lambda int⁻ mutants are able to lysogenize at a frequency of approximately 10^{-6} per infected cell. Such lysogens apparently can be formed due to the general rec system of the host cell. GOTTESMAN and YARMOLINSKY (1968b) demonstrated that in these lysogens the prophage was not inserted at the B.B′ site, but rather at random locations on the host chromosome. Thus the high frequency and specificity of the prophage insertion depend on the very efficient integration system that is coded for, at least in an essential part, by the int gene of phage lambda.

When the primary λ attachment site (B.B′) in Escherichia coli was deleted, λ integrated at secondary sites. Some of these lysogens became mutants. This mutagenesis was not random. The inserted prophage reduces the expression of distal genes in the same operon (SHIMADA et al., 1973).

How does the int function recognize specific nucleotide sequences at the site of integration? Do the recognition sites harbor palindromic sequences which proved so important as recognition signals for restriction enzymes? The bipartite structure of the attachment site (P.P′) on the lambda chromosome has been elucidated by PARKINSON and collaborators (PARKINSON and HUSKEY, 1971; PARKINSON, 1971; DAVIS and PARKINSON, 1971).

Integration resembles genetic recombination in that it proceeds via breakage and rejoining, but differs from general recombination by its requirement for the int function. DNA synthesis is not required for insertion or excision. However, lambda mutants deficient in DNA replication lysogenize less efficiently than wild type lambda (BROOKS, 1965).

Excision is the reversal of integration and entails recombination of the P and P′ sites of the prophage. For efficient excision the products of both the int and xis genes are necessary. In rare cases, faulty excision occurs as a result of illegitimate recombination between sites in the prophage and the bacterial chromosome (CAMPBELL, 1962) giving rise to phage particles which are defective in phage genes, but carry bacterial markers (e.g. the gal operon in λdg). Such phage particles can transduce bacterial markers, e.g. various parts of or the entire gal operon.

The expression of the int and xis genes is under the control of the N gene product and therefore regulated by the leftward operator and promoter. The interesting observation was made that after infection of *Escherichia coli* with a λ N⁻ mutant, integration cannot occur, but that the λ N⁻ genome replicates once with each cellular replication, and that the λ N⁻ DNA persists in the cell as a non-integrated plasmid (SIGNER, 1969). Many details of the differential control of genes int and xis are still unknown. One of the paradoxes of regulation in λ centers around the question of how a phage genome manages to stay integrated, since both integration and excision are highly efficient processes

and the int and xis genes both belong to the N operon. Two mechanisms have been discussed, but neither is entirely satisfactory:

a) WEISBERG and GOTTESMAN (1971) provided evidence that the xis gene product is relatively unstable; b) SHIMADA and CAMPBELL (1974a) have discovered a weak leftward promoter, pI, which is probably located in the xis gene and may be independent of immunity. This secondary promoter may uncouple the otherwise obligatory co-transcription of the int and xis functions and thus in effect drive the insertion-excision reaction in the direction of integration.

Recently, mutations in the vicinity of xis were isolated which may define a third gene, called hen which also appears to be involved in the integration process (CHUNG, GREEN, and ECHOLS, cited by SHIMADA and CAMPBELL, 1974b). GOTTESMAN and GOTTESMAN (Abstracts, Phage Meeting, Cold Spring Harbor, 1974) reported that they succeeded in showing *in vitro* recombination between the att region in λ DNA and bacterial DNA.

GUARNEROS and ECHOLS (1973) have provided evidence for a thermolabile element required for integration, but not in excision. FREIFELDER et al. (1975), GOTTESMAN and GOTTESMAN (1975), and NASH (1975) have carried out detailed studies on the requirements for integration.

5. Conclusion

This brief glimpse at some of the highlights of bacteriophage lambda must necessarily remain fragmentary. However, it should serve to remind us that there is a phage encoded, highly efficient recombination system which leads to the insertion of the phage DNA into the bacterial chromosome at one specific site. Discounting rare errors, the system of excision is equally effective and specific. The phage DNA is integrated in toto and its gene order becomes permuted in the process. The regulatory mechanisms behind the insertion-excision pathways are just beginning to be unraveled. It is also difficult to understand what factors, cellular and/or viral, influence the outcome of an infection of *E. coli* by bacteriophage lambda. Lastly, the consequences of integration of the viral genome for the host cell in a stable lysogen are practically unknown.

For the study of integrated viral genomes in eukaryotic cells, it is extremely useful to understand the lambda system. On the other hand, it would be unwarranted to become completely enthralled by the appealing analogies which this well analyzed model offers.

B. Bacteriophage Mu

In 1963, TAYLOR discovered phage Mu in K-12 strains of *Escherichia coli*. The striking feature of this phage was that it caused polar mutations in a large number of host genes (TAYLOR, 1963). In prototrophic *E. coli* lysogenized by

phage Mu, 2% of the survivors were auxotrophic. The site of phage induced mutation could be correlated with the location of insertion of the phage genome into the host chromosome (JORDAN et al., 1968; TOUSSAINT, 1969; MARTUSCELLI et al., 1971; BORAM and ABELSON, 1971; BUKHARI and ZIPSER, 1972). Thus, it became likely that phage Mu could integrate its genome into the *E. coli* chromosome practically at random. In this respect, the temperate *E. coli* phage Mu differed markedly from other temperate phages, like phage lambda or P22 (LEVINE, 1972) which have one specific site of integration or phage P2 with a limited number of attachment sites (CALENDAR and LINDAHL, 1969).

1. The DNA of Phage Mu

The morphology of phage Mu was described by To et al. (1966) and by MARTUSCELLI et al. (1971). The phage particle resembles phage T4 in certain aspects. The DNA of the phage is a double-stranded molecule of 28×10^6 daltons (TORTI et al., 1970). The length of the linear DNA molecule as measured in electron micrographs ranged between 12.9 ± 0.1 μm (MARTUSCELLI et al., 1971) and 14.5 ± 0.7 μm (TORTI et al., 1970). More detailed information about the anatomy of the DNA of phage Mu was obtained by denaturation and renaturation of the DNA and subsequent electron microscopy: In addition to regular duplex molecules, more complex structures were observed. Most of the renatured molecules carried split ends on one terminus, and about 50% of the renatured molecules had a split end and a loop (the so-called G loop) on the same terminus (BADE, 1972; DANIELL et al., 1973a, b) (Fig. 5b). The split ends of Mu DNA are probably due to host DNA which remains after the excision of the prophage (DANIELL et al., 1973a, b). The split ends are not detectable in the prophage form of Mu DNA (HSU and DAVIDSON, 1974). The G loop is explained by an inversion of some of the phage DNA sequences (HSU and DAVIDSON, 1974). This inversion was also found in the prophage (HSU and DAVIDSON, 1972). In the region of the G loop the sequence occurs either in the direct or the inverted order and is bracketed by inverted repeat sequences of approximately 50 nucleotides (HSU and DAVIDSON, 1974). The inversion is thought to be generated by reciprocal recombination between the inverted repeat sequences (HSU and DAVIDSON, 1974). The G loop was also observed in the DNA of defective lambda phage which carried 14% of Mu DNA on one terminus (DANIELL et al., 1973b). The G loop was also found when phage Mu was replicated in rec A^- and rec B^-C^- hosts (WIJFFELMAN et al., 1972; DANIELL et al., 1973b).

Except for a minor fraction (1.45% of the late RNA) of the phage genome, the RNA is transcribed from that strand of Mu DNA which binds more poly U,G (BADE, 1972).

2. The Genetics of Phage Mu

A large number of mutants of phage Mu have been isolated and 21 cistrons have been identified (ABELSON et al., 1973). Only two of the phage functions

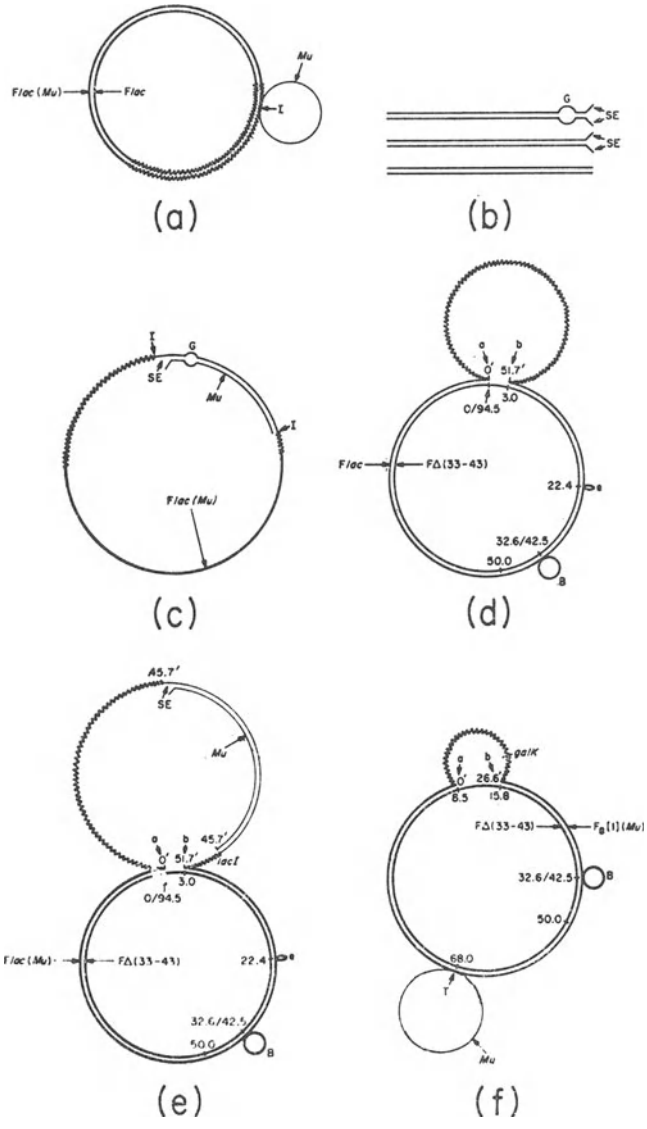
are known, c is responsible for immunity and lys is required for the lysis of the host. There is agreement among several laboratories that the gene orders in the prophage and the vegetative phage are identical and that there is a specific site on the phage genome which undergoes recombination with an unlimited number of sites in the host chromosome (BUKHARI and METLAY, 1973; FAELEN and TOUSSAINT, 1973; HOWE, 1973a; WIJFFELMAN et al., 1973). Moreover, it is apparent from a genetic analysis of the prophage that it can be inserted in either of two orientations relative to the orientation of the host gene in which the prophage integrates (BORAM and ABELSON, 1973; BUKHARI and METLAY, 1973; HOWE, 1973a; HSU and DAVIDSON, 1972; WIJFFELMAN et al., 1973). Phage Mu has been recognized to be capable of generalized transduction of host markers at a frequency of 10^{-7} to 10^{-9} transductants per plaque forming unit (HOWE, 1973b).

3. Integration of Phage Mu

Phage Mu induces strong polar mutations in approximately 2% of the lysogens (TAYLOR, 1963; TAYLOR and TROTTER, 1967; JORDAN et al., 1968). These mutants have a very low reversion frequency of 10^{-10} or less. Linear insertion of the DNA of phage Mu-1 was directly demonstrated by MARTUSCELLI et al. (1971). These authors isolated covalently closed circular DNA molecules from *E. coli* strains carrying the episome F' lac or F' lac Mu^+ and compared the lengths of these molecules. The mean length of the F' lac episome of *Escherichia coli* was 37.6 ± 0.4 μm and that of the F' lac Mu^+ lysogen 53.2 ± 0.4 μm. The difference of 15.6 μm was close to the values of 12.9 μm and 14.5 μm for the DNA of vegetative phage Mu determined by MARTUSCELLI et al. (1971) and TORTI et al. (1971), respectively.

Insertion of the phage genome was also documented genetically. BORAM and ABELSON (1971) analyzed a Mu-induced chl-D mutant located between the insertion site of lambda and the gal operon, and demonstrated that lambda did not transduce gal genes from this mutant and that transducing particles of lambda isolated from this strain contained parts of the Mu genome. BUKHARI and ZIPSER (1972) investigated 76 different Mu-induced mutations in the Z gene of the lac operon of *E. coli* and provided powerful evidence that the insertion of the Mu genome in the Z gene was random. It could also be demonstrated that there were no major deletions of host DNA at the site of integration.

Little is known about the enzymatic mechanism that is responsible for the insertion of Mu DNA, except that it is phage encoded, recognizes a specific site on the phage DNA and has apparently no specific requirements as to recognition of host sequences. The integration mechanism of Mu can catalyze the insertion of the DNA of a lambda N^- mutant in an *E. coli* rec A^- host, but this mechanism is not operative in a Mu lysogen (FAELEN and TOUSSAINT, 1971). This artificial insertion of lambda into the host chromosome is not site-specific. Phage Mu does not have a recombination system of its own, since in a rec A^- host, recombination frequencies are extremely low. However, phage Mu can utilize the recombination system provided by coinfection with phage



Figs. 5a—f. Schematic representation of heteroduplex molecules. (a) F lac (Mu)/F lac heteroduplex. At I Mu inserted into F lac. (b) Self renatured Mu DNA molecules; SE = split ends; G = internal nonhomology loop of 3.0 kb (kilobases) length. (c) Flac (Mu)/Mu heteroduplex. SE = split ends; G = internal nonhomology loop. (d) F lac/F Δ (33–43) heteroduplex. Straight lines indicate F sequences, sawtooth lines bacterial DNA. The numbers indicate kilobases; a and b are the junctions between F and bacterial DNA. Loop B is due to a deletion between kilobase 33 and 43 [F Δ (33–43)]. Loop E is characteristic for this F lac. (e) F lac (Mu)/F Δ (33–43)/Mu diheteroduplex. The Mu prophage is inserted at 45.7 kb of the bacterial DNA. (f) F_8 (1) Mu/F Δ (33–43) heteroduplex. This Figure was taken from HSU and DAVIDSON (1972)

lambda (WIJFFELMAN et al., 1972). In a rec A^- host, phage Mu can also promote the integration of an F^+ episome into the host chromosome (VAN DE PUTTE and GRUIJTHUIJSEN, 1972).

HSU and DAVIDSON (1972) demonstrated by electron microscopy of heteroduplex DNA molecules that the Mu genome was linearly inserted into the F′ episome of *E. coli* and that the prophage contained the same sequences in identical order as in the vegetative phage (Fig. 5).

After induction of cells lysogenic for phage Mu, covalently closed circular DNA molecules were observed (HSU and DAVIDSON, 1972; WAGGONER et al., 1974). The lengths of these molecules ranged from 36.5 to 156.7 kilobases. These heterogeneous molecules were not simply mono- or oligomers of the phage chromosome, but contained phage genomes linked to cellular DNA (WAGGONER et al., 1974).

Genetic evidence for asymmetric excision of the prophage Mu was published by SCHROEDER and VAN DE PUTTE (1974). According to this study the prophage DNA is excised very precisely at a specific site on one end and unspecifically on the other end. This finding explains the occurrence of the split ends of the vegetative phage DNA. Using physical methods, SCHROEDER (1975a) was able to show that the prophage is excised from an *F′ lac pro Mu* molecule 10 min after thermal induction. The vegetative phage DNA apparently originates by cleavage from intracellular intermediates which consist of alternating host and phage sequences (SCHROEDER et al., 1974). This model would explain the occurrence of host DNA sequences on the variable terminus of the phage DNA and the phenomenon of generalized transduction in phage Mu. SCHROEDER (1975b) could also demonstrate that the parental DNA from Mu particles infecting a host cell was converted into supercoiled circular molecules and that large amounts of the parental phage DNA were covalently linked to cellular DNA.

4. Conclusions

The most striking and unique property of bacteriophage Mu is that it can integrate its DNA into the host genome randomly and cause polar mutations at the sites of insertion. In this respect Mu contrasts with other temperate *E. coli* phages like P2 and P22. Phage Mu codes for its own integration system which facilitates recombination between a specific site on the circularized phage genome and randomly selected sites on the host chromosome.

C. Adenovirus

In discussions on the interactions of oncogenic viruses with mammalian cells, the assumption is frequently made that integration of the viral DNA into the host genome is one of the essential steps in the process that eventually leads to the transformation of cells infected with oncogenic viruses. Although there is as yet little evidence for the validity of this hypothesis, it has stimulated a great deal of work which has suggested that the genomes of most DNA and RNA oncogenic viruses can be inserted into the host chromosome. It is well documented that the genetic information encoded in the genome of RNA tumor viruses is transcribed by the reverse transcriptase into DNA (TEMIN and BALTIMORE, 1971) prior to integration into the host genome (VARMUS et al., 1974).

Considering all the evidence on integrated viral genomes, it appears conceivable that insertion of viral DNA is a general phenomenon and that, in some cases, integration may be required for the replication of the viral genetic material. There must be additional, possibly very complex steps linking the event of integration to the transformed state of the cell. The phenomena characteristic for transformed cells (altered physiology of growth, controlled transcription of viral genes, occurrence of new antigens, specific surface changes etc.) must be connected to the altered genetic condition of the cell. Investigations of this "missing link" have now come to the fore of research on oncogenic viruses.

In principle, two approaches have been taken to examine the integrated state of viral genetic material:

1. At early times after infection of cells with oncogenic DNA or RNA viruses, the fate of the parental or newly synthesized viral DNA was followed and viral DNA was shown to be integrated into the host chromosome (DOERFLER, 1968; 1970; ZUR HAUSEN and SOKOL, 1969; BURLINGHAM and DOERFLER, 1971; HIRAI et al., 1971; COLLINS and SAUER, 1972; HIRAI and DEFENDI, 1972; RALPH and COLTER, 1972; MANOR et al., 1973; VARMUS et al., 1973b; BURGER and DOERFLER, 1974; HÖLZEL and SOKOL, 1974; DOERFLER et al., 1974; VARMUS et al., 1974). This type of study approaches the problem from a general point of view and aims at an investigation of the mechanism of integration

2. The integrated state of the viral DNA in cells transformed by oncogenic viruses was the subject of equally intensive research (SAMBROOK et al., 1968; DULBECCO, 1968; GREEN, 1970b; GELB et al., 1971; HIRAI and DEFENDI, 1971; SMITH et al., 1972; GELB and MARTIN, 1973; MARKHAM and BALUDA, 1973; PETTERSSON and SAMBROOK, 1973; VARMUS et al., 1973b; HILL et al., 1974; SAMBROOK et al., 1974; VARMUS et al., 1974; GALLIMORE et al., 1974). This approach focuses on the virus-transformed cells which, in a sense, represent a terminal state and probably a special case in the gamut of interactions between susceptible cells and oncogenic viruses.

1. Adenovirus-Cell Systems

Adenoviruses were discovered in 1953 by ROWE et al. in human adenoids. There are 33 human types of adenoviruses and numerous types were isolated from other species. Human adenoviruses can be propagated to very high titers in human cells growing in tissue culture (GREEN and PIÑA, 1963) and cause characteristic cytopathic effects (BOYER et al., 1959). Some types of human adenoviruses (types 2 and 5) can also replicate in hamster cells. In 1962, TRENTIN et al. and HUEBNER et al. discovered that human adenovirus types 12 and 18 caused tumors in newborn hamsters. POPE and ROWE (1964) and MCBRIDE and WIENER (1964) demonstrated that hamster cells could be transformed in tissue culture by adenovirus types 12 and 18. In subsequent years, a large number of cell types were transformed by human adenoviruses, e.g.

rat embryo cells by adenovirus type 2 (FREEMAN et al., 1967; GALLIMORE, 1974), baby hamster kidney (BHK 21) cells by adenovirus type 12 (STROHL et al., 1967), rabbit cells (LEVINTHAL and PETERSON, 1965) by adenovirus type 12, and hamster cells by adenovirus type 2 (LEWIS et al., 1974).

PIÑA and GREEN (1965), and HUEBNER (1967) have divided the human adenoviruses into three classes according to their oncogenic potential: The highly oncogenic group comprises adenovirus types 12, 18 and 31; the group of intermediate oncogenicity adenovirus types 3, 7, 8, 11, 14, 16, 21, and the non-oncogenic or rather weakly oncogenic group the remaining adenovirus types (MCALLISTER et al., 1969). Adenovirus types 2 and 5, initially thought to be non-oncogenic, were subsequently shown to transform cells in tissue culture (FREEMAN et al., 1967; LEWIS et al., 1974; GALLIMORE, 1974), although these adenovirus types do not lead to tumor formation upon injection into animals. PIÑA and GREEN (1965) detected an interesting correlation between the degree of oncogenicity of human adenoviruses and the G + C content of the DNA of these adenoviruses. The highly oncogenic group had G + C contents of 48–49%, the intermediate group G + C contents of 50–53%, and the weakly oncogenic group G + C contents of 56–60%. The biological significance of this discovery remains unknown.

It becomes increasingly clearer that the so called transformed state of a cell does not necessarily guarantee that it will behave like a malignant cell when reinjected into animals.

Adenovirus-transformed cells do not produce virions or viral structural proteins, they do, however, contain the T-antigen (HUEBNER, 1967) which is presumably virus-coded. Viral DNA sequences are efficiently transcribed in transformed cells and the transcripts become associated with polysomes in the cytoplasm of transformed cells (FUJINAGA and GREEN, 1966, 1967a, b, 1968, 1970; FUJINAGA et al., 1969; GREEN et al., 1970). The genes transcribed in transformed cells correspond to genes transcribed early in productive infection (GREEN et al., 1970). Late viral genes are not expressed in transformed cells. GREEN et al. (1970) examined cell line 8617, a rat cell line transformed by adenovirus type 2 (FREEMAN et al., 1967), and found RNA sequences homologous to 4–10% of both strands of the viral DNA. GALLIMORE (1974) isolated and characterized nine independent lines of rat cells transformed by adenovirus type 2. SAMBROOK et al. (1974), GALLIMORE et al. (1974), and SHARP et al. (1974a) determined the fragments of the viral genome which persisted in these transformed cell lines (Section C., 4.). These fragments corresponded to the left, GC-rich terminus of the viral genome. In accordance with these results, SHARP et al. (1974b) observed that the viral messenger RNA sequences isolated from transformed rat cell lines corresponded to those viral DNA sequences shown to persist in these cells (SAMBROOK et al., 1974; GALLIMORE et al., 1974; SHARP et al., 1974a).

Adenoviruses are able to infect cells abortively; the best studied system is that of BHK21 cells infected with adenovirus type 12 (DOERFLER, 1968, 1969, 1970; STROHL, 1969a, b; DOERFLER and LUNDHOLM, 1970; DOERFLER

et al., 1972b; RAŠKA and STROHL, 1972; WEBER and MAK, 1973; ORTIN and DOERFLER, 1975). In this system the block in viral replication is early, i.e. viral DNA replication is not detectable (DOERFLER and LUNDHOLM, 1970; RAŠKA et al., 1971). An exceedingly small fraction (2×10^{-5}) of the BHK21 cells infected with adenovirus type 12 is transformed (STROHL et al., 1967; STROHL, 1969a; STROHL et al., 1970).

Adenoviruses and cells infected or transformed by adenoviruses have proven to be extremely useful systems in the study of the interaction between oncogenic viruses and host cells. As it is impossible to give a complete summary on this interesting virus system, I should like to refer the reader to several excellent reviews of this field (SCHLESINGER, 1969; GREEN, 1970; PHILIPSON and PETTERSON, 1973; STROHL, 1973; TOOZE, The Molecular Biology of Tumor Viruses, 1973; LINDBERG and PHILIPSON, 1974; PHILIPSON et al., 1975).

2. Integration of Viral DNA in Cells Productively Infected with Adenovirus

Human KB cells (EAGLE, 1955) are productively infected by adenovirus type 2, and each cell produces in excess of 10^6 virus particles. In spite of considerable efforts by many laboratories, the mechanism of replication of the viral DNA is known only in outline. Adenovirus type 2 DNA is a linear, double-stranded molecule of 22×10^6 molecular weight (GREEN et al., 1967; DOERFLER and KLEINSCHMIDT, 1970). As could be shown by the technique of denaturation mapping, the DNAs of adenovirus type 2 (DOERFLER and KLEINSCHMIDT, 1970) and type 12 (DOERFLER et al., 1972a) exhibit unique denaturation patterns, and hence, the gene order cannot be circularly permuted. The DNAs of all adenoviruses examined have a peculiar inverted terminal repetition (GARON et al., 1972; WOLFSON and DRESSLER, 1972; ROBINSON et al., 1973) in which the two termini of one strand of adenovirus DNA carry complementary sequences comprising 1–4%, in some cases up to 20% of unit genome length, depending on the serotype. Due to this terminal repetition, adenovirus DNA can form hydrogen-bonded, single-stranded circular molecules. Some of the viral DNA can be isolated from purified virions in a circular configuration when the use of proteolytic enzymes is avoided (ROBINSON et al., 1973; DOERFLER et al., 1974; BROWN et al., 1975). These circles appear to be stabilized by a protein link and not by covalent bonds.

Several laboratories have reported the isolation of partly single-stranded structures as possible intermediates in viral DNA replication (VAN DER VLIET and SUSSENBACH, 1972; SUSSENBACH et al., 1973; VAN DER EB, 1973; PETTERSSON, 1973; ROBIN et al., 1973; DOERFLER et al., 1973; ELLENS et al., 1974). There is at present no evidence for the involvement of covalently closed supercoiled circular forms of viral DNA in the replication process (HORWITZ, 1971; DOERFLER et al., 1973). It is conceivable that circular viral DNA molecules stabilized by a protein link (ROBINSON et al., 1973) play a role in viral DNA

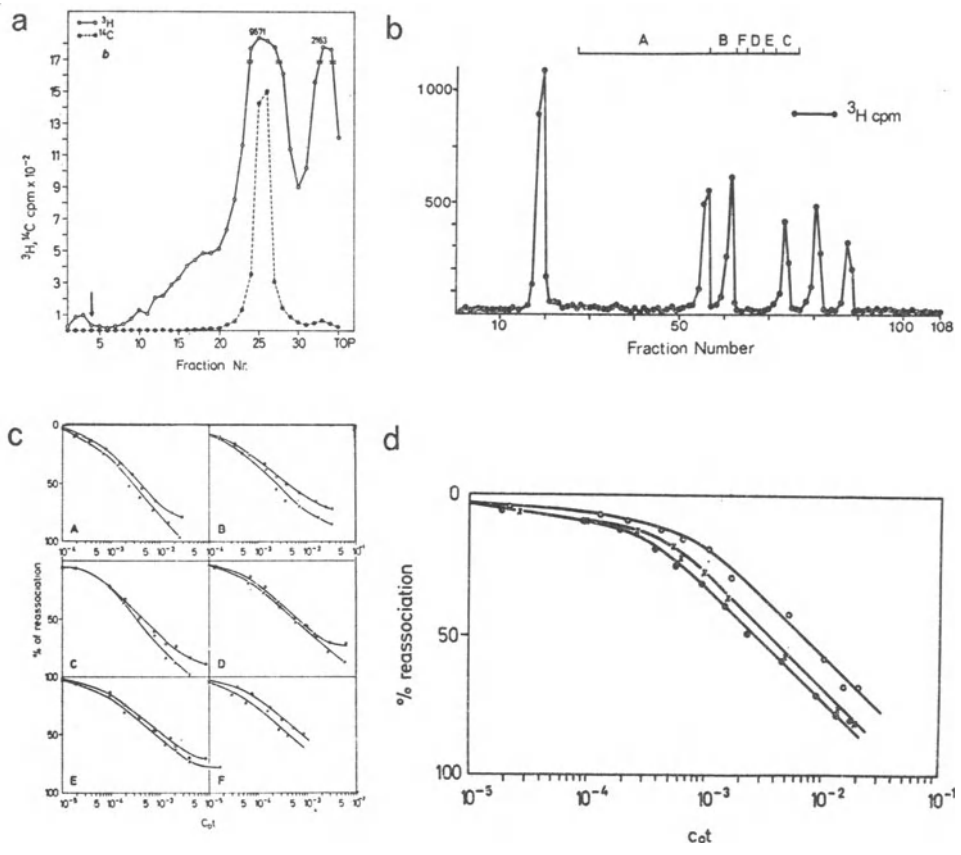


Fig. 6a—d. Integration of adenovirus DNA in productively infected cells. (a) Human KB cells growing in monolayers were infected with adenovirus type 2, and the newly synthesized DNA was labeled with ^{3}H-thymidine from 14–17 h postinfection. At the end of the labeling period, the cells were lysed in alkali for 18 h together with ^{14}C-labeled adenovirus 2 marker DNA on top of an alkaline sucrose gradient. The DNA was sedimented in the SW41 rotor of a Spinco L2–65B ultracentrifuge at 4° C and 35000 rpm for 140 min. This graph was taken from BURGER and DOERFLER (1974). (b) Adenovirus type 2 DNA was cleaved by the R.RI restriction endonuclease from E. coli and the fragments were separated by electrophoresis on polyacrylamide agarose gels as described by DOERFLER et al. (1974). The direction of electrophoresis is to the right. The largest fragment (A) is in fractions 18–19, the smallest fragment (F) in fractions 87–88. The insert localizes the fragments on the adenovirus 2 DNA map (PETTERSON et al., 1973). (c) Reassociation kinetics: Each of the ^{3}H-labeled R.RI restriction endonuclease fragments of adenovirus 2 DNA was reannealed with itself (o—o) and in the presence of unlabeled, fast sedimenting DNA from adenovirus 2-infected KB cells (×—×) (see Fig. 6a). The conditions of reassociation have been described by DOERFLER et al., 1974. (d) Enhancement of the reassociation rate of ^{3}H-labeled adenovirus 2 DNA by fast-sedimenting DNA from adenovirus 2-infected KB cells. o—o Reassociation of the ^{3}H-labeled adenovirus 2 DNA with itself; ×—×, •—• reassociation of ^{3}H-labeled adenovirus 2 DNA in the presence of different amounts of unlabeled, fast-sedimenting DNA. This figure was taken from DOERFLER et al. (1974)

replication. However, so far such structures have not been isolated from infected cells. Adenovirus DNA replication is discontinuous (WINNACKER, 1975) and there seems to be more than one point of origin of replication (PEARSON, 1974; WINNACKER, 1974). SUSSENBACH et al. (1972) have suggested a model of adenovirus DNA replication according to which replication starts at the right molecular end of adenovirus DNA and proceeds by displacement of that parental strand which has the higher buoyant density in alkaline CsCl density gradients (SUSSENBACH et al., 1973). Although this model has stimulated a great deal of experimentation, definite answers to the problem of adenovirus DNA replication are not yet available.

An analysis of the different forms of newly synthesized adenovirus DNA in KB cells productively infected with adenovirus type 2, revealed a fast sedimenting (50–90S), alkali stable form of viral DNA (Fig. 6a) which contained both viral and cellular sequences (BURLINGHAM and DOERFLER, 1971; DOERFLER et al., 1971; BURGER and DOERFLER, 1974). Extensive control experiments showed that this fast sedimenting viral DNA was not due to unspecific inclusion of viral DNA into the bulk of cellular DNA, to association of viral DNA with protein or RNA, or to a supercoiled, covalently closed circular form of viral DNA. Since the fast sedimenting viral DNA rebanded in alkaline CsCl density gradients at a density stratum intermediate between that of the viral and cellular marker DNAs, it was concluded that the fast sedimenting viral DNA represented an integrated form of adenovirus DNA. Moreover, upon ultrasonic treatment of the ^{3}H-labeled, fast sedimenting DNA prior to resedimentation in alkaline CsCl density gradients, the labeled DNA shifted to the density positions of viral and cellular DNAs (BURGER and DOERFLER, 1974). This result is consistent with the model of integrated viral DNA.

The interpretation that the fast sedimenting viral DNA contained an integrated form was supported by the finding that the rate of reassociation of viral DNA (Fig. 6d) was enhanced by fast sedimenting DNA, and the rate of reassociation of fast sedimenting DNA by cellular DNA. A quantitative analysis of results obtained from different experiments indicated that in KB cells infected with adenovirus type 2 at a multiplicity of infection of 100 PFU/cell, between 2000 and 7000 copies of viral DNA per cell were present in the fast sedimenting form at 16–18 h postinfection (DOERFLER et al., 1974). The fast-sedimenting form of newly synthesized viral DNA was detectable by DNA-DNA hybridization experiments starting 2–4 h postinfection (SCHICK et al., 1975). Control experiments using the technique of reassociation kinetics demonstrated that viral DNA was not detectable in the fast sedimenting DNA extracted from uninfected KB cells (DOERFLER et al., 1974).

The DNA of adenovirus type 2 can be cleaved by the R.RI restriction endonuclease from *E. coli* into six specific fragments which can be separated on polyacrylamide-agarose gels (Fig. 6b) (PETTERSSON et al., 1973). The fast sedimenting DNA from Ad2-infected KB cells enhanced the rate of reassociation of each of the six R.RI fragments of adenovirus type 2 DNA (Fig. 6c) sug-

gesting that each of the six fragments of viral DNA was represented in the integrated viral DNA (DOERFLER et al., 1974). It could not be decided whether all of the fragments were present in equimolar amounts, nor whether all the fragments were integrated contiguously.

There is preliminary evidence that the parental viral DNA can also become integrated rather early (2–6 h) after infection of KB cells with adenovirus type 2, and after infection with extracted DNA of adenovirus type 2 (GRONEBERG et al., 1975).

The biological function of the integrated form of adenovirus DNA in the productive system is not known at present. It is striking though that both parental and newly synthesized viral DNA in the integrated form is detectable early after infection, and that a surprisingly large number of viral DNA copies are inserted per cell. It is therefore conceivable that the fast sedimenting form of viral DNA plays an important role both in early and late transcription of viral DNA.

3. Integration of Viral DNA in Cells Abortively Infected with Adenoviruses

Adenovirus type 12 infects BHK 21 cells abortively (DOERFLER, 1968, 1969; STROHL, 1969a, b). Viral particles can be detected electron microscopically in the cytoplasm 10–20 min after inoculation (BROWN and DOERFLER, unpublished results). Viral DNA replication is not detectable in these cells (DOERFLER, 1969, 1970; DOERFLER and LUNDHOLM, 1970; RAŠKA et al., 1971), even with the most sensitive technique of reassociation kinetics (FANNING and DOERFLER, unpublished results). The DNA of adenovirus type 12 is transcribed in BHK 21 cells and the polysome-associated viral sequences correspond to early regions of the viral genome (RAŠKA et al., 1971; RAŠKA and STROHL, 1972). Viral RNA is found in association with polysomes starting 5–7 h postinfection with a maximum between 10 and 12 h postinfection (ORTIN and DOERFLER, 1975). The DNA of adenovirus type 12 can also be cleaved into six specific fragments with the R.RI restriction endonuclease from *Escherichia coli* (MULDER et al., 1974). There is evidence that the polysome-associated viral RNA is derived from fragments A and C, the terminal fragments of adenovirus 12 DNA (ORTIN and DOERFLER, unpublished results). The maximal size of the polysome associated, adenovirus 12-specific RNA in BHK 21 cells is approximately 1.5×10^6–1.9×10^6 daltons (ORTIN and DOERFLER, 1975). A complex containing parental viral DNA, protein and RNA was isolated starting 2 h after infection of BHK 21 cells with adenovirus type 12. This complex may represent a transcription complex (DOERFLER et al., 1972b).

In BHK 21 cells infected with adenovirus type 12 at sufficient multiplicities, 100% of the cells became positive for T-antigen (STROHL, 1969a). In BHK 21 cells blocked in DNA replication by serum depletion, cellular DNA synthesis was induced in the adenovirus type 12 infected cells with about the same efficiency as was T-antigen synthesis (STROHL, 1969b). The newly synthesized

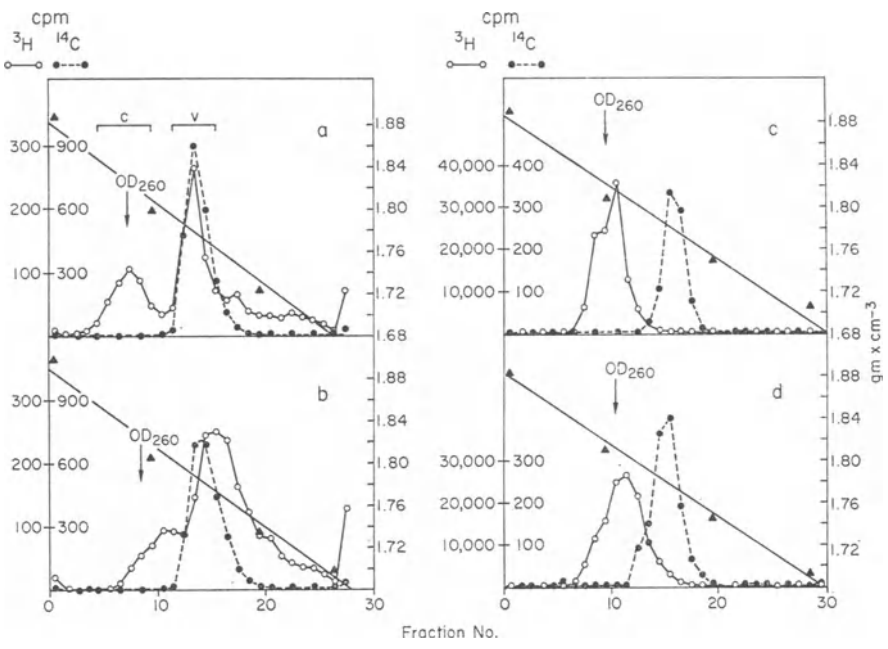


Fig. 7a—d. Integration of the parental viral DNA in BHK21 cells abortively infected with adenovirus type 12. Equilibrium sedimentation patterns in alkaline CsCl of DNA from ^{3}H-Ad12-infected 5BU-BHK21 cells (a, b) and from mock-infected 5 BU-BHK21 cells (c, d) before and after fragmentation. 5 BU-BHK21 cells were infected with ^{3}H-Ad12. At 42 h after infection, the cells were washed. Mock-infected 5 BU-BHK21 cells were grown in medium containing 2 μCi of ^{3}H-thymidine per ml. The DNA was extracted and analyzed by equilibrium sedimentation in alkaline CsCl density gradients. To each gradient ^{14}C-labeled Ad12 DNA was added as density marker. (a) DNA from Ad12-infected 5 BU-BHK21 cells was sedimented to equilibrium without prior fragmentation. The horizontal bars indicate the fractions of cellular (C) and viral (V) DNA. The arrows indicate the positions of the peaks of the OD_{260} in each of the graphs. (b) DNA extracted from the Ad12-infected cells was first fragmented by ultrasonic treatment; then ^{14}C-labeled Ad12 DNA was added and the mixture was sedimented to equilibrium. (c) DNA from mock-infected 5 BU-BHK21 cells which had been labeled with ^{3}H-thymidine was centrifuged to equilibrium in an alkaline CsCl density gradient. (d) DNA from mock-infected 5BU-BHK21 cells was fragmented by ultrasonic treatment; then ^{14}C-labeled Ad12 DNA was added and the mixture was centrifuged to equilibrium. This figure was taken from DOERFLER (1970)

DNA had a molecular weight between 5×10^6 and 10×10^6 (STROHL, 1969b; DOERFLER, 1969).

The DNAs of BHK21 cells and adenovirus type 12 can be separated either by size in alkaline sucrose density gradients or by equilibrium centrifugation in neutral and alkaline CsCl density gradients, provided the cellular DNA is substituted with the thymidine analogue 5-bromodeoxyuridine. When BHK21 cells are grown in the presence of 5 μg of 5-bromodeoxyuridine per milliliter for 4 days, approximately 50% of the thymidine residues in the cellular DNA

are replaced by 5-bromodeoxyuridine (DOERFLER, 1968). Such cells were infected with high multiplicites of ^{3}H-thymidine labeled adenovirus type 12 and the distribution of the parental label between the viral and cellular density positions was determined in neutral and alkaline CsCl density gradients at various times after infection. Starting approximately 16 h postinfection, parental viral label was detected in the cellular density position (DOERFLER, 1968). Upon ultrasonic treatment of the DNA prior to equilibrium sedimentation, the labeled DNA banded in a buoyant density position intermediate between that of viral and cellular DNA in both neutral and alkaline CsCl density gradients indicating that the parental label in the cellular DNA position was due to viral DNA integrated into cellular DNA by covalent linkage (DOERFLER, 1968; 1970) (Fig. 7). These results represented the first evidence for integration of viral DNA in mammalian cells.

These interpretations were confirmed by the results of experiments in which the parental viral ^{3}H-label isolated from the cellular density position in alkaline CsCl density gradients could be shown to hybridize to both viral and cellular DNA fixed to membrane filters (DOERFLER, 1970). Similar results were obtained with adenovirus type 12-infected Nil-2 cells (a hamster cell line) by ZUR HAUSEN and SOKOL (1969).

Since the specific radioactivity of the parental viral DNA could be determined accurately in the inoculum, the number of viral genome equivalents per cell could be calculated; 6–24 copies per cell were found in the integrated state, 140–430 copies were found free at 28 h postinfection, 5–55 viral DNA copies were found integrated at 45–47 h postinfection (DOERFLER, 1970). This estimate of the number of viral genome copies was not based on DNA-RNA calibration hybridization which might be subject to the criticism that hybrids were lost from the filters (HAAS et al., 1972).

Since the fate of parental viral DNA was followed in infected cells, it had to be ascertained that the ^{3}H-label incorporated into cellular DNA was not due to reincorporation of solubilized mononucleotides. This possibility was ruled out (1) by the finding that the parental label incorporated into cellular DNA, hybridized with viral and cellular DNA, and (2) by the observation that the same relative amounts of integrated viral genomes were found when DNA replication was chemically inhibited to $>96\%$ by cytosine arabinoside from 2 h before to 44 h after infection (DOERFLER, 1970). Unlike in the case of phage lambda, inhibition of protein synthesis by cycloheximide affected integration only slightly. After fragmentation of the intracellular DNA to pieces of $1\text{–}2\times10^6$ daltons by ultrasonic treatment, 75% of the parental viral label in the density stratum of cellular DNA shifted to an intermediate density position. This result indicates that fragments of viral DNA are integrated, rather than the entire adenovirus type 12 genome (DOERFLER, 1970).

In BHK21 cells abortively infected with adenovirus type 12, approximately 30% of the cell-associated parental viral DNA is linked covalently to cellular DNA. Seventy percent remain as free viral DNA. The viral DNA is fragmented prior to integration. The time course of appearance of viral DNA fragments

argues in favor of a precursor role of viral DNA fragments for the integrated form of viral DNA (BURLINGHAM and DOERFLER, 1971). It is conceivable that the fragments of the parental viral DNA are generated by the adenovirus-associated endonuclease (BURLINGHAM and DOERFLER, 1972). This endonuclease was found in association with the penton capsomer of the adenovirion (BURLINGHAM et al., 1971); however, recently it was possible to separate the endonuclease from the penton (DOERFLER and PHILIPSON, 1973; REIF et al., 1975). The actual role of the adenovirus-associated endonuclease for the integration process remains uncertain. Similarly, there is only suggestive evidence that this endonuclease may be responsible for the fragmentation of cellular DNA (DOERFLER, 1969; STROHL, 1969b; BURLINGHAM and DOERFLER, 1971).

Evidence has been published that the DNA of adenovirus 12 can become associated with chromosomes of human embryonic kidney cells (ZUR HAUSEN, 1967) and of BHK21 and Nil-2 hamster cells (ZUR HAUSEN, 1968). There was, however, no indication that this association was specific for one particular chromosome (ZUR HAUSEN, 1973). STICH and YOHN (1967) reported pulverization of chromosomes in hamster cells infected with adenovirus type 12.

MCDOUGALL et al. (1972) used the technique of *in situ* hybridization of adenovirus-specific RNA and detected adenovirus type 12-specific sequences in chromosomes of productively infected human cells and of cells transformed by adenovirus types 2 and 12.

In a later report, MCDOUGALL et al. (1973) demonstrated in the WL 24a-2-A cell line, a clonal derivative from the fusion of WI 38 cells (human lung fibroblasts) and LMTK$^-$ cells (thymidine kinase negative mouse cells), that infection with adenovirus 12 caused a specific lesion on human chromosome 17 and that this defect was close to the TK (thymidine kinase) locus. Adenovirus infection, at the same time, enhances the production of the host thymidine kinase (KIT et al., 1970). MCDOUGALL et al. (1972) considered it unlikely that adenovirus DNA was integrated at the altered chromosome site, but preferred the hypothesis that a virus coded protein "uncoiled" the chromosome at a site adjacent to the TK locus (MCDOUGALL et al., 1973).

YAMAMOTO et al. (1972) isolated less tumorigenic mutants of adenovirus type 12, and tested these mutants for the capability to integrate into the genome of nonproductively infected rat brain cells. Using gradient techniques (DOERFLER 1968, 1970), YAMAMOTO et al. (1972) found that the less tumorigenic mutants (particularly the mutant 1t3) were able to integrate, although they did not transform cells.

4. Persistence of Viral Sequences in Adenovirus Transformed Cells

At the present time, there is no conclusive evidence that adenovirus DNA is integrated in cells transformed by this virus, although, after the foregoing discussion it is reasonable to assume that viral genetic material persists in transformed cells in the integrated state. GREEN et al. (1970), and GREEN (1970b; 1972) have published results which support this conclusion.

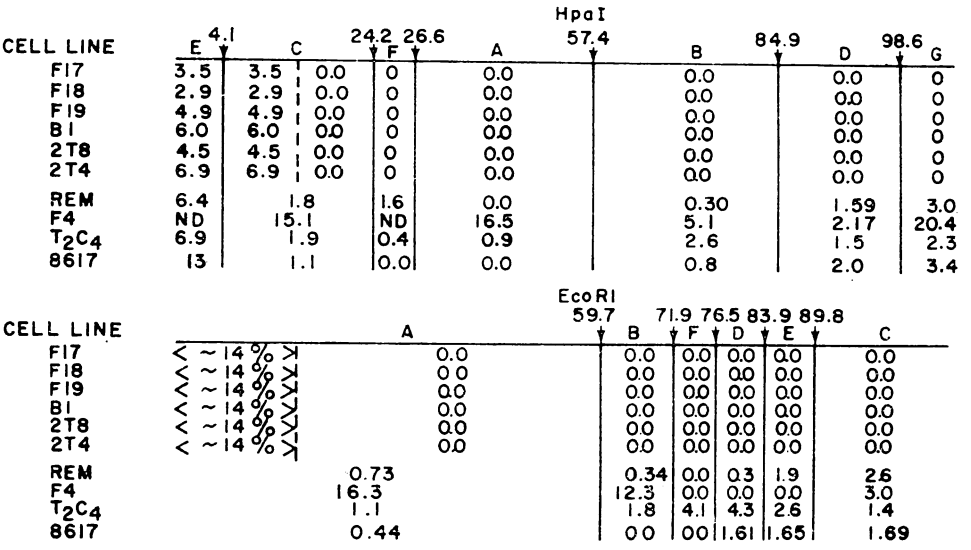


Fig. 8. Persistence of adenovirus type 2 sequences in adenovirus 2-transformed rat cells. Radioactively labeled adenovirus type 2 DNA was cleaved with the restriction endonucleases from Hemophilus influenzae (HpaI) and R.RI from E. coli (Eco RI), respectively. The sites of cleavage by these enzymes are indicated on the adenovirus 2 genome by arrows and the fractional lengths of the fragments are shown by percentage figures. Ten different rat cell lines transformed by adenovirus type 2 (GALLIMORE, 1974) were examined by the reassociation technique for the presence of adenovirus 2 genetic material. Each of the fragments indicated was annealed with itself and in the presence of DNA from the different rat cell lines. From the enhancement of the reassociation rate the number of fragment copies per cell were calculated for each of the different lines. The numbers of copies for each of the cell lines are listed under the respective fragments. This figure was taken from SAMBROOK et al. (1974)

The most accurate measurements of the number of viral genome copies per transformed cell came from determining the enhancement of the rate of reassociation of radioactively labeled viral DNA in the presence of the DNA from transformed cells (GELB et al., 1971). The first results of PETTERSSON and SAMBROOK (1973) using adenovirus transformed cells estimated that there was close to one copy of adenovirus 2 DNA per transformed rat cell. More precise data were recently obtained by SAMBROOK et al. (1974), GALLIMORE et al. (1974), and SHARP et al. (1974a; 1974b). These authors used specific fragments of adenovirus 2 DNA generated by restriction enzymes R.RI from *E. coli* and Hpa I from *Hemophilus parainfluenzae*. Each of these fragments was reassociated in the presence and absence of the DNA from ten lines of adenovirus 2 transformed rat cells. The scheme presented in Fig. 8 summarizes the data obtained by SAMBROOK et al. (1974), SHARP et al. (1974a), and GALLIMORE et al. (1975). In each adenovirus 2-transformed cell line a specific set of viral genes is present. It is common to all of the cell lines examined that the left, GC-rich molecular end of the viral DNA persists; only in one cell line is the entire genome represented. At this point, it is interesting to recall

the data obtained with hamster cells abortively infected with adenovirus type 12 where fragments of viral DNA are integrated (DOERFLER, 1970). Moreover, the pattern of persisting viral genetic material explains the observations that infectious virus cannot be rescued from adenovirus transformed cells by treatment with ultraviolet light, chemicals (LANDAU et al., 1966) or by fusion with permissive cells (cited in TOOZE, 1973, p. 451; WEBER, 1974).

GRAHAM et al. (1974a, b) were able to demonstrate that a fragment on the left terminal of the adenovirus 2 DNA molecule comprising the segment between 1 and 7.5% on the fractional length scale was sufficient to transform rat embryo cells, when these cells were directly infected with specific fragments of viral DNA (GRAHAM and VAN DER EB, 1973a, b). These data are consistent with those of SAMBROOK et al. (1974), SHARP et al. (1974a) and GALLIMORE et al. (1974). Furthermore, the data of GRAHAM et al. (1974a, b) confirm the notion that fragments of adenovirus DNA might be integrated, as was outlined in Section C., 3.

Recently, evidence was obtained that a fragment 300 nucleotide pairs away from the left end of the Ad 2 genome and 1500 nucleotide pairs in length was sufficient for transformation (VAN DER EB, personal communication).

There is yet another line of evidence in support of the model of integrated viral genomes in adenovirus transformed cells: Nuclear RNA isolated from adenovirus transformed cells was shown to contain both viral and cellular sequences in the same molecule (TSEUI et al., 1972; DARNELL et al., 1974). These molecules seemed to be precursors for messenger RNA, since they contained sequences of polyadenylic acid which were added during the processing of adenovirus messenger RNA molecules (PHILIPSON et al., 1971; WALL et al., 1972; DARNELL et al., 1974). The presence of viral and cellular sequences in the same RNA molecule suggests that these molecules are derived from viral sequences integrated into the host genome. SHARP et al. (1974) reported that in adenovirus transformed rat cells the virus specific RNA carries sequences complementary to only the segment of adenovirus 2 DNA between 0.03 and 0.1 fractional units on the physical map.

With the technique of *in situ* hybridization and autoradiography, LONI and GREEN (1973) detected virus-specific sequences over the nuclei and chromosomes of cells transformed by adenovirus types 2, 7, and 12. Similar results were obtained by MCDOUGALL et al. (1974). A correlation of viral sequences to specific chromosomes could not be established. Obviously, this experimental approach is of limited value to prove the integration of viral genomes.

Further work will still be needed to determine whether the adenovirus-specific genes shown to persist in adenovirus-transformed cells are indeed integrated.

D. Simian Virus 40 (SV40)

Among the DNA tumor viruses, simian virus 40 (SV40) and cells infected or transformed by this virus are the most extensively investigated systems.

There is evidence that the viral genome can be inserted into the host genome of productively or abortively infected and transformed cells.

1. The SV40 System

Simian virus 40 was discovered in 1960 by SWEET and HILLEMAN as a contaminant in monkey kidney cells which were used in the preparation of vaccines against poliomyelitis virus. EDDY et al. (1961, 1962) demonstrated that SV40 was oncogenic in newborn hamsters, and SHEIN and ENDERS (1962) were able to transform cells in tissue culture. SV40 infects monkey cells productively; whereas mouse cells are nonpermissive for this virus, and human and hamster cells are semipermissive for SV40, i.e. some cells in semipermissive cultures produce virus, while others are infected abortively. Rat cells, guinea pig cells, rabbit cells and bovine cells are infected non- or semipermissively. All these different cells can be transformed in tissue culture by the virus.

The parameters influencing viral transformation were studied most extensively in the 3T3 mouse cell line infected with SV40. When very high multiplicities of infection were used (10^6 PFU/cell), up to 40% of the cells were transformed (TODARO and GREEN, 1966a). For the fixation of the transformed state of the cell it was necessary that the SV40-infected 3T3 cells replicated at least once (TODARO and GREEN, 1966b). The genetic properties of the host cell seemed to play a decisive role in the frequency of transformation. Cells obtained from patients with Fanconi's anemia, Down's syndrome or Klinefelter syndrome had considerably increased transformation frequencies (TODARO et al., 1966; POTTER et al., 1970; MUKERJEE et al., 1970). A careful analysis of the properties of SV40-transformed cells has recently been published by RISSER and POLLACK (1974).

There is now very good evidence that SV40 gene A function is required for the maintenance of transformation (MARTIN and CHOU, 1975; TEGTMEYER, 1975; BRUGGE and BUTEL, 1975; OSBORN and WEBER, 1975).

The DNA of SV40 is a supercoiled, covalently closed circular molecule of molecular weight $3.0–3.6 \times 10^6$ (CRAWFORD and BLACK, 1964; TAI et al., 1972). The sedimentation behavior of SV40 DNA has been analyzed in detail (for review, see CRAWFORD, 1969): Form I DNA, the supercoiled, covalently closed circular molecule, sediments at 20 S in neutral gradients. Form II, the uncoiled circular molecule in which one of the strands has been nicked, sediments at 16S. Component III, sedimenting more slowly than form II, is present in some preparations of SV40 and represents linear DNA molecules of host origin. Recently, it has been very elegantly demonstrated that form I of SV40 DNA contains 20–24 superhelical turns per molecule (KELLER and WENDEL, 1974).

The DNA of SV40 has been extensively mapped with the aid of restriction enzymes (Fig. 9). DANNA and NATHANS (1971) discovered that the restriction enzyme from *Hemophilus influenzae* (SMITH and WILCOX, 1970; KELLY and SMITH, 1970) cut both strands of SV40 DNA at 11 specific sites which were mapped on the DNA relative to each other (DANNA et al., 1973) and relative

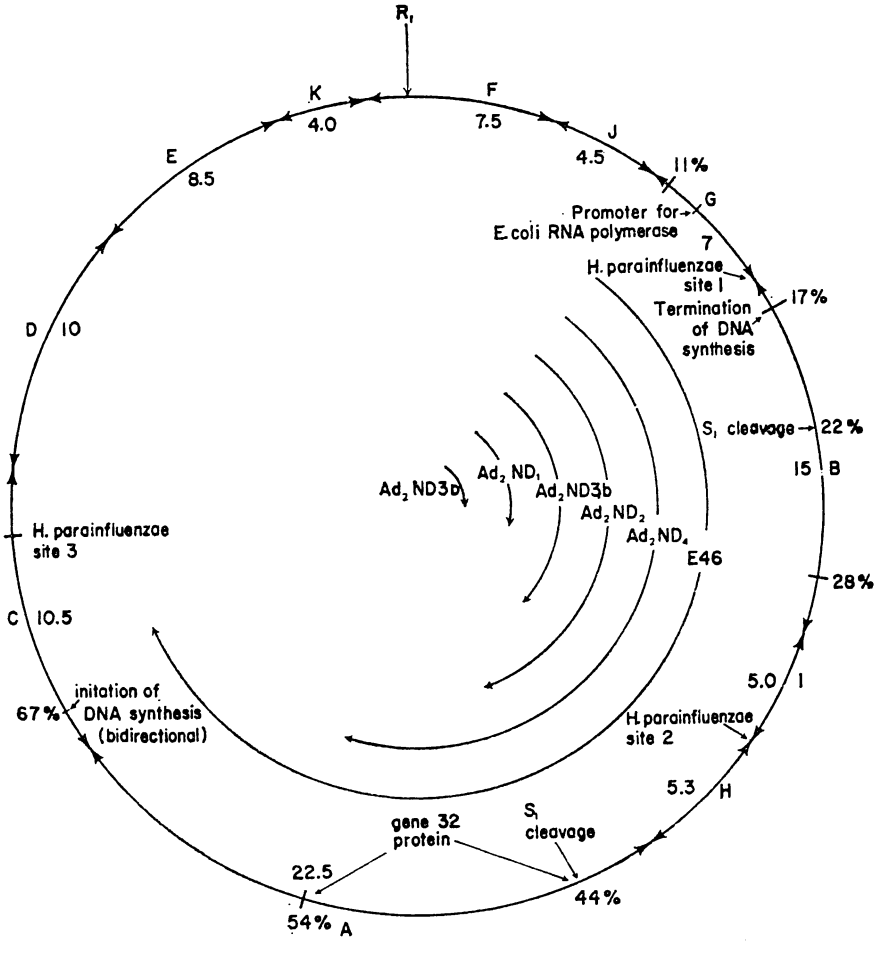
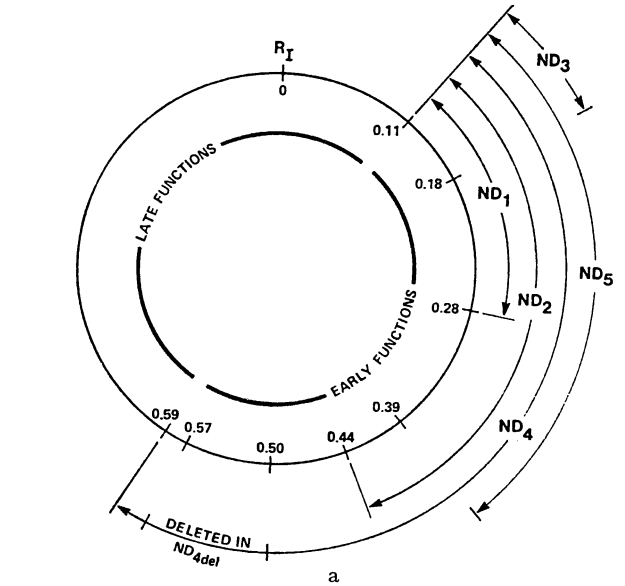


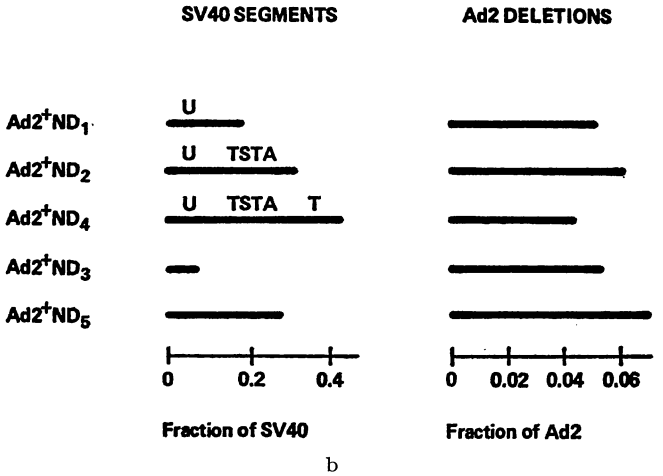
Fig. 9. The physical map of the SV40 genome. The sites at which bacterial restriction endonucleases cleave the viral genome are indicated with R_I, H. parainfluenzae, and by double arrow heads for the EcoRI endonuclease, the endonuclease from Hemophilus parainfluenzae, and the endonuclease from Hemophilus influenzae, respectively. The sites at which SV40 DNA replication is initiated and terminated, the site of a promoter for the E. coli RNA polymerase, and the site of binding to the gene 32 protein of phage T4 are also indicated. The segments of the SV40 genome integrated into defective and nondefective adenovirus-SV40 hybrids are also shown. This Figure was taken from TOOZE (1973)

to the unique cleavage site of the restriction enzyme $R{\cdot}R_I$ from *Escherichia coli* (MULDER and DELIUS, 1972; MORROW and BERG, 1972). The cleavage sites on SV40 DNA of the restriction endonuclease from *Hemophilus parainfluenzae* have also been mapped (SACK and NATHANS, 1973) (Fig. 9). The SV40 DNA fragments in six adenovirus-SV40 (nondefective ND_1–ND_5, and the defective E46+) hybrids (LEBOWITZ et al., 1974) (Fig. 10a, b) have been related to the SV40 DNA map (Fig. 10a, b). Compare also section F.

Several template functions and the regions of early and late transcription have been localized on the SV40 genome (DANNA et al., 1973; KHOURY et al.,



a



b

Fig. 10. (a) The SV40 genome. On the circular map the EcoRI cleavage point is the origin. The fragments of the SV40 genome inserted in the non-defective adenovirus-SV40 hybrids are designated by arrows. The common point of origin of the known hybrids lies at 11%, in the Hin-G fragment. This Figure was taken from MORROW et al. (1973). (b) SV40 segments of the non-defective adenovirus 2-SV40 hybrids. The SV40 segments form an overlapping series. The letters above each hybrid segment designate the SV40-specific antigens induced by each hybrid. The deletions in the adenovirus 2 genome for each of the hybrids are also shown. The common end point for the deletions is 0.14 fractional adenovirus 2 length units from one end of each hybrid. This Figure was taken from MORROW et al. (1973) (a) and KELLY and LEWIS (1973) (b)

1973; LEBOWITZ et al., 1974). Detailed analyses of the SV40 genome are presently being continued in several laboratories using restriction endonucleases from many different microorganisms. This analysis is also aided by the isolation

of SV40 variants with specifically altered genomes (BROCKMAN and NATHANS, 1974; MERTZ et al., 1974). The laboratories of FIERS (FIERS et al., 1974) and of WEISSMAN (DHAR et al., 1974) are presently sequencing the entire SV40 genome.

TEGTMEYER and OZER (1971) have isolated temperature sensitive mutants of SV40 which fall into four complementation groups. The defects in five of these mutants have been mapped on the SV40 genome by marker rescue experiments using *Hemophilus influenzae* and *Hemophilus parainfluenzae* restriction enzyme fragments of wild-type SV40 DNA (LAI and NATHANS, 1974).

SV40 DNA replication is initiated at the 67% point of the viral genome, i.e. in the *Hemophilus influenzae* C fragment and proceeds bidirectionally (DANNA and NATHANS, 1972; NATHANS and DANNA, 1972; FAREED et al., 1972). Temperature-sensitive mutants of SV40 (TEGTMEYER and OZER, 1971) which are defective in DNA replication at the nonpermissive temperature all fall into one complementation group. The enzymatic mechanism of SV40 DNA replication is not understood. One enzymatic function that must be involved in DNA replication of supercoiled circular molecules is an "untwisting" activity which converts form I DNA into a relaxed covalently closed circular molecule (LEVINE et al., 1970; FAREED et al., 1972). This activity has been demonstrated in nuclei of mouse embryo cells (CHAMPOUX and DULBECCO, 1972) and of KB cells (KELLER, 1974). During the replication of SV40 DNA a small amount of oligomeric forms of viral DNA is formed (JAENISCH and LEVINE, 1971).

Infection with SV40 and polyoma virus (see Section E.) induces cellular DNA synthesis in stationary cultures (DULBECCO et al., 1965; RITZI and LEVINE, 1970). This induction is dependent at least in part on a viral function, since the ts-3 mutant of polyoma virus fails to induce cellular DNA synthesis at the non-permissive temperature (DULBECCO and ECKHART, 1970; ECKHART and DULBECCO, 1974). To a certain extent, the temperature sensitivity of the ts 3 mutant can be diminished by the addition of fresh medium and serum to the culture (ECKHART and DULBECCO, 1974). The importance of the induction of cellular DNA synthesis for viral DNA replication cannot be assessed with certainty. Concomitant with or following the induction of cellular DNA synthesis, SV40 or polyoma infection leads to an increase in the activity of numerous enzymes in the infected cells. Most of these enzymes are involved in DNA replication. WEIL et al. (1974) have postulated that SV40 and polyoma infection exert a pleiotropic effect on the host genome thus leading to induction of cellular DNA synthesis, induction of host enzymes and the complex events that manifest themselves in the transformed state of the cell.

2. Integration in the Productive System

Evidence for the integration of SV40 DNA into the host genome in permissively infected African green monkey kidney (CV-1) cells was first presented by HIRAI and DEFENDI (1972). Insertion of viral DNA began 20 h postinfection and remained at a constant level up to 48 h postinfection. In this study viral

and cellular DNAs isolated from nuclei of infected cells were separated by precipitation of the high molecular weight DNA (HIRT, 1967) and by zone velocity sedimentation in alkaline sucrose density gradients. Viral sequences were detected by nucleic acid hybridization using ^{3}H-labeled c-RNA. Inhibition of DNA synthesis by D-arabinosyl cytosine did not interfere with integration.

In productively infected CV-1 cells, WALDECK et al. (1973) found SV40 DNA to be preferentially integrated into cellular DNA of 1.5 to 2 times the contour length of SV40 DNA.

HIRAI et al. (1974a) proposed that induction of cellular DNA synthesis might be a consequence of viral DNA integration. When CV-1 cells were infected with the temperature-sensitive early SV40 mutant ts 101 (ROBB and MARTIN, 1972), induction of cellular DNA synthesis and synthesis of the T, V and U antigens, and of viral DNA were blocked at the non-permissive temperature. Under these conditions, integration of viral DNA was absent in productively infected CV-1 cells (HIRAI et al., 1974b). However, the ts 101 mutant behaves very differently in nonproductive hamster cells: The ts 101 mutant induced T antigen and integrated into the host genome, but did not induce cellular DNA synthesis. Thus, there must be a block in this mutant uncoupling integration and induction of cellular DNA synthesis in hamster cells.

The number of integrated viral genomes in productively infected cells was estimated by HÖLZEL and SOKOL (1974). HÖLZEL and SOKOL (1974) devised an elaborate procedure to ascertian that CV-1 cellular DNA was not contaminated with free viral DNA: Cellular DNA was first precipitated by the HIRT method (1967) and subsequently subjected to velocity sedimentation in alkaline sucrose gradients, equilibrium centrifugation in ethidium bromide/CsCl density gradients and an additional velocity sedimentation in an alkaline sucrose density gradient. From the results of reconstitution experiments in which CV-1 DNA and free viral DNA had been artificially mixed the authors of this report calculated that cellular DNA extensively purified by this procedure contained as little as 0.006% free viral DNA. The cellular DNA isolated by the same procedure from CV-1 cells productively infected with SV40 contained more than 20000 integrated viral genome equivalents per cell. If these data are correct, one has to conclude that in the course of productive infection with SV40 massive recombination between the viral and host genomes occurs. On the other hand, it is difficult to rule out rigorously that the presumptive integrated SV40 genomes are not in fact concatenated linear or circular forms of viral DNA. Evidence for such oligomeric forms of SV40 DNA has been recently adduced (MARTIN; RIGBA, personal communication).

The mechanism by which the viral genomes are integrated and the significance of the integration process for viral DNA replication are under intensive investigation in a number of laboratories. Little definitive information is available as yet. The observation that in ts 101 infected monkey cells viral DNA replication and integration are absent (HIRAI et al., 1974b) could still be coincidental and does not necessarily prove that these events are closely linked.

An adenosine triphosphate-dependent polynucleotide ligase was detected in uninfected chicken, hamster, mouse, monkey, and human cells, as well as in mouse embryo, monkey kidney, and HeLa cells infected with polyoma virus, SV40, and vaccinia virus, respectively (SAMBROOK and SHATKIN, 1969). Presumably, a ligase could be involved in the integration event. However, the presence of a ligase in uninfected and infected cells obviously does not mean that this ligase is necessarily involved in the process of integration of viral DNA.

3. Substituted SV40 Genomes

When SV40 was serially passaged at high multiplicity of infection, non-infective virus particles were formed which contained supercoiled circular DNA which was heterogeneous in size and shorter than the DNA from SV40 passaged at low multiplicity (YOSHIIKE, 1968). Shortly after YOSHIIKE's 1968 report, it was demonstrated that SV40 grown in BSC-1 cells, a monkey cell line, contained closed circular viral DNA which hybridized also to cellular DNA (ALONI et al., 1969). Apparently, host sequences had been covalently linked to SV40 DNA. Virus particles containing DNA with both viral and cellular sequences covalently linked have to be distinguished from the "pseudovirions" which occur in SV40 preparations grown on African green monkey kidney cells (LEVINE and TERESKY, 1970) and in polyoma virus (MICHEL et al., 1967; WINOCOUR, 1967). In pseudovirions linear host cell DNA is encapsidated into virus particles.

The first systematic investigation of SV40 particles carrying host sequences in the viral DNA was presented by LAVI and WINOCOUR (1972). When BSC-1 monkey cells were infected with SV40 at low multiplicity of infection (0.032 PFU/cell), hardly any hybridization of this SV40 DNA to host cell DNA was observed. However, with increasing multiplicities of infection (0.16 to 3000 PFU/cell), an increasing amount of the newly synthesized SV40 DNA hybridized to cell DNA. This result was obtained, regardless of whether the SV40 DNA was extracted from purified virions or directly from the infected cells. The DNA from plaque purified virus did not contain cellular sequences. However, upon passage at high multiplicity, the viral DNA acquired host sequences. To explain the occurrence of these "substituted" SV40 genomes, LAVI and WINOCOUR (1972) postulated that this recombination event between the SV40 and host genomes was the consequence of integration and excision of the viral DNA during lytic infection. The substituted viral genomes which arose in this way might have an advantage during DNA replication, since they are shorter than intact viral DNA. Thus, the substituted genomes would be amplified after their generation by faulty excision. An alternate mechanism envisaged an increase in the frequency of recombination between viral and host genomes with increasing amounts of cellular DNA incorporated into the viral DNA.

The SV40 DNA from virions obtained after infection at low and high multiplicities were also analyzed for sequence homogeneity by the technique

of heteroduplex mapping (TAI et al., 1972). The DNA from virus passaged at low multiplicity carried deletions in 2% of the molecules, but substitutions could not be detected. The DNA derived from virions passaged seven times undiluted or at high multiplicities carried substitutions in 12 or 7%, and deletions in 13 or 11% of the molecules, respectively. More than 80% of the molecules carrying substitutions were shorter than SV40 DNA. The substitutions comprised about 20–30% of the wild type SV40 sequences.

Substituted SV40 DNA could be isolated starting with four different single plaque isolates from two different SV40 strains (777 and 776), when these were serially passaged undiluted (LAVI et al., 1973). The majority of the substituted genomes were non-infectious and were shorter than wild type SV40 DNA without homology to host DNA. Evidence was presented that the substituted SV40 genomes were able to replicate and incorporate ^{3}H-thymidine into host sequences.

Substituted SV40 DNA in which 66% of the molecules contained host DNA was digested with the restriction endonuclease from *Hemophilus influenzae* and subsequently analyzed by electrophoresis on polyacrylamide gels. Nine fragments could be resolved which had molecular weights different from the fragments obtained by digestion of wild type SV40 DNA. Two of the nine fragments contained highly reiterated cellular sequences. The majority of the fragments was thought to carry unique host sequences (ROZENBLATT et al., 1973).

More recent results from WINOCOUR's laboratory (FRENKEL et al., 1974; WINOCOUR et al., 1974) indicated that the integrated host sequences were not a random selection of all host sequences, and that the cellular sequences were predominantly of the non-reiterated type. Starting from a single plaque isolate, the host sequences were similar in substituted genomes arising from a particular set of serial passages, but they were different for genomes derived from passages originating from different single plaque isolates. There was no homology between the cellular sequences in substituted genomes of polyoma virus (LAVI and WINOCOUR, 1974) and SV40.

In certain instances, substituted SV40 genomes also contained a reiteration of the sequences in the Hin C and D fragments of the SV40 genome. An extreme example of this type was observed by BROCKMAN et al. (1973) in the sV particles from late passage virus. These particles carried DNA in which non-reiterated host DNA and an SV40 segment equivalent to part of the *Hin*-C fragment (site of initiation of DNA replication) were tandemly repeated. SV40 variants with specifically altered genomes were isolated by BROCKMAN and NATHANS (1974). The locations of rearrangements in the DNA of defective SV40 particles have also been mapped using the R.RI restriction endonuclease and heteroduplex formation with wild type SV40 DNA (RISSER and MULDER, 1974). Triplications of specific viral sequences in SV40-like DNA have been reported by FAREED et al. (1974) and KHOURY et al. (1974).

Of all substitutions in the SV40 genome, 75–80% were found to occur at specific sites, i.e. at the 25, 29, and 31% points relative to the Eco RI cleavage

site. These data suggested that there was some specificity with respect to the site of integration on the viral genome (CHOW et al., 1974).

Recently, YOSHIIKE et al. (1974) were able to rescue a defective SV40 genome from a transformed mouse cell line. The rescued genome had a deletion and an insertion at two different sites.

Considering all the evidence available at present, the most likely mechanism for the generation of substituted SV40 genomes is that of integration and faulty excision of the viral genome during lytic infection (see also preceding section). Integration may occur at only a limited number of sites in the host chromosome. There is evidence that the site of recombination on the viral genome is specific. It is still an open question whether, upon repeated passage, the substituted viral DNA molecules have a selective advantage during replication due to their reduced length or whether substituted genomes accumulate due to an enhanced capacity for recombination with the host, once host sequences have been incorporated into the SV40 genome.

4. Mixed Transcripts Containing Viral and Host Sequences

Further evidence for the notion of integrated SV40 DNA in productively infected and transformed (see also Section D. 5.) cells, comes from the analysis of the virus-specific RNA in these cells. LINDBERG and DARNELL (1970) reported that the heterogeneous nuclear RNA from SV40 transformed cells contained sequences homologous to SV40 DNA and was considerably longer than the virus-specific RNA isolated from polysomes. Presumably, high molecular weight RNA was processed to the m-RNA associated with polysomes and was possibly transcribed from integrated viral genomes. The RNA isolated from SV40-transformed 3T3 cells (mouse cells) was 0.75 to 3 times the length of single-stranded SV40 DNA. Similar results and conclusions were presented for the SV40-specific RNA isolated from SV40-transformed and lytically infected cells by DARNELL et al. (1970), TONEGAWA et al. (1970), and WEINBERG et al. (1972).

The conclusion that this high molecular weight RNA contained both viral and cellular sequences covalently linked was further supported by the results of DNA-RNA hybridization experiments. The high molecular weight nuclear RNA was hybridized to viral DNA, eluted from the filters and subsequently hybridized to cellular DNA in a second reaction. Very similar results were described for SV40 transformed cells (WALL and DARNELL, 1971) and for lytically infected cells (ROZENBLATT and WINOCOUR, 1972). JAENISCH (1972) chose a slightly different approach and demonstrated that a large portion of the SV40-specific RNA of longer than genome length was sensitive to digestion by pancreatic RNase after annealing to viral DNA.

Thus, it appears very likely that integrated SV40 genomes are co-transcribed with adjacent cellular sequences both in lytically infected and in transformed cells. We do not understand at present what role cellular and/or viral regulatory functions play in the transcription process.

5. Integration in SV40-Transformed and Abortively Infected Cells

Cell lines transformed by SV40 do not produce virus. However, the virus can be rescued from some of these lines by cocultivation with permissive cells or by fusion of the transformed cells with permissive cells (GERBER, 1966; KOPROWSKI et al., 1967; WATKINS and DULBECCO, 1967; TOURNIER et al., 1967). Fusion is usually induced by UV-inactivated Sendai virus (HARRIS and WATKINS, 1965). CROCE and KOPROWSKI (1973) and POSTE et al. (1974) have demonstrated that rescue of SV40 from SV40-transformed cells is possible by fusion with anucleate monkey cells and that nondividing confluent cultures of monkey cells are most efficient in the rescue process. SV40 complementary ^{3}H-labeled RNA (c-RNA) was synthesized *in vitro* with the DNA-dependent RNA polymerase from *E. coli*, and this RNA was used in DNA-RNA hybridization experiments for the quantitation of the number of viral genome copies in transformed cells (WESTPHAL and DULBECCO, 1968; SAMBROOK et al., 1968). (It should be mentioned that WESTPHAL and KIEHN (1970) demonstrated that the *E. coli* polymerase transcribed one strand of the SV40 genome faithfully.) Although hybridization methods gave qualitatively satisfactory results, this procedure proved to be problematic for the exact quantitation of the number of viral genome copies per cell. HAAS et al. (1972) found that DNA-RNA hybrids failed to be retained on filters when the DNA was saturated with c-RNA. Thus, the calibration curves on which the enumeration of viral gene copies was based, have to be corrected.

The state of the viral DNA in SV40-transformed cells was elucidated by SAMBROOK et al. (1968). High molecular weight cellular DNA was isolated in alkaline sucrose density gradients and shown to contain viral DNA covalently linked to cellular DNA. Free supercoiled circular SV40 DNA could not be found in these cells. This study, however, could not rigorously rule out the possibility that circular oligomers of free viral DNA cosedimented with high molecular weight cellular DNA. The number of integrated viral gene copies reported by SAMBROOK et al. (1968) were overestimates due to the above mentioned limitations of the filter hybridization method used. Nevertheless, this report presented evidence for the integrated state of SV40 DNA in transformed cells. BENJAMIN (1968) found no homology between SV40 or polyoma virus and the mitochondrial DNA of cells transformed by these viruses.

The presence of the SV40 surface (S) and tumor (T) antigens could be correlated with the persistence of SV40 genomes in SV40-transformed cells (LEVINE et al., 1970). Furthermore, these workers used improved hybridization techniques for the quantitation of SV40 genomes and arrived at values considerably lower than those reported by WESTPHAL and DULBECCO (1968) and SAMBROOK et al. (1968).

Further improvements in the techniques to quantitate viral genomes in transformed cells came with the application of DNA reassociation kinetics (BRITTEN and KOHNE, 1968; WETMUR and DAVIDSON, 1968) to the measurement of viral gene equivalents in transformed cells (GELB et al., 1971). In four

out of five SV40 transformed 3T3 cell lines, GELB et al. (1971) detected only one SV40 genome equivalent, and 3 SV40 equivalents were measured in the fifth cell line. In African green monkey kidney cells transformed by SV40, less than one viral genome equivalent was observed. Similar quantitative data were obtained by OZANNE et al. (1973). In SV40-transformed cells the viral DNA appeared to be linked to the nonreiterated sequences of cellular DNA (GELB and MARTIN, 1973).

In SV40-transformed cells, different segments of the SV40 genome were found at different frequencies by the technique of reassociation kinetics using SV40 DNA fragments which were produced by sequential cleavage with the restriction endonucleases Eco RI and Hpa I (SAMBROOK et al., 1974). Thus, transformed cells may contain one or several entire viral genome equivalents in addition to various fragments at different frequencies. The SVT2 line of SV40-transformed mouse cells contains six copies of a segment of SV40 DNA which carries the early region of the SV40 genome and one copy of the late region per cell. The early genes were found to be transcribed (BOTCHAN et al., 1974).

BOTCHAN and MCKENNA (1973) investigated the integrated SV40 genome in high molecular weight DNA from SV40-transformed mouse cells (SVT2 cells) with the restriction endonuclease Eco RI. Since this enzyme cleaves SV40 DNA only at one site, one would expect two fragments containing viral sequences, if there were one Eco RI cleavage site in the cellular DNA on either side of the viral genome. SVT2 DNA was cleaved with the Eco RI endonuclease, the fragments were resolved by electrophoresis on agarose gels, and the SV40-specific sequences were localized by hybridization with ^{3}H-labeled cRNA. Two peaks of SV40 DNA were found. The SV40-containing fragments had molecular weights of 3.1×10^6 and 1.8×10^6 daltons. The sum of these molecular weights is higher than the molecular weight of SV40 DNA. This finding suggests that there is covalent linkage between viral and host DNA. There are probably a limited number of integration sites on the host chromosome.

The integration of SV40 DNA was also demonstrated in SV40-transformed Chinese hamster embryo cells (HIRAI and DEFENDI, 1971). Hybridization of SV40 cRNA with the nucleolus-associated DNA and the total nuclear DNA from SV40-transformed Chinese hamster cells revealed SV40 specific sequences in both DNA fractions. These results are consistent with multiple integration sites of the SV40 genome (HIRAI et al., 1974b).

Moreover, it was shown that SV40 DNA became associated in an alkali-stable form with the DNA of abortively infected Chinese hamster embryo cells 15–20 h postinfection and that integration was not inhibited by D-arabinosyl cytosine and only partly inhibited by cycloheximide (HIRAI et al., 1971). COLLINS and SAUER (1972) investigated the fate of SV40 DNA infecting non-permissive 3T3 cells. At 48 h postinfection with viral DNA, a large number of genome equivalents had become associated with cellular DNA in an alkali-stable form. Cellular DNA replication did not appear to be a prerequisite for the integration process.

Some SV40-transformed cells, the "flat transformants" or abortively transformed cells, differ from regular transformants in that they do not grow in multiple layers of cells, and upon repeated passage, loose the characteristics of transformed cells altogether. SMITH et al. (1972) claimed that some of these abortively transformed cell lines contained as many as five SV40 genome equivalents per diploid cell.

In human cells transformed by SV40, the chromosome was identified which carried the SV40 genome (CROCE et al., 1973; CROCE et al., 1974a and b). These authors used techniques developed by WEISS (1970) and fused mouse cells deficient in thymidine kinase with an SV40 transformed human cell line which was deficient in hypoxanthine phosphoribosyltransferase. A concordant segregation of the gene(s) for SV40 T-antigen and human chromosome C-7 was observed. By fusing hybrid clones with the CV-1 cell line which is permissive for SV40 it could be shown that the genes for the viral capsid proteins were present only in those cells which were T-antigen positive. Furthermore, it was possible to rescue defective SV40 virions from hybrid subclones containing human chromosome 7, but not from those subclones which had lost that chromosome (CROCE et al., 1974a). These data indicated linkage between human chromosome C-7, the gene for the SV40 T-antigen and the integrated SV40 genome. Furthermore, CROCE et al. (1974b) concluded that the SV40 genome in human chromosome C-7 introduced gene(s) which were mandatory for continued cell division and might code for "transforming factors". Monkey cells can be transformed by the adenovirus 7-SV40 hybrid virus (see Section F.) (JENSEN and DEFENDI, 1968). In such transformants the expression of the SV40 T-antigen is linked to the monkey chromosome which corresponds to human chromosome C-7 (CROCE et al., 1974c).

These data present conclusive evidence for the chromosomal location of the integrated SV40 genomes and correlate the presence of the viral genome in a specific chromosome with the characteristics of virus-transformed cells.

Before concluding this section, the experiments of JAENISCH and MINTZ (1974) should be mentioned. These authors infected isolated mouse embryos at the early blastula stage with SV40 DNA, reimplanted these embryos into pregnant animals and demonstrated the persistence of SV40 genomes in several organs of the newborn mice originating from the infected blastulae. So far, no information is available as to whether the persisting viral genomes have any effect on the development of the first or future generations of these persistently infected mice or on the occurrence of tumors in these animals.

E. Polyoma Virus

In the history of the work on DNA tumor viruses, polyoma virus has played a major role and many important contributions have been made and continue to be made with this virus.

The first evidence for the persistence of the genome of polyoma virus (and of SV40) in virus-transformed cells came from experiments in which a small portion of the RNA from transformed cells was found to hybridize specifically

to viral DNA (BENJAMIN, 1966). In mouse kidney cells productively infected with polyoma virus, ACHESON et al. (1971) detected, late after infection, giant RNA molecules which were larger than the viral genome. At that time, it could not be decided whether these RNA molecules stemmed from multiple cycles of transcription or represented molecules transcribed jointly from cellular DNA and adjacent integrated polyoma genomes.

RALPH and COLTER (1972) isolated cellular DNA from mouse cells productively infected with polyoma virus by gradient centrifugation. The cellular DNA, presumably uncontaminated by free polyoma DNA, was hybridized with ^{3}H-labeled polyoma c-RNA. The experimental evidence suggested that polyoma genomes were present in the integrated form. Following infection of mouse and hamster (BHK21) cells, both the parental and the newly synthesized polyoma DNA was found associated with cellular DNA in an alkali stable form (BABINK and HUDSON, 1972). In human cells little, if any, polyoma DNA could be detected in the integrated form. Integrated viral sequences in polyoma-transformed cells were detected by MANOR et al. (1973).

Thus, evidence for integration of the polyoma genome in lytically infected and transformed cells has been obtained in several laboratories. However, the work has been less extensive than with SV40 or adenoviruses.

F. The Adeno-SV40 Hybrid Viruses

As has been demonstrated in the sections on adenoviruses and SV40, these viruses are able to integrate their genomes into the host chromosome both in productive and abortive infections, and the viral genomes persist in transformed cells in the integrated state as well. The adeno-SV40 hybrid viruses represent a group of viruses in which SV40 genes are integrated into the DNA of another virus, viz. the adenovirus genome. The study of these hybrid viruses in which the DNA's of two oncogenic viruses are covalently linked (BAUM et al., 1966) has yielded a great deal of information about the anatomy of the hybrid DNA and the function of the SV40 genes incorporated into the adenovirus DNA. However, very little if any information is available as yet on the mechanism of the integration event. Obviously, two different mechanisms can be envisaged:

1. Direct recombination between the viral genomes.

2. Integration of the two viral genomes into the host chromosomes at the same site or a closely linked site followed by faulty excision of the viral genomes.

The adeno-SV40 hybrid viruses could serve as a model for the study of integrated viral genomes.

The first adeno-SV40 hybrid viruses which were discovered (HUEBNER et al., 1964; ROWE and BAUM, 1964; RAPP et al., 1964) were defective viruses which could not be propagated without a nonhybrid adenovirus as a helper. The defectiveness hampered the detailed genetic analysis of the hybrids. In 1969, LEWIS et al. isolated the first nondefective adenovirus 2-SV40 hybrid ($Ad2^+$ ND_1 = nondefective hybrid 1) which was able to replicate with one-hit kinetics both in human embryonic kidney cells and in African green monkey

kidney cells (LEWIS et al., 1969). In cytolytic infections the Ad2$^+$ ND_1 hybrid virus induced a new SV40-specific antigen, the U antigen, but not the SV40-specific T antigen (LEWIS and ROWE, 1971). The U antigen was detected by using sera from SV40 tumor bearing hamsters and was stable to heating at 50° C for 30 min.

The DNA molecules from the Ad2$^+$ ND_1 hybrids contained Ad2 and SV40 sequences covalently linked (CRUMPACKER et al., 1970; LEVIN et al., 1971), as in the adenovirus 7-SV40 defective hybrid virus (BAUM et al., 1966). The hybrid virions did not contain supercoiled circular SV40 DNA (CRUMPACKER et al., 1970). Only a fraction of the Ad2$^+$ ND_1 genome consisted of SV40 sequences which corresponded to an equivalent of 17% of the SV40 genome (Figs. 9, 10). The biophysical characterization of the Ad2$^+$ ND_1 DNA revealed that it was practically indistinguishable from Ad2 DNA (CRUMPACKER et al., 1971). An analysis of the SV40 genes transcribed in the Ad2$^+$ ND_1 genome in lytically infected cells demonstrated that the SV40 segment present in the Ad2$^+$ ND_1 DNA corresponded to a portion, but not all, of that part of the SV40 genome which was transcribed early (OXMAN et al., 1971).

In 1973, LEWIS et al. reported the isolation of four new nondefective adenovirus 2-simian virus 40 hybrid viruses, designated Ad2$^+$ ND_2, Ad2$^+$ ND_3, Ad2$^+$ ND_4, and Ad2$^+$ ND_5 which were all derived from the same Ad2-SV40 hybrid population, as Ad2$^+$ ND_1 was. These hybrids differed significantly in their biological properties. The hybrids Ad2$^+$ ND_1, Ad2$^+$ ND_2, and Ad2$^+$ ND_4 grew both in human embryonic kidney (HEK) and primary African green monkey cells, whereas Ad2$^+$ ND_3 and Ad2$^+$ ND_5 could replicate only in HEK cells. Moreover, the nondefective hybrids differed in the spectrum of the SV40-specific antigens which they induced in productively infected cells (LEWIS et al., 1973; KELLY and LEWIS, 1973) (Fig. 10b).

The biophysical properties of the DNA's of the nondefective hybrids Ad2$^+$ ND_1–Ad2$^+$ ND_5 were very similar to those of Ad2 DNA. Ad2 and SV40 genes were linked covalently in all of the nondefective hybrid DNAs. The fraction of SV40 genes in each hybrid DNA was determined by hybridization with SV40 complementary RNA (HENRY et al., 1973), by competition hybridization of the RNAs which were synthesized in cells lytically infected with each of the hybrids (LEVINE et al., 1973), and by heteroduplex mapping of the hybrid DNA molecules with the RI restriction endonuclease fragments of SV40 DNA (KELLY and LEWIS, 1973; MORROW et al., 1973). The schemes in Figs. 9 and 10a summarize the results of these experiments and indicate precisely which sections of the SV40 genome were inserted into the adenovirus genome. The SV40 genes integrated into the Ad2 genome corresponded exclusively to early SV40 genes (Figs. 9, 10a) (LEVINE et al., 1973; MORROW et al., 1973; LEBOWITZ et al., 1974). Depending on the size of the inserted SV40 fragment, the deletions in the Ad2 genome varied in size (Fig. 10b). All the fragments of SV40 DNA present in the hybrid viruses had a common left end in the Hin-G fragment of SV40 and represented an overlapping series (Figs. 9, 10) (KELLY and LEWIS, 1973; LEBOWITZ et al., 1974). It is interesting to note that the

common end points of all the nondefective hybrids and of the E46+ hybrid lie in the Hin-G fragment which contains also the site of termination of SV40 DNA replication (DANNA and NATHANS, 1972).

Although the nondefective hybrid Ad2+ ND_5 apparently contains the SV40 genes responsible for the U and TSTA antigens, as can be seen in Fig. 10b, cells infected with the Ad2+ ND_5 hybrid do not produce these antigens (LEVINE et al., 1973; KELLY and LEWIS, 1973). There is no definitive explanation for this phenomenon at the moment. LEWIS and ROWE (1973) suggested that a frameshift caused by the insertion of the SV40 genes might be responsible for the lack of antigen expression. The same authors calculated the genes for the SV40-specific transplantation antigen (TSTA) to be located between 0.17 and 0.43 fractional map units on the SV40 map.

The synthesis of the SV40-specific T-antigen was found to be very sensitive to interferon inhibition in cells infected with SV40, whereas the adenovirus 2-specific T-antigen is expressed in interferon-treated cells infected with adenovirus type 2. When cells were infected with the non-defective hybrid Ad2+ ND_4, the synthesis of both the SV40 and the adenovirus 2 T-antigens became interferon resistant. This result was interpreted to indicate that the expression of the SV40 T-antigen in the Ad2+ ND_4 was under the control of adenovirus 2 genes (OXMAN et al., 1974). To substantiate this conclusion, OXMAN and colleagues (1974) examined the RNA transcribed in Ad2+ ND_4-infected cells and were able to demonstrate RNA molecules which consisted of adenovirus 2 and SV40 sequences covalently linked. Hence, transcription of the hybrid virus DNA had to be initiated in the adenovirus 2 or SV40 genome and had to continue across the point of linkage between the two genomes.

In the Ad2+ ND_1 and Ad2+ ND_3 hybrid virus DNAs, a preferred site for the initiation of transcription was found in *in vitro* transcription with *Escherichia coli* DNA-dependent RNA polymerase (ZAIN et al., 1973). So far, this initiation site could not be correlated with the site for initiation of transcription of the hybrid virus DNA in infected cells.

Adenovirus 2 and the nondefective hybrid viruses were also examined as to their oncogenic potential (LEWIS et al., 1974a and b). Adenovirus 2, and the Ad2+ ND_1–Ad2+ ND_5 hybrid viruses failed to produce tumors when injected into newborn hamsters. They did, however, transform hamster kidney cells in tissue culture. The transformed cells contained the adenovirus 2-specific T-antigen and adenovirus 2-specific RNA. Upon injection into hamsters, the transformed cells produced tumors with the histopathology characteristic for adenovirus-induced tumors. There was no evidence whatsoever that the SV40 genetic material integrated into the adenovirus 2 genome was involved in the transformation process and only some of the SV40-specific antigens (U, TSTA or T) were expressed in the cells transformed by the hybrid viruses. Thus, adenovirus type 2 was able to transform both rat (FREEMAN et al., 1967; GALLIMORE, 1974) and hamster cells (LEWIS et al., 1974a and b), and it therefore appears clear that adenovirus type 2 has oncogenic potential the expression of which might depend on the host and/or the experimental conditions employed.

G. Herpes Viruses

The problem of integration of viral DNA in herpes virus-infected cells has been investigated with a number of different herpes viruses. The most extensively studied system was that of the Epstein-Barr virus (EB virus) DNA persisting in human lymphoblastoid cell lines (for review, see KLEIN, 1973; ZUR HAUSEN, 1975). This virus has been implicated in the causation of Burkitt's lymphoma (BURKITT, 1962, 1963), infectious mononucleosis (HENLE et al., 1968) and nasopharyngeal carcinoma (OLD et al., 1966) in humans. Burkitts' lymphoma is a lymphoreticulo-proliferative disease which occurs mainly in children and which is endemic in the equatorial zones of Africa.

The synthesis of EB viral capsid antigens and of virus particles could be induced by 5-bromodeoxyuridine treatment of the Raji line and the NC 37 line (GERBER, 1972; HAMPAR et al., 1972). The Raji line (PULVERTAFT, 1965) and the NC 37 line were derived from patients with Burkitt's lymphoma (BURKITT, 1962, 1963), and these cells did not detectably produce EB virus or viral antigens prior to induction. An enhanced induction of EB virus was observed when cells in the early S phase of the cell cycle were treated with inhibitors of DNA synthesis, like 1-β-D-arabinofuranosylcytosine, hydroxyurea, or excess thymidine (HAMPAR et al., 1974).

In 1970, ZUR HAUSEN and SCHULTE-HOLTHAUSEN demonstrated by DNA-DNA hybridization that EB virus DNA persisted in the "non-producing" Raji line. With the same technique, the EB genome could be detected in cells derived from other cases of Burkitt's lymphoma and nasopharyngeal carcinoma, whereas cells from control tumors were free of EB DNA (ZUR HAUSEN et al., 1970). Estimates of the number of viral DNA copies came from studies employing DNA-RNA hybridization techniques using Epstein-Barr virus DNA-complementary RNA (NONOYAMA and PAGANO, 1971; ZUR HAUSEN, 1972). According to NONOYAMA and PAGANO (1971), the Raji line contained 65 Epstein-Barr virus genome equivalents, line F 265, 100 equivalents, and line NC 37, 80 genome equivalents. ZUR HAUSEN (1972) detected hybridizable material in cells derived from 27 cases of Burkitt's lymphoma and from 6 cases of nasopharyngeal carcinoma and came to comparable quantitative estimates. For reasons discussed above (page 40), these estimates must be regarded with reservations. NONOYAMA and PAGANO (1973) further refined the analysis by using the technique of reassociation kinetics for the investigation of Epstein-Barr virus DNA in cells from Burkitt's lymphomas and nasopharyngeal carcinomas and could confirm their earlier estimates. Recently, KLEIN et al. (1974) described human lymphoblastoid cell lines which did not contain detectable amounts of Epstein-Barr virus DNA or virus-specific antigens.

The physical state of the Epstein-Barr virus genome was investigated by techniques described in previous sections. The results of *in situ* cRNA-DNA hybridization experiments led ZUR HAUSEN (1972) to conclude that the Epstein-Barr virus DNA was associated with the chromosomes of Raji cells. In the non-producing Raji line, viral DNA was presumably not covalently linked to the host genome and it was suggested that the viral genome might be linked

to the host genome by alkali-labile bonds. (NONOYAMA and PAGANO, 1972; ADAMS et al., 1974; TANAKA and NONOYAMA, 1974).

The DNA of Epstein-Barr virus ($\varrho = 1.718$ gm/cm^3) (SCHULTE-HOLTHAUSEN and ZUR HAUSEN, 1970) and of human cells ($\varrho = 1.700$ gm/cm^3) can be separated by equilibrium centrifugation in CsCl density gradients. When the DNA from Raji cells containing 50–60 copies of Epstein-Barr virus DNA per cell is analyzed by equilibrium sedimentation in neutral CsCl density gradients, a large proportion of the intracellular Epstein-Barr virus DNA remains associated with cellular DNA. This association is stable towards pronase and phenol treatment. Part of the viral DNA linked to cell DNA shifts to intermediate density positions upon shear breakage of the cellular DNA (ADAMS et al., 1973). However, after alkali denaturation, the viral DNA separates from the host chromosome (JEHN et al., 1972; ADAMS et al., 1973; TANAKA and NONOYAMA, 1974). It was suggested that the Epstein-Barr virus DNA was linked to the host chromosome by alkali-labile bonds.

One out of three cottontop marmosets inoculated with Epstein-Barr virus developed lymphoproliferative disease. Between 1 and 2 viral genome equivalents per cell were detected in tumors and infiltrates of the spleen from these animals (ZUR HAUSEN et al., 1974).

The role of herpes simplex virus 2 in human cancers, particularly in human cervical cancers has been an ardently debated issue (NAIB et al., 1966; RAWLS et al., 1969) and is far from being settled. Experimental tumors have been produced by human herpes simplex virus in hamsters and in mice (NAHMIAS et al., 1970, 1971). DUFF and RAPP (1971) demonstrated that hamster cells could be transformed *in vitro* by UV-inactivated herpes virus type 2 and that the transformed cells did not contain leukosis virus markers (RAPP et al., 1972). Transformation of rodent cells by UV-inactivated herpes simplex virus types 1 and 2 was shown by GARFINKLE and MCAUSLAN (1974), whereas DARAI and MUNK (1973) reported that human embryonic lung cells exhibited properties of transformed cells after infection with UV-inactivated herpes virus type 2. FRENKEL et al. (1972) reported that in a single case of a human cervical tumor which did not contain infectious herpes simplex virus 2, the tumor cells contained 39% of the viral DNA covalently linked to highly repetitive sequences of host DNA. This finding has not yet been repeated in other laboratories. At the time of this writing, the role of herpes viruses in human cancers appears to be a completely open question.

Before concluding this section, another tumor disease caused by a herpes virus has to be mentioned, namely Marek's disease (BIGGS et al., 1968; BIGGS, 1973). Marek's disease is a lymphoproliferative illness in chicken which is caused by a herpes virus (CALUCK et al., 1970; NAZERIAN and WITTER, 1970). NAZERIAN et al. (1973) established by DNA-RNA hybridization experiments using RNA complementary to Marek's disease virus DNA that in cells isolated from five different Marek's disease tumors (derived from ovary, liver and testis), 3–15 viral genome equivalents per cell were present. The state of these viral genomes in the cells has not yet been determined.

In summary, one can conclude that in herpes virus-transformed cells and in cells from tumors in the etiology of which herpes viruses are incriminated, viral genetic material has been shown to persist and may be associated with cellular DNA by alkali-labile bonds. The molecular state of the viral genomes in the cell could also be comparable to that of a bacterial plasmid. The role of herpes viruses in the causation of human malignancies will require further investigations.

H. RNA Tumor Viruses

It is impossible to summarize in this article the entire RNA tumor virus field. Moreover, many extensive reviews on RNA tumor viruses have been published (GREEN, 1970; VIGIER, 1970; TEMIN, 1971; TEMIN and BALTIMORE, 1972; GALLO, 1972; HILL and HILLOVA, 1974). Therefore, this chapter will be limited to a survey of the evidence for integration of the RNA tumor virus genomes.

1. The Provirus Hypothesis

In 1964, TEMIN proposed the provirus hypothesis to explain the replication of the RNA tumor virus genome. In its simplest form, the provirus hypothesis states that after infection, the genome of RNA tumor viruses is transcribed into a DNA copy, that this DNA is integrated into the host chromosome, and that the progeny RNA is synthesized on this DNA template. This at the time daring hypothesis was based on the observation that the replication of and transformation by Rous sarcoma virus (RSV) could be blocked by actinomycin D (TEMIN, 1963) and by inhibitors of DNA replication (TEMIN, 1964). Further evidence for the involvement of DNA in the replication of the RSV genome was published by BADER (1964), BADER and BADER (1970), BALDUZZI and MORGAN (1970), and BOETTIGER and TEMIN (1970). These authors demonstrated that transformation of chicken embryo fibroblasts by RSV was blocked 50–90% when RSV-infected cells were maintained for 18–24 h in medium containing 5-bromodeoxyuridine and then exposed to visible light.

TEMIN's provirus hypothesis met skepticism for many years, mainly because it postulated a direct violation of the much revered dogma of molecular biology. This dilemma was resolved when TEMIN and MIZUTANI (1970), and independently BALTIMORE (1970), discovered the enzyme reverse transcriptase in Rous sarcoma and Rauscher mouse leukemia virions, respectively. The reverse transcriptase was able to catalyze the synthesis of DNA on a natural or synthetic RNA template and required all four deoxyribonucleoside triphosphates. The properties of the reverse transcriptase and the details of the reaction have been extensively studied, and have been reviewed by TEMIN and BALTIMORE (1972). Shortly after the discovery of the reverse transcriptase, it became clear that several enzymes were associated with RNA tumor viruses (MIZUTANI et al., 1970) and were apparently involved in the replication of the RNA tumor virus genome. It was also recognized that a DNA-RNA hybrid was formed as an intermediate in the reverse transcription process (ROKUTANDA

et al., 1970; SPIEGELMAN et al., 1970). In the meantime, a huge amount of information has accumulated on reverse transcriptase and its possible role in RNA tumor virus-infected and RNA tumor virus-transformed cells, as well as in normal cells from early stages of development.

2. Virus-Specific DNA in RSV-Transformed Cells

Physical evidence for the occurrence of Rous sarcoma virus-specific DNA in RSV-transformed cells came from the work of VARMUS et al. (1973a). Using the reassociation technique, these authors could demonstrate that Rous sarcoma virus-specific DNA was present in RSV-transformed rat and mouse cells, but not in normal cells. Two populations of double-stranded DNA were identified, corresponding to approximately 5 and 30% of the 70 S RSV RNA genome. The RSV-specific DNA was detected in the non-repetitive fraction of the DNA from transformed cells. These results bridged the gap at that time between the abundance of information on the mechanism of the reverse transcriptase reaction and TEMIN's provirus hypothesis (TEMIN, 1964) postulating a DNA intermediate in cells infected with and/or transformed by RSV.

A DNA provirus has also been demonstrated in the lytic cycle of Visna virus which is not known to be oncogenic (HAASE and VARMUS, 1973).

3. Integrated RSV DNA

BRITTEN and KOHNE (1968) had demonstrated that when unsheared cellular DNA was incubated to c_0t values which allowed the reassociation of repeated, but not unique sequences, "networks" of DNA were formed which precipitated and could be separated from the remainder of the DNA by low speed centrifugation. Using this technique, VARMUS et al. (1973b) were able to show that RSV-transformed permissive (duck) and non-permissive (mammalian) cells contained RSV-specific DNA covalently integrated into reiterated sequences of the host genome. In mouse 3T3 cells, 0.8 copies of RSV-specific DNA per cell were found in the integrated state 12 h postinfection; in duck cells 4–6 integrated copies of RSV-specific DNA per cell were detected between 6 and 24 h postinfection. The time course of appearance of integrated RSV-specific DNA was very similar to that reported in cells infected with adenovirus type 12 (DOERFLER, 1968) and SV40 (HIRAI and DEFENDI, 1971).

In a recent report, VARMUS et al. (1974) outlined the sequence of steps early after infection of permissive cells by Rous sarcoma virus. Within the first 3 h postinfection, double-stranded, RSV-specific DNA of molecular weight 6×10^6 was synthesized in the cytoplasm of infected cells by the RSV-associated DNA polymerase. The RSV-specific DNA was presumably converted into a supercoiled, covalently closed circular molecule. This conclusion was still tentative, as it rested solely on the results of equilibrium sedimentation experiments in CsCl-ethidium bromide density gradients and of velocity sedimentation in alkaline sucrose gradients. Integration into the nuclear host DNA began early after infection. Integration of the RSV-specific DNA could be inhibited to

83% by ethidium bromide (1.0 μg/ml). The effect of ethidium bromide on cell transformation by RSV was not yet clear; preliminary results suggested however that transformation was suppressed by ethidium bromide.

Evidence for the covalent linkage of avian myeloblastosis virus (AMV)-specific DNA to the DNA of chicken embryo fibroblasts and to the DNA of AMV-transformed cells was presented by MARKHAM and BALUDA (1973). Free AMV-specific DNA was not found in these cells.

An elegant way of demonstrating the association of RSV-specific DNA with the DNA of RSV-transformed cells was worked out by HILL and HILLOVA (1972, reviewed in 1974) and by HILL et al. (1974). These authors isolated the cellular DNA from XC rat cells transformed by the Prague strain of RSV, purified the DNA after alkali denaturation and neutralization by equilibrium centrifugation in neutral CsCl density gradients, and demonstrated that this DNA, both in the native and denatured forms, had the capacity to transform chicken embryo fibroblasts in culture. These results provided further support for the replication scheme of RNA tumor viruses via a DNA intermediate and demonstrated that the entire genetic equivalent of RSV required for the transformation event was associated with the genome of RSV-transformed mammalian cells.

I. Endogenous Viral DNA

Several lines of evidence support the conclusion that probably all avian and murine cell lines contain the genome of RNA tumor viruses either in part or in toto. In many instances, this genetic information can be activated by various physical and chemical agents to produce infectious virions. It is likely, albeit not proven in all cases, that the virus-specific information is transmitted vertically from generation to generation in the form of the provirus, i.e. virus-specific DNA integrated into the host genome. The postulate of vertically transmitted virus-specific DNA is part of the so-called oncogene hypothesis proposed by HUEBNER and TODARO (1969). The evidence for endogenous viruses and their activation was extensively reviewed by ROWE et al. (1972a), by ROWE (1973), by LILLY and PINCUS (1973), and by HANAFUSA et al. (1974).

It had been observed by several investigators that supposedly virus-negative strains of mice or cells derived from these animals contained virus particles of the C-tpye (BERNHARD, 1960), particularly after induction of tumors in the animals by physical or chemical agents. Furthermore, it became apparent that a large number of cell lines established from presumably normal animals contained the group specific, gs, antigen (HUEBNER et al., 1964) or virion antigens (GEERING et al., 1966; STOCKERT et al., 1971) of known RNA tumor viruses. The widespread presence of the gs antigen in normal murine tissues could be demonstrated by the very sensitive radioimmune assay (PARKS et al., 1973).

1. Induction of RNA Tumor Viruses in "Normal" Cells

When BALB/c mouse embryo cells were maintained in culture by frequent passage at high cell density (3T12 line), some of the established cell lines started

to release murine leukemia viruses (AARONSON et al., 1969). This observation, amongst others, led to the hypothesis that the virus-specific genetic information was transmitted vertically as part of the host genome (HUEBNER and TODARO, 1969). Very similar results were reported for noninfectious AKR mouse embryo cell lines after repeated transfer in culture (ROWE et al., 1971). The detection of virus induction became possible with the development of a quantitative assay for murine leukemia viruses (ROWE et al., 1970). The induction of virus replication could be enhanced in several clonally derived sublines of AKR cells by irradiation with X-rays or ultraviolet light, or by transformation with SV40. Virus could also be induced by X-irradiation in BALB/3T3 cells (POLLACK et al., 1970). ROWE et al. (1971) concluded from these observations that probably all the cells in the AKR lines contained the entire viral genome. At the same time, WEISS et al. (1971) were able to demonstrate that avian tumor viruses could be induced in normal, gs antigen negative chicken cells after exposure to ionizing radiation, chemical carcinogens or mutagens. These authors established the identity of the induced virions with the known avian tumor viruses by comparing several physical, chemical and genetic parameters. TODARO (1972) found a striking correlation between the transformed state of "spontaneously" occurring transformed clones of BALB/c 3T3, 3T6 and 3T12 cells and the release of high titers of type C virions and concluded that the control of endogenous type C viruses was affected by the transformed state.

More quantitative investigations became possible after the discovery that treatment of AKR and BALB/c cells with 5-iododeoxyuridine (5-IUDR) or 5-bromodeoxyuridine (5-BUDR) induced murine leukemia viruses in 0.1–0.5 percent of the cells (LOWY et al., 1971; AARONSON et al., 1971). This induction occurred as early as 3 days after the addition of 5-IUDR or 5-BUDR. The halogenated pyrimidines have to be incorporated into DNA in order to exert the virus-inducing effect (TEICH et al., 1973).

2. Virus-Specific DNA and RNA in "Uninfected" Cells

With the availability of in vitro synthesized, radioactively labeled DNA-copies of the RNA tumor virus genomes,it became possible to screen the DNA from "normal", i.e. nonproducing avian and murine cell lines for the presence of virus-specific nucleotide sequences. Using a radioactively labeled viral DNA probe, it was determined by the reassociation technique that chicken cells which did not synthesize RSV-specific gs antigen or the chick helper factor (HANAFUSA et al., 1970) contained up to 16 viral gene copies per diploid cell (VARMUS et al., 1972). A similar number of viral gene equivalents was measured in gs antigen positive cells which were also RAV-O (Rous associated virus) positive. Similar results were reported by BALUDA (1972) and also by NEIMAN (1973), who found with the DNA-RNA hybridization technique that normal chicken embryos and adult chickens contained 1.7–4.6 viral genome equivalents per cell. Leukemia cells and cells infected with RSV or AMV carried 4 and 13 viral DNA equivalents, respectively.

The number of murine leukemia virus DNA equivalents in "normal" AKR, C58 and NIH/3T3 cells was measured by reassociation experiments using ^{3}H-labeled Kirsten murine leukemia virus DNA (GELB et al., 1973), and was found to range from 9.5 to 13.8 equivalents per cell. The number of viral DNA copies could not be correlated with differences in leukemia incidence among different mouse strains. GELB et al. (1973) concluded that the murine leukemia virus-specific DNA was probably covalently linked to cellular DNA, since the viral DNA co-sedimented with high molecular weight chicken cell DNA. This conclusion was premature, as the cellular DNA had been isolated from neutral sucrose gradients.

In addition to RNA tumor virus-specific DNA, viral RNA was also found in uninfected chicken (HAYWARD and HANAFUSA, 1973) and mouse cell (BALB/c 3T3) lines (BENVENISTE et al., 1973). Cells producing the avian tumor virus group-specific antigen and the virus-related helper factor carried 3–40 copies of viral RNA, whereas RSV-infected cells contained 3000–4000 viral RNA molecules per cell (HAYWARD and HANAFUSA, 1973). Similarly, a low level of viral-related RNA was present in BALB/c 3T3 cells and the amount of RNA was markedly increased in murine leukemia virus transformed cells (BENVENISTE et al., 1973).

More detailed studies, in which the kinetics of reassociation of a virus-specific, ^{3}H-labeled DNA probe were analyzed in the presence of DNA from "normal" AKR mouse cells and from "normal" NIH Swiss mouse cells, revealed that the AKR cells contained two sets of DNA sequences, one represented ten times, the other one four times per haploid AKR cell genome. The NIH Swiss cells carried 15 copies of only one set of DNA sequences (CHATTOPADHYAY et al., 1974).

In "normal" cells, the integrated form of the endogenous avian myeloblastosis virus DNA was associated with cell sequences which were reiterated 1200 times (EVANS et al., 1974). Each DNA equivalent corresponded in size to the 35S RNA subunit of the virion. In AMV-infected cells, however, additional viral sequences might be integrated adjacent to unique cellular sequences, in tandem with endogenous viral DNA.

3. Genetic Analysis

Several laboratories have been using classical Mendelian genetics to investigate host control factors which influence the susceptibility to infection with murine leukemia viruses (for review, see ROWE, 1973). The 23 strains of naturally occurring murine leukemia viruses which were tested fell into two classes: They were able to infect mouse embryo cells either of NIH Swiss mice (N-tropic viruses) or of BALB/c mice (B-tropic viruses). When cells from the F_1 hybrid mouse generation (N-type × B-type) were tested, they were resistant to both N-type and B-type viruses. Backcross studies suggested that a single genetic locus was responsible for the resistance towards virus infection (PINCUS et al., 1971a). Further studies indicated that this N-B locus was identical to the Fv-1 locus (LILLY, 1970) which had been found to determine sensitivity

of cells to infection with Friend leukemia virus. Sensitivity of certain mouse strains to infection with Friend leukemia virus is also determined by a second genetic locus, Fv-2 (LILLY, 1970) which does not seem to be effective in tissue culture (PINCUS et al., 1971b). All mouse strains found to be sensitive at the Fv-1 locus were susceptible to N-type virus.

The analysis of genetic crosses between different mouse strains for the expression of infectious murine leukemia virus became feasible after a reliable plaque assay had been developed (ROWE et al., 1970), and after the discovery that virus was present in the tail tissue of mice starting 2–6 weeks of age (ROWE and PINCUS, 1972). These studies led to the conclusion that AKR mice had two unlinked, autosomal loci either of which sufficed to induce infectious virus when crossed into Fv-1^n strains which did not produce virus. One of these loci (V_1) was located on linkage group I, 25–30 map units from the locus for albino (ROWE, 1972). The Fv-1 locus suppressed the expression of infectious virus. This suppresion could be overcome by induction with 5-iododeoxyuridine. The host range of the virus acquired by the hybrid generation from crosses with the AKR strain was always of the AKR type. These results were interpreted as evidence that the virus-inducing loci contained the murine leukemia virus genome (ROWE and HARTLEY, 1972). The virus-inducing locus, designated AKv-I, which presumably carries the integrated viral DNA, was located on the genetic map of AKR mice. This locus was found to map on linkage group I, about 12 map units from Gpi-1, the locus for the isozymes of glucose phosphate isomerase (ROWE et al., 1972b). More recent studies seem to suggest that there are at least 4 different sites for virus induction in the mouse genome (ROWE, 1973; LILLY and PINCUS, 1973).

Combining genetic analyses and reassociation kinetics, CHATTOPADHYAY et al. (1975) presented conclusive evidence that the C-type virus inducing locus Akv-1 was viral genetic material.

IV. Conclusions

A. Facts

Integration of viral into host genomes has been well established for many years in the bacteriophage systems, most notably for bacteriophages λ, P22, P2 and Mu. Among animal viruses, integration is well documented for the adenovirus and SV40 DNAs, and to a lesser extent for polyoma virus DNA. There is a peculiar case of an alkali-labile association of Epstein-Barr virus DNA with host DNA in lymphoblastoid cells. Moreover, convinving evidence has been presented for the covalent insertion of the reverse transcripts of tumor virus RNA, particularly in the Rous sarcoma, the avian myeloblastosis, and the murine leukemia virus systems. Complete copies of the DNA of C-type viruses were found in the somatic cell DNA of the chicken, Chinese hamster, Syrian hamster, mouse, rat, cat, pig, and baboon (TODARO et al., 1974). In the case of the RNA tumor viruses, it is now clear that the DNA genomes of these viruses or parts of them can be vertically transmitted in animals from one

generation to the next. It has been suggested that the genomes of RNA tumor viruses may have been transmitted from one species to another at some time during evolution (TODARO et al., 1974). Thus, it may be appropriate to view genes of this nature from a dualistic point of view, since it may become difficult to designate a given gene as viral or as belonging to the host.

Only in one instance can the site of integration of viral genes be localized on a specific chromosome of eukaryotic cells: SV40 DNA can become associated with human chromosome C-7 (CROCE et al., 1973). In human cells infected with adenovirus type 12, a specific break on chromosome 17 close to the thymidine kinase locus has been reported (MCDOUGALL et al., 1973). However, there is no evidence to suggest that the site of this break is identical with the site of integration.

The persistence of the viral genome in virus-transformed cells has been amply demonstrated. Integration of viral genetic material in transformed cells has been shown in SV40-transformed cells, in cells transformed by RNA tumor viruses, and there is circumstantial evidence for integration in cells transformed by adenoviruses. In most cases of virus-transformed cells, the persisting viral genes continue to be transcribed, at least in part, and virus-specific messenger-RNA, frequently of high molecular weight, is found associated with the polysomes. Thus, it is clear that viral genes are translated in transformed cells. Perhaps, the integrated viral genes are transcribed together with host genes. Thus, it is conceivable that transcription of viral genes becomes subject to host control or *vice versa*.

A very well studied example of integration is that of the integration of the genome of one virus into the DNA of another virus. The different nondefective SV40-adenovirus 2 hybrid viruses contain in their DNAs an overlapping series of SV40 fragments. These fragments represent 7, 17, 28, 33, and 48% of the SV40 genome in the $Ad2^+ ND_3$, $Ad2^+ ND_1$, $Ad2^+ ND_4$, $Ad2^+ ND_2$, and $Ad2^+ ND_5$ hybrids, respectively (Fig. 10a). The fraction of the adenovirus 2 genome which becomes deleted as a consequence of integration, is indicated in Fig. 10b. SV40 and adenovirus genes are cotranscribed in cells infected with the nondefective hybrid viruses, and there is evidence that the expression of some of the SV40 genes is subject to control by the adenovirus genome.

It has been clearly shown that in substituted genomes of SV40, host DNA is covalently linked to viral DNA. It is possible, albeit not proven, that these substituted viral genomes are generated by faulty excision of the SV40 DNA from the host genome and subsequent amplification of these genomes by preferential replication due to their lower molecular weight. Such faulty excision has been extensively studied in the defective lambda genomes.

B. Problems

Above all, it is mandatory for a review to point out open problems, in particular those which will become amenable to experimental analysis in the future.

1. Number of Viral Gene Copies Per Cell

In several systems, the number of viral gene copies persisting per virus-transformed cell has been determined with great accuracy. Considerably less reliable are the estimates of the number of viral gene copies integrated into the host genome in productively infected cells. Here the additional problem arises of distinguishing between "replication monsters" and truly integrated viral genomes. The technology for determining the exact number of integrated viral genome equivalents is available and is being refined. Hence, this type of analysis should be completed for most virus cell systems in the near future. Furthermore, the availability of a large number of restriction endonucleases permits a precise determination of those segments of the viral genome which persist in the integrated form in cells infected or transformed by viruses.

2. Site of Integration

One of the most important questions in work on integration in eukaryotic cells remains open, namely whether there is a specific site or sites of insertion into the host chromosome. With the advanced state of bacterial genetics, detailed information is available for the site(s) of integration of bacteriophage lambda or Mu. In eukaryotic cells, some time will be required, before a comparable level of genetic sophistication will be accomplished, although the use of hybrids between restriction enzyme fragments of eukaryotic DNA and of bacterial plasmids may lead to progress in this field. Moreover, recent developments in chromosome banding techniques (CASPERSSON, 1970) and chromosomal analysis (RUDDLE, 1971; BOONE et al., 1972; RUDDLE, 1972) point out new approaches for investigations on the sites of interaction with viral genomes. Using classical genetic techniques, rapid progress in the localization of the integrated viral genome on the host chromosome has been made for murine leukemia virus DNA (ROWE, 1973; LILLY and PINCUS, 1973).

Moreover, the availability of the restriction endonucleases may permit specific excision of integrated viral genes and possibly, an analysis of the adjacent host genetic material. The knowledge of the sites of integration is an important precondition for an understanding of the role that integration may play for the transcription and/or replication of the viral genome.

A corollary to that problem is the question, whether the viral genome is integrated as a whole or in a fragmented form, and whether different sections of the viral genome are localized at different sites on the host chromosome. In those instances where integration of a very large number of viral genomes has been implicated, it will have to be investigated whether integration occurs in tandem or in a more complicated way, e.g. according to the onion skin model (SMITHIES, 1973).

3. The Mechanism of Integration

The mechanism of integration is not well understood in the case of the animal viruses. The circular genomes of the papova viruses would satisfy one of the requirements of the CAMPBELL (1962) model. In adenoviruses an intra-

cellular circular intermediate has not been observed as yet. There is circumstantial evidence that fragments of adenovirus DNA (of $1-5 \times 10^6$ daltons size) serve as precursors in the integration process.

Even in the bacteriophage systems (λ and Mu) the enzymatic mechanism of integration has been incompletely studied, although it is known that the integration apparatus is, at least partly, encoded in the viral genome. In cells infected with SV40 and adenovirus, ligase (SAMBROOK and SHATKIN, 1969) and endonuclease (BURLINGHAM and DOERFLER, 1971) activities have been described. Although it is reasonable to assume that these enzymes are involved in the mechanism of integration, direct proof is not available at the moment.

Integration of the genome of bacteriophage λ is mediated by the int system which catalyzes reciprocal recombination between one specific site on the host chromosome (B.B') and one specific site on the circularized phage genome (P.P'). The genome of phage P22 on the other hand has several specific integration sites. In contrast, phage Mu has one specific locus on its chromosome which is able to recombine at random with any site on the *E. coli* chromosome.

For the adenovirus and SV40 genomes it is not known whether specific recombination sites exist on the viral and host DNAs. In SV40-transformed human cells, chromosome C-7 contains the SV40 genetic information (CROCE et al., 1973).

In the substituted SV40 genomes, which conceivably arise from faulty exision in productive infection, the host sequences are often of the unique type. This result suggests that viral DNA may have become integrated into unique host sequences.

It is tempting to raise the question whether certain palindromic and/or repetitive sequences known to abound in mammalian cell DNA (PYERITZ and THOMAS, 1973; WILSON and THOMAS, 1974) could function as recognition sites in the host chromosome for the integration event, and whether palindromic sequences which exist in the SV40 and adenovirus genomes might serve as the viral counterparts in the reciprocal recombination event.

4. Is there a Repressor-Like Mechanism in Virus-Transformed Eukaryotic Cells?

The question whether a virus specific, repressor-like substance can be detected in virus-transformed cells remains unresolved. Virus-free extracts of SV40-transformed (CASSINGENA and TOURNIER, 1968; CASSINGENA et al., 1969) and adenovirus type 12-transformed (CHAMPE et al., 1972) cells were shown to contain a factor which inhibits viral replication in permissive cells infected with SV40 and adenovirus type 2, respectively. Similarly, extracts of Burkitt's lymphoma cells were shown to block the replication of herpesvirus hominis in permissive cells (RABSON et al., 1971). On the other hand, several laboratories have reported that SV40 was able to replicate in SV40-transformed monkey cells (BARBANTI-BRODANO et al., 1970; RAPP and TRULOCK, 1970; SAUER and HAHN, 1970). This latter result is not consistent with the presence

of a repressor in the classical sense, but does not conclusively rule out this possibility.

5. Integration and Transformed State

At this point, the discussion enters into the realm of working hypotheses which are not always supported by experimental facts. Such hypotheses may help in planning new experimental approaches to distinguish between a number of models. Several such hypotheses have been proposed to explain how tumor viruses cause malignant transformation of cells (HUEBNER and TODARO, 1969; LEVINE and BURGER, 1972; COMINGS, 1973).

There is no proof for a causal relationship between the integrated state of viral genetic material into the host DNA and the altered metabolic conditions of cells malignantly transformed by oncogenic viruses. If one wanted to subscribe to a mutational theory of cancer, it would be appealing to postulate that the integration of viral DNA could permanently alter host genes which have crucial regulatory functions. In this context, we have already invoked the analogy to bacteriophage Mu (see Introduction).

Elaborating on this model, one has still to differentiate between several possible mechanisms which relate integration to transformation:

a) Integration alters host regulatory genes directly, due to somatic mutation. Transformation would then be the direct consequence of the loss of essential functions or the acquisition of new functions.

b) Integration serves mainly to fix the viral genome in the cell, and thus allows the continued synthesis of virus-specific functions which elicit processes leading to the transformation of the cell. Such virus-specific "transformation protein(s)" have been postulated to exert a pleiotropic effect (WEIL et al., 1974).

c) Obviously, these two mechanisms are not mutually exclusive, but may both be operative.

A mutational theory of viral transformation would offer a unifying concept for malignant transformation of cells by chemical, physical, and viral agents. In this context, it is interesting to mention that a wide variety of carcinogens have been shown to be mutagens (see KIER et al., 1974 for a recent report).

And yet, a mutational theory in its simplest form will not suffice to explain all the phenomena in viral transformation. It is becoming clear that "normal", i.e. non-transformed cells of many, perhaps all species carry viral genetic material in an integrated form. In some cases, the viral genes are transcribed and translated. Furthermore, it has been shown that in many instances the viral genetic material can be induced chemically and that the cells will be transformed only after induction of viral genes (oncogene-virogene concept, HUEBNER and TODARO, 1969).

This dual role of viral genetic material supports the notion that viral genomes do indeed "have their exits and their entrances", while on the other hand, they can exhibit a "truly fixed and resting quality". Perhaps, such endogenous viral genomes play a role also in cells latently infected with viruses

and in certain cases of genetic disease. The possibility has to be considered that in the course of repeated exists and entrances, host genetic material may be transferred from one cell to another, as has been demonstrated for transducing bacteriophage. It is not known whether such a mechanism is operative in eukaryotes, although substituted genomes of SV40 contain cellular sequences (LAVI and WINOCOUR, 1972). Moreover, MUNYON et al. (1971) have demonstrated that thymidine kinase-less cells can be transformed to thymidine-positive cells by inactivated herpes simplex virus.

In this context, one should remember that in prokaryotes strong polar mutations can be caused by the insertion of host DNA sequences (800 and 1400 nucleotide pairs long) into the control region of certain operons (for review see STARLINGER and SAEDLER, 1972). These authors have pointed out that such transmissible genetic elements could play an important role in many different systems.

For the viral genome, insertion into the host chromosome may offer a number of advantages. For viral transcription, particularly of early genes, it might be essential to link viral genes to cellular sites at which the cellular polymerase(s) can bind. Even if this linkage to cellular genes were not an essential step, it might render viral transcription much more efficient. Under conditions unfavorable for viral replication, integration might salvage the viral genome from degradation. Lastly, one has to ask, whether the site(s) of viral insertion could have any relation to the origin of the viral genetic material in evolution.

On the cellular side, integration of viral genes could bestow upon the transformed cell properties which would make it fit to survive selective pressure which is exerted either by the specific culture conditions employed in vitro (e.g. agar technique) or by the immunological defense systems in an intact organism.

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Leukemic Transformation with Avian Myeloblastosis Virus: Present Status[1]

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Table of Contents

I. Introduction

Many RNA and DNA tumor viruses induce neoplastic transformation *in vitro*, but few of these are leukemia viruses acting on hematopoietic target cells. The virus of avian myeloblastosis (AMV) is such an agent, capable of producing morphological changes in hematopoietic tissues grown *in vitro*, and thus provides an excellent model to study leukemia induction at the cellular level.

This review will provide the reader with the following information: (1) a comprehensive summary of the avian leukemia viruses which are found in nature or are carried in the laboratory; (2) the biological characteristics of these viruses; and (3) some guidelines which this system can provide for further

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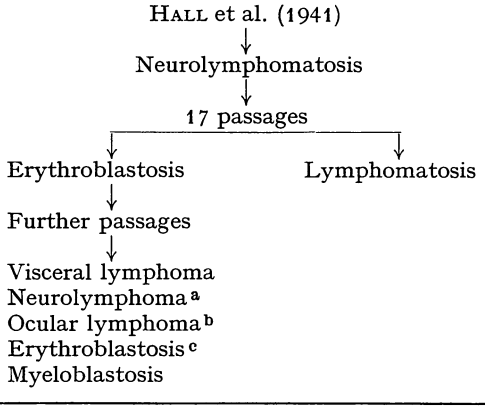
investigations in the field of leukemia and cell differentiation. Comprehensive reviews on avian RNA tumor viruses by Beard (1963), Vogt (1965a), Ponten (1971), Vigier (1970), and Temin (1971) are available.

II. Origin of Avian Myeloblastosis Virus (AMV)

The origin of the most widely employed AMV stocks is summarized in Table 1. Ellerman and Bang (1908) were the first to report isolates of avian myeloblastosis virus from several cases of visceral lymphomatosis and erythroblastic as well as myeloblastic leukemia. Unfortunately there was no follow-up study made on the original isolates and we assume that they were lost.

Table 1. Passage history of AMV



a Johnson, 1941; b Eckert et al., 1951; c Burmester, 1957.

The virus stock currently used in many laboratories is BAI strain A (BAI denotes Bureau of Animal Industry) which is a derivative of the virus isolated and described by Hall et al. (1941). This isolate was obtained from two birds with neurolymphomatosis. Both showed gross enlargement of the brachial nerves and one was found to have a marked increase in the number of lymphoid cells in the peripheral blood. After several passages from pooled nerve and bone marrow, it was possible to obtain a strain with leukemogenic activity. This strain was then studied in more detail by Beard (1963) and his group at Duke University, and aliquots of this virus were subsequently made available to several laboratories.

III. Biological Properties of AMV and its Associated Viruses (MAVs)

Since 1950, the BAI strain A has been regarded as a pure strain of myeloblastosis virus. More recently, however, it was shown that stock preparations of AMV contained at least two distinct antigenic types of virus, designated

as AMV-1 and AMV-2 (VOGT, 1965b). These two agents were further studied and purified by terminal dilution on selectively resistant chick fibroblasts and found to belong to avian tumor virus subgroup A and B, respectively (VOGT and ISHIZAKI, 1966a; VOGT and ISHIZAKI, 1966b). AMV-1 and AMV-2 lacked leukemogenic activity; however, when injected into day-old chicks, both strains were able to cause osteopetrosis and kidney tumors (SMITH and MOSCOVICI, 1969). Because AMV-1 and AMV-2 were unable to induce leukemia in the chicken, the term myeloblastosis virus seemed inappropriate for both agents and they were renamed myeloblastosis-associated virus MAV-1 (subgroup A) and MAV-2 (subgroup B). It was also proposed to call the original heterogenous BAI-A strain of AMV *standard AMV* which refers to the virus stock containing the cell transforming and leukemogenic principle associated with the two non-transforming strains of MAV-1 and MAV-2 (MOSCOVICI and VOGT, 1968). A leukemogenic preparation also exists which is free of subgroup A virus. It is referred to as subgroup B AMV (MOSCOVICI and ZANETTI, 1970)[3]. No subgroup A AMV with leukemogenic activity has been described. Attempts to free the transforming principle of AMV of associated viruses have so far been unsuccessful.

IV. Brief Synopsis of Diseases Produced by AMV and MAVs

Standard AMV causes myeloblastosis, osteopetrosis, lymphoid leukosis, and nephroblastoma in chickens (BEARD, 1963). Earlier investigations indicated that AMV was pluripotent, in that a single virus particle was thought to be able to cause several different types of neoplasms (BALUDA, 1962). However, BURMESTER'S group has demonstrated that AMV lost the potential to cause leukemia when renal tumor extracts were passaged in line 15I chickens (WALTER et al., 1962). Subsequently, it was found that two leukosis viruses were present in the AMV stock (MOSCOVICI and VOGT, 1968). MAV-1 and MAV-2 were further purified and their oncogenic spectrum was determined. At the same time, passage of AMV into Japanese quail led to the isolation of MAV-1 subgroup A (MOSCOVICI and MCINTYRE, 1966). When injected again in chick embryos, an abnormally high incidence of osteopetrosis was observed. Another isolate obtained from a quail rhabdomyosarcoma caused a high incidence of lymphoid leukosis when reinjected in chick embryos. All of the quail viruses derived from the quail tumors were of the A subgroup and none were able to induce leukemia in the chick nor cellular transformation of chick cell cultures. At the same time, a strain of MAV-2 subgroup B was obtained from the serum of a leukemic bird infected with standard AMV (VOGT, personal communication). When chick fibroblasts susceptible to all avian tumor virus subgroups (type C/0 cells) were infected with this serum, only MAV-2 was recovered from

3 As is described later (Chapter IX and X), AMV contains replication-defective particles which require a helper virus. It is not known, however, whether all or only some AMV particles are replication-defective. Therefore, the term AMV-B which will be used in this review has been retained bearing in mind that the subgroup B properties of AMV-B may be helper-dependent. The same remark will apply to AMV-C and AMV-D (Chapter X).

the supernatant fluid. This MAV also caused a high incidence of osteopetrosis when injected intravenously into chick embryos (Smith and Moscovici, 1969). A low incidence of other types of tumors were found in addition to osteopetrosis. This could be ascribed to the inadequate methods of viral isolation available at that time. Recent techniques have been described based on the property of some of the associated viruses of avian tumor viruses to produce cytopathic plaques under agar (Graf, 1972; Moscovici et al., in preparation). This method would allow the preparation of virus stocks from single plaques and reexamination of the oncogenic spectrum of each isolate. Unfortunately only members of subgroup B and D have plaque-forming ability.

V. Spontaneous Regression of Leukemia

AMV-induced leukemia is nearly always of a progressive nature. However, some investigators have reported occasional spontaneous recovery from leukemia in experimental birds. Olson (1940) reported four cases of spontaneous regression of leukemia in 98 experimental birds. One of the four birds was found to be viremic. Oberling et al. (1934) reported a 10% incidence of spontaneous recovery following inoculation with a leukemic strain developed by them.

Studies of spontaneous regression have almost exclusively been confined to Rous sarcoma virus (RSV)-induced tumors (Rubin, 1962). Rous sarcomas grow and regress as a function of virus inoculum and age of the host. The lowest incidence of regression was found in the youngest animal injected with high viral doses. Stenkvist and Ponten (1963) did not find any difference between the growth rate, virus content, or histology of progressively growing Rous tumors which later regressed, and tumors which continued to progress. Tumors which were regressing contained little or no infectious RSV and were heavily infiltrated by lymphocytes.

In contrast to the extensive literature on regression of sarcomas, few similar studies on tumors of the hematopoietic system have been conducted. Our laboratory has recently attempted to analyze more systematically the phenomenon of regression in chickens infected with AMV as embryos (Silva and Moscovici, 1973). The i.v. route of inoculation was found to be more efficient in inducing leukemia than the i.p., yolk sac, or allantoic routes, respectively. The rate of regression was found to be a function of the age of the embryo at the time of AMV inoculation. When 12-day-old chicken embryos (type C/0 white leghorn line of Kimber Farms, Niles, Calif.) were injected i.v. with 50 transforming units (TFU) of AMV, about 35% (76/218) of the hatched chicks developed leukemia within 6 weeks from hatching, and no regressions were observed (Table 2). When 17-day-old embryos of the same chicken line were injected i.v. with a 10-fold higher virus dose, approximately 33% (64/196) of the hatched chicks developed leukemia, of which 15% (10/64) regressed. Chicks injected i.p. on the day of hatching with 5×10^3 TFU of AMV showed a slightly lower incidence of leukemia (21%) with about the same rate of regression (17%). In spite of several attempts to reproduce leukemia and regression in a group

Table 2. Spontaneous regression of AMV-induced leukemia in type C/0 chickens[a]

Route of injection	Dose of virus (TFU)[b]	Age of embryo in days	No. injected	No. with leukemia (% with leukemia)	No. regressed	% of leukemic birds that regressed
Yolk sac	5×10^3	12	89	13 (15)	1	7
Allantoic	5×10^3	12	14	1 (7)	0	—
Intravenous	50	12	218	76 (35)	0	—
Intravenous	5×10^2	17	196	64 (33)	10	15
Intraperitoneal	5×10^2	day of hatching	327	70 (21)	12	17

[a] White leghorn line of Kimber Farms, Niles, California, susceptible to all avian tumor virus subgroups.

[b] Transforming units determined by end-point dilution in chicken yolk-sac cultures susceptible to all avian tumor virus subgroups (type C/0).

Table 3. Spontaneous regression of AMV-induced leukemia in type C/ABE chickens[a]

Route of injection	Dose of virus (TFU)[b]	Age of embryo in days	No. injected	No. with leukemia (% with leukemia)	No. regressed	% of leukemic birds that regressed
Allantoic	5×10^3	12	12	0	0	—
Intravenous	5×10^3	12	8	4 (50)	1	25
Intravenous	5×10^3	17	70	26 (37)	9	35
Intraperitoneal	5×10^3	day of hatching	185	45 (24)	13	29

[a] Fibroblast cultures of type C/ABE chickens are genetically resistant to avian tumor viruses of subgroups A, B and E but can be infected by subgroup C viruses.

[b] Transforming units determined by end-point dilution in type C/0 yolk-sac cultures.

injected via the yolk sac with 5×10^3 TFU of AMV, only one bird spontaneously regressed (Table 2).

Similar experiments were also performed with chick embryos which are genetically resistant to avian tumor viruses of subgroup A, B, and E but can be infected by subgroup C. Such chickens are referred to as type C/ABE (DUFF and VOGT, 1969; CRITTENDEN et al., 1973). C/ABE chickens were partially but not completely resistant to AMV, and all viral inoculations were performed with a 10-fold higher concentration of virus than the one used for the 17-day-old type C/0 embryos (Table 3). The allantoic route of inoculation produced no leukemia. Intravenous injection of AMV into 12-day-old embryos was found to be the most efficient way of inducing leukemia. The percentage of inoculated birds developing leukemia decreased as the age of the bird at the time of inoculaton increased. The regression rate for type C/ABE chicks was significantly higher than the regression rate for type C/0 chickens. Spontaneous regression occurred nearly twice as frequently in leukemic C/ABE as it did in leukemic C/0 chickens (Tables 2 and 3).

Regressed C/0 and C/ABE birds were sacrificed at various intervals and examined grossly and microscopically. Gross examinations occasionally revealed lymphoma-like tumors of the spleen, liver, kidney, or lung. Histological examinations of these tumors revealed that normal tissue had been replaced by blast-like cells. Some of the tumors were found to be of lymphoid origin, but other tumors could not be conclusively identified. In some cases, examination of bone marrow from regressed birds revealed that 30% of the marrow had also been replaced by these blast-like cells. The C/0 chickens were viremic during the entire period of study, even after the leukemia had regressed. In contrast, the plasma of leukemic or regressed C/ABE chickens was always negative for AMV when tested on C/0 yolk-sac cultures.

Attempts were made to determine whether am immunological mechanism was involved in the regression of AMV-induced leukemia. The role of the bursa of Fabricius was investigated earlier to study the influence of humoral immunity on resistance to AMV infection (BALUDA, 1967). A shift to the more virulent forms of leukemia in hormonally bursectomized birds inoculated with AMV was observed. If susceptible chickens were injected with highly concentrated AMV 42 days after hatching, only 4.5 % eventually developed myeloblastic leukemia. If injected on day one, the incidence of leukemia approached 60%. Hormonal bursectomy was obtained by injecting 12-day-old embryos with 19-nortestosterone. Such bursectomized birds injected 42 days after hatching were as suceptible to myeloblastic leukemia as normal birds injected on the day of hatching.

Our own preliminary studies indicate that regression can occur in the absence of humoral immune system. It was found that two out of five bursectomized chickens injected with AMV at hatching developed leukemia and later both regressed. In addition, 20 five-week-old chickens bursectomized either by hormonal treatment or with cyclophosphamide never developed myeloblastic leukemia when injected with high doses of AMV. This failure to confirm BALUDA'S observations may have resulted from the use of different chicken flocks, and it is obvious that further studies are needed to clarify this matter.

A nonimmunological mechanism might also be considered to explain regression of myeloblastic leukemia: controlling humoral factors are released by leukemic cells *in vivo* as well as *in vitro*. One of these factors, known as Colony Stimulating Factor (CSF), regulates granulopoiesis and monocyte formation (METCALF, 1973). As is shown later in this review, the leukemic cells respond to CSF and can be induced to differentiate into mature nondividing progeny.

VI. Transformation of Hematopoietic Cells in Culture

The first succesful attempt to infect and transform chick bone-marrow cells *in vitro* with AMV was made by DOLJANSKI and PIKOWSKI (1942), and later confirmed by the studies of BEAUDREAU et al. (1960) and LAGERLÖF (1960). Transformation of these cells into leukemic myeloblasts was shown to

be accompanied by continuous virus release at a rate of about 30 physical particles per cell per hour (BEAUDREAU et al., 1960).

More detailed studies of the transforming effect of AMV on several chick and other avian hematopoietic cells were reported later by BALUDA and GOETZ (1961), BALUDA et al. (1964), and MOSCOVICI and VOGT (1968). These studies have focused on the transformation of yolk-sac cells. The yolk sac represents the major site of blood formation during the stage of embryonic development. Hematopoiesis in the yolk sac is maximal between the 10th and 15th day and then declines, ending just before hatching. Thereafter, throughout adult life, the bone marrow becomes the chief locus of hematopoietic activity (for further details see ROMANOFF, 1960). In studies with AMV yolk-sac cultures are preferred to bone-marrow cultures, mainly because the yolk sac yields a larger number of cells and because the culture of yolk-sac cells has been standardized. The technique of yolk-sac culture has been recently described in detail by MOSCOVICI and MOSCOVICI (1973). Culture of yolk sac from chicken embryos of different genetically determined susceptibility to avian tumor virus has been performed according to this technique. Variations in the number of cells to be seeded and in the cultural behavior of these cells *in vitro* were observed sometimes from embryo to embryo and always from chicken line to chicken line. Moreover, as is described later, the response of yolk-sac cells to AMV transformation is not uniform when cells derived from embryos of different lines are considered, even though fibroblasts of such embryos may show the same pattern of susceptibility for avian tumor viruses.

Yolk-sac cells in culture have all the characteristics of typical macrophages. They vary in size and in shape, from elongated to round cells, having a round and eccentric nucleus. Their cytoplasm contains dense perinuclear granules (phagosomes) and lipid droplets. Moreover, yolk-sac cells display a high phagocytic acticity, as shown by the rapid ingestion of colloidal carbon particles.

When primary or secondary chick yolk-sac cells were exposed to a multiplicity of 5–10 infectious units per cell, morphological changes could be seen as early as 3 days post infection. Round, refractile cells appeared and grew rapidly occupying a large area of the culture dish. These cells were histologically identified as myeloblasts.

Table 4 shows that in addition to yolk-sac cells, other cells derived from embryonic and adult hematopoietic tissues can be transformed by AMV

Table 4. Transformation of chicken cell cultures derived from hematopoietic tissues. Cell cultures exposed to standard AMV

Embryonic					Adult[a]				
Yolk sac	Bursa of Fabricius	Bone marrow	Liver	Spleen	Buffy coat	Thymus	Bone marrow	Liver	Spleen
+	+	+	+	+	+	+	+	+	+

[a] Age of birds, 6 to 8 weeks.
+ =Transformation accompanied by proliferation of myeloblasts.

(Baluda et al., 1964; Moscovici, unpublished). The study of the transforming ability of AMV has been mainly confined to cells derived either from yolk-sac or bone marrow. No information is available with cells derived from other tissues. Transformation of chicken fibroblastic cells by AMV was described by Moscovici et al. (1969). This matter, however, needs further investigation since contradictory results have been reported (Graf, 1973).

VII. Titration of AMV

Several different methods exist for the titration of AMV. However, most of these techniques have a major drawback: they measure activity of the myeloblastosis-inducing virus together with the associated transformation-defective viruses MAV-1 and MAV-2, which are found in excess in stocks of AMV obtained from the plasma of leukemic chickens (Moscovici and Vogt, 1968; Smith and Moscovici, 1969), but do not measure the transforming activity of AMV.

A. Adenosine Triphosphatase Assay

This assay measures the adenosine triphosphatase (ATPase) present at the surface of virions (Mommaerts et al., 1954; Beaudreau and Becker, 1958). The technique is suitable for rapid screening of leukemic plasma but requires relatively high concentrations of virus. Only virions produced by myeloblasts carry ATPase which appears to be derived from the host cell (de-Thé, 1964), and infectious as well as noninfectious particles register in this assay.

B. Interference Assay

This assay is based on the strong resistance to superinfection with Rous sarcoma virus developed by chick-embryo fibroblasts infected with AMV (Vogt and Rubin, 1963). Although very sensitive, this technique is somewhat laborious because it requires repeated transfers of the assay cultures.

C. Fluorescence Assay

Viral antigens appearing on the surface of chick-embryo fibroblasts infected with AMV can be stained with specific fluorescent antisera (Vogt, 1963; Vogt and Rubin, 1963). This fluorescence can occur in discrete foci when cells are infected at the appropriate multiplicity. These foci are proportional in number to the concentration of the inoculum. This technique is, however, very difficult to recommend as a routine titration assay because it is cumbersome and requires skill in handling the fluorescence technique.

D. Plaque-Formation Assay

Chicken fibroblasts infected by avian leukosis viruses of subgroups B and D develop plaques after addition of neutral red to the agar overlay (Dougherty and Rasmussen, 1964; Graf, 1972; Moscovici et al., in preparation). This technique is the simplest method of measuring infectivity in AMV preparations but will only detect replicating viruses of subgroups B and D.

E. Quantitative Assays of AMV-Induced Cellular Transformation

These techniques are based on the leukemogenic activity of AMV *in vivo* and *in vitro*.

AMV Titration in Day-old Chickens. The technique was developed by ECKERT et al. (1951–1954) at the time when tissue culture assays for leukemogenic activity were not available. This *in vivo* titration of AMV is based on the incidence of leukemia and requires several weeks for completion as well as extensive animal facilities.

AMV Titration in Chick Embryos (BALUDA and JAMIESON, 1961). This assay presents the same drawbacks as the preceding technique but is more sensitive due to the use of embryos.

Titration of AMV in Yolk-sac Cultures by End-point Dilution. When cultures of chicken hematopoietic cells (derived from the yolk-sac or the bone marrow) are infected with AMV, small, rounded cells appear, which are identified as myeloblasts by light microscopy. In 1961 BALUDA and GOETZ, exploiting this observation, developed an end-point titration assay in which individual cell cultures were scored as either positive or negative and virus titers were computed according to the formula of REED and MUENCH (1938). This assay suffers from the lack of precision inherent in end-point procedures (DAVIS et al., 1973).

Agar Overlay Assay of AMV. In 1967, MOSCOVICI developed a focus assay based on the enumeration of discrete myeloblastic areas in AMV-infected hematopoietic tissue cultures, overlayed with semi-solid purified agar. This technique has been modified and improved.

Primary yolk-sac cells are prepared according to published techniques (MOSCOVICI and MOSCOVICI, 1973) from 13-day-old chicken embryos. These primary cultures are used between 7 and 9 days to prepare secondary yolk-sac cells at a density of about 150 cells/mm^2 which are infected 2–4 hours post-seeding with different dilutions of AMV.

VIII. Envelope Classification of AMV

It has long been known that individual chickens differ in their sensitivity to natural infection by avian leukemia-sarcoma viruses (GREENWOOD et al., 1948). Breeding has produced flocks with a different susceptibility to various members of avian tumor viruses (WATERS and BURMESTER, 1961). Differences in genetic susceptibility between chicken embryos contributed to the availability of Rous sarcoma virus pock titration on the chorioallantoic membrane and it was not until genetically uniform embryo cultures became available that Rous sarcoma virus could be titrated quantitatively (TEMIN and RUBIN, 1958; VOGT, 1969).

Susceptibility and resistance of birds to infection by avian RNA tumor viruses has been the object of extensive analysis by several investigators. These studies have revealed that resistance against virus infection is cellular and is genetically determined (CRITTENDEN et al., 1963). A single dominant gene controls cellular susceptibility to viruses of subgroup A and a second gene with

similar characteristics controls the suceptibility to members of subgroup B (Rubin, 1965; Crittenden et al., 1967.) Cellular resistance to subgroup C leukosis viruses has also been found to be a recessive trait controlled by a single autosomal locus (Payne and Biggs, 1970). Based on these initial discoveries, an attempt was made to classify the numerous viruses of the leukosis-sarcoma complex (Vogt and Ishizaki, 1965). The classification was achieved by the use of selectively resistant cells and by the antigenic differences of the viral envelope. Three major properties are used for the classification, namely, host range, viral interference, and antigenic cross reactions. They led to a relatively complete classification which now comprises all avian leukosis and sarcoma agents falling in 7 distinct subgroups named A, B, C, D, E, F, and G (Vogt, 1967; Weiss, 1967; Duff and Vogt, 1969; Vogt, 1970; Hanafusa and Hanafusa, 1973; Fujita et al., 1974).

Viruses of subgroups A, B, C, and D represent the field and laboratory isolates, while viruses of subgroups E, F, and G have the same envelope antigens of the endogenous virus that is induced or rescued from three different avian species—chicken, ringnecked pheasant, and golden pheasant, respectively.

Recent studies have shown that susceptibility of chicken yolk-sac cells to avian sarcoma and leukosis viruses may differ from that of fibroblasts of the same animal (Gazzolo et al., 1974). In particular, yolk-sac cells derived from type C/0 embryos and expected to be susceptible to all avian tumor virus subgroups unexpectedly failed to support the growth of subgroup A virus including MAV-1 while subgroup B agents, such as MAV-2 were readily produced. These results must be taken into consideration when the subgroup classification of AMV by its host range is examined.

A. Host Range of AMV

The host range of the leukemogenic agent of AMV stocks has been studied in yolk-sac cultures derived from chicken strains of known genetic background (Moscovici and Vogt, 1968). The results of these studies and of additional unpublished observations are schematically represented in Table 5. They show that variations in the titers of standard AMV and AMV-B occur from culture to culture, from line to line, even if these lines belong to the phenotype C/0[4], i.e. are genetically susceptible to all subgroups of avian tumor viruses as tested in the Rous sarcoma focus assay with fibroblast cultures. These variations may be explained by the different behavior of yolk-sac cells in culture according to their origin. The highest titers of standard AMV and AMV-B are obtained with yolk-sac cells of Line 6 (C/E). Titers 10–100 times lower were obtained with Spafas line (C/E); the same results were obtained with Line 7, Subline 1 (C/A), and Line 151 (C/C). A 10-fold decrease in titer was obtained with yolk-sac cells from chick embryos of phenotype C/BE and C/ABE. This resistance of C/BE and C/ABE cultures to AMV indicates that AMV probably belongs to subgroup B.

4 In the nomenclature of chicken cells, C denotes chicken cell and the bar is followed by the viral subgroup which is genetically excluded from the cell. Thus C/A cells are resistant to subgroup A but can be infected by viruses of other subgroups. C/0 cells are not resistant to any of the avian tumor virus subgroups.

Table 5. Average titer of standard AMV and AMV-B in chicken hematopoietic cells

Phenotype of cell and chicken line[a]	Standard AMV	AMV-B
C/0 Kimber	2.8×10^{4}[b]	2.5×10^{4}
C/E Line 6	6.0×10^{5}	3.0×10^{5}
C/E Spafas	3.2×10^{3}	3.5×10^{4}
C/E Bantam	5.0×10^{4}	1.6×10^{4}
C/A Line 7—Subline 1	1.8×10^{4}	8.5×10^{3}
C/BE Kimber	ND[c]	6.0×10^{1}
C/BE Bantam	3.0×10^{1}	1.0×10^{0}
C/C Line 15 I	8.0×10^{4}	1.6×10^{4}
C/ABE	2.0×10^{1}	ND

[a] In the nomenclature of chicken cells, C denotes chicken cell and the bar is followed by the virus subgroup which is genetically excluded from the cell. Thus C/A cells are resistant to subgroup A but can be infected by viruses of other subgroups. C/0 cells are resistant to not any of the avian tumor virus subgroups.

[b] Titer expressed as focus forming units/ml.

[c] ND = not done.

B. Interfering Properties

Viral interference has been one of the methods used to classify avian RNA tumor viruses (VOGT and ISHIZAKI, 1965). Previous studies using chicken fibroblasts have led to the discovery of two associated viruses present in the stocks of standard AMV, i.e. MAV-1 and MAV-2 (MOSCOVICI and VOGT, 1968). However, to determine whether the two associated viruses which do not induce myeloblastosis *in vivo* or transform hematopoietic cells *in vitro* have interfering activity against the transforming component of AMV, the use of yolk-sac cells

Table 6. Interference of avian leukosis viruses with AMV-B and standard AMV[a]

Interfering virus	Subgroup of interfering virus	Relative efficiency of plating[b]	
		AMV-B	Standard AMV
MAV-1	A	0.60	0.50
MAV-2	B	*0.01*[c]	*0.10*
RAV-1[aa]	A	0.80	1.00
RAV-2	B	0.60	1.00
tdB77[bb]	C	0.65	ND
CZAV[cc]	D	0.95	ND

[a] Yolk-sac cells were infected with avian leukosis viruses at a multiplicity of infection of 0.5-interfering unit (i.u.) per cell, then challenged 6 days later with AMV-B and standard AMV.

[b] Plating efficiency in control cells not preinfected with avian leukosis virus = 1.0.

[c] Italics = significant reduction of focus formation.

[aa] RAV = Rous associated virus.

[bb] tdB77 = transformation defective derivative of avian sarcoma virus B77 subgroup C.

[cc] Carr Zilber associated virus subgroup D; ND = not done.

was necessary. For this purpose, C/E yolk-sac cells were pre-infected with MAV-1 and MAV-2, respectively. One week later, the cultures were challenged with several dilutions of standard AMV or AMV-B, overlayed with agar, and transformed foci were scored 12 days later (Table 6). MAV-1, a subgroup A virus, failed to interfere with the production of myeloblastic foci by standard AMV. This result can be explained by the failure of subgroup A viruses to replicate in yolk-sac cells (Gazzolo et al., in preparation). MAV-2, a subgroup B virus, interfered with standard AMV and AMV-B, suggesting that the myeloblastosis-inducing component of AMV should be assigned to subgroup B. Later (Section XI) we discuss whether or not MAV-2 plays a role in the replication of AMV.

C. Envelope Antigenicity

The results of studies shown in Table 7 confirm the affiliation of AMV with subgroup B. In fact, treatment of standard AMV by specific immune sera prepared against MAV-2 neutralized all the transforming activity of this virus as tested by the focus assay. The same results were obtained with AMV-B. No neutralization of standard AMV nor AMV-B was observed with immune sera prepared against RAV-1 (subgroup A), against RAV-2 (subgroup B), and td-B77 (subgroup C), respectivley. The failure of anti RAV-2 serum to react with AMV is probably due to the lack of cross reaction between subgroup B virus (Vogt and Ishizaki, 1966a).

In conclusion, the results of the classification of AMV suggest that this virus is probably a member of subgroup B. These studies also indicate that subgroup A components present in standard AMV play no role in the leukemic process.

Table 7. Neutralization of AMV-B and standard AMV by different immune sera

Immune serum[a]	Fraction of survivors	
	AMV-B	Standard AMV
Anti-RAV-1	0.77	1.54
Anti-RAV-2	1.32	1.20
Anti-MAV-2	*0.001*	*0.001*
Anti-standard-AMV	*0.001*	*0.001*
Anti-tdB77	0.52	1.0

[a] Neutralizing sera are obtained from hyperimmunized chickens. A 1/100 dilution of serum was incubated with 200 focus forming units of standard AMV or AMV-B at 37° C. After 40 min, surviving virus was plated. Italics = significant neutralization of focus formation.

IX. Defectiveness of AMV

During the past few years, several studies have concentrated on AMV and its interaction with cells *in vitro* and in particular, on the question of whether transformed myeloblasts remain as virus producers or if it would be possible

to obtain virus-free leukemic cells. This question is directly related to the defectiveness of AMV in a way similar to that originally demonstrated by the Bryan high-titer strain of Rous sarcoma virus (HANAFUSA, 1964–1965; HANAFUSA et al., 1970a, 1970b; WEISS, 1969).

Previous studies (MOSCOVICI and ZANETTI, 1970) indicated that nonproducer myeloblasts could be obtained by infecting cells derived from two cell phenotypes, namely C/E and C/ABE. In the first case, clones of virus-free transformed myeloblasts were obtained when a low multiplicity of infection was used. With C/ABE cultures only high doses of virus caused transformation and foci of myeloblasts were free of infectious virus. Rescue of transforming AMV was possible when nonproducing myeloblasts of C/E or C/ABE cultures were superinfected with several strains of helper viruses of subgroups C or D. More detailed studies on the defectiveness of AMV are described in the following sections.

A. Isolation of Nonproducers

Secondary yolk-sac cells were infected by standard or AMV-B, at a multiplicity of infection, allowing the formation of about 5 foci per dish. Because of the differences in genetic susceptibility to AMV, the virus dose varied according to the cell phenotype—low for C/E cells of line 6 and high for C/ABE (Table 8). The infected cells were then overlaid with nutrient agar and single foci of myeloblasts were picked 12–15 days after infection and seeded on a feeder layer of Japanese-quail fibroblasts. The myeloblasts proliferated and formed clonal cultures containing about 1×10^6 cells/ml. Nonproducer clones were identified by infectivity tests for MAV-1, MAV-2, and transforming AMV. In some cases the data were supplemented by electron microscopy and tests for a viral peak at a density of 1.16 by centrifugation in sucrose gradients. Table 8 summarizes the results and shows that all type C/ABE foci were nonproducers,

Table 8. Isolation of AMV nonproducer clones from different chicken cell phenotypes

Phenotype of cell and chicken cell line[a]	Virus	Virus dose (focus forming units per cell)	Number of clones studied	Number of virus producers		Number of nonproducers
				MAV-2[b]	AMV + MAV-2[c]	
C/0 Kimber	Standard AMV	5×10^{-4}	25	5	2	18
C/E Line 6	AMV-B	5×10^{-4}	14	0	13	1
C/E Spafas	AMV-B	5×10^{-3}	49	17	8	24
C/A Line 7	AMV-B	5×10^{-2}	20	0	1	19
C/ABE	Standard AMV	5×10^{-2}	21	0	0	21

[a] In the nomenclature of chicken cells, C denotes chicken cell and the bar is followed by the virus subgroup which is genetically excluded from the cell. Thus C/A cells are resistant to subgroup A but can be infected by viruses of other subgroups. C/0 cells are not resistant to any of the avian tumor virus subgroups.

[b] MAV-2 was detected by plaque assay.

[c] Detected by formation of myeloblastic foci in yolk-sac cultures and by plaque formation in fibroblasts.

while only 7% of the foci obtained from C/E Line 6 fell into this category. The percentage of nonproducers isolated from C/0 Kimber and C/E Spafas cultures was 70% and 50%, respectively.

B. Rescue of AMV from Nonproducers

Rescue of transforming AMV was attempted by challenging nonproducer myeloblasts with leukosis viruses belonging to 4 subgroups, namely A, B, C, and D. The myeloblast cultures were divided in equal amounts containing approximately 5×10^5 myeloblasts and distributed in small plastic tubes to which the helper viruses were added at a multiplicity of infection of 2–3. The mixtures were kept for one hour at 37° and each mixture was then seeded on a feeder layer of quail fibroblasts. Proliferation of myeloblasts under these conditions continued. Supernatant fluids were harvested from each culture at various intervals and then pooled, filtered, and tested for biological activity. Each harvest was tested for transforming activity on susceptible yolk-sac cultures. In addition, interference tests were performed to detect the growth of the helper virus.

In previous experiments (Moscovici and Zanetti, 1970), it was found that only helper viruses of subgroup C were able to rescue transforming AMV regardless of the phenotye of the chicken embryo from which the culture was derived. These studies were carried further and the rescuing ability of additional helper viruses was discovered (Moscovici and Gazzolo, unpublished results). Table 9 shows representative examples of these rescue experiments. These results indicate that helper viruses of subgroups C and D are most effective in activating the production of infectious AMV. Subgroup B viruses occasionally rescue AMV from nonproducer clones, but subgroup A viruses are unable to rescue

Table 9. Rescue of AMV from nonproducer clones after challenge with avian leukosis virus

Phenotype	Clones challenged	Challenging viruses and subgroups									
		MAV-1 A	RAV-1 A	MAV-2 B	RAV-2 B	RAV-7 C	tdB77 C	RAV-49 C	CZAV D	RAV-50 D	None
C/0	645/4	–[a]	ND[b]	–	ND	+	ND	+	ND	ND	–
Kimber	645/7	–	ND	–	ND	–	ND	–	ND	ND	–
	645/11	–	ND	–	ND	–	ND	–	ND	ND	–
C/E Spafas	189/3	–	–	–	+	ND	+	ND	+	ND	–
	189/17	–	–	+	+	ND	+	ND	–	ND	–
	189/27	–	–	–	–	ND	–	ND	–	ND	–
	189/37	–	–	–	–	ND	–	ND	–	ND	–
C/ABE	987/3	–	ND	–	ND	+	+	+	+	+	–
	987/4	–	ND	–	–	–	–	–	–	–	–
	987/6	–	ND	–	–	–	–	–	–	–	–
	987/9	–	ND	–	ND	+	+	+	+	+	–

[a] – = No transforming activity detected. + = Transforming activity recovered.
[b] ND = Not done.

AMV. Certain nonproducer clones could not be rescued by any of the helper viruses indicating that AMV exists in different states of defectiveness similar to Rous sarcoma virus (HANAFUSA, 1970). The replication of helper viruses in nonproducer cultures was followed by independent tests. Growth of helper viruses followed the known pattern of genetic susceptibility to avian tumor virus subgroups with the exception of subgroup A helpers.

These data support the conclusion that stocks of AMV-B and of standard AMV contain replication defective particles which are able to transform yolk-sac cells. Some, but not all of these replication-defectives can be rescued with helper viruses. The rescuing ability of helpers is so far confined to viruses of subgroups B, C, and D.

C. Host-Range Expansion of AMV with Envelopes Provided by Subgroup C and D Helper Viruses

Some of the AMV progeny virus recovered after addition of subgroup C and D helpers to nonproducer myeloblasts was further analyzed. Firstly, it was determined whether these agents with C and D envelopes were leukemogenic when injected i.v. into embryonated eggs. Secondly, host-range studies in yolk-sac cells of chickens and other avian species were performed.

The data shown in Table 10 indicate that the titer of AMV-C[5] is about 1000 times higher than the titer of AMV-B when these viruses are assayed on C/BE and C/ABE yolk-sac cells. AMV rescued with subgroup D helper viruses showed homologous interference with subgroup D sarcoma viruses, but not, as other subgroup D leukosis viruses, with a subgroup B challenge. AMV with a subgroup D envelope deserves further study in view of the fact that subgroup D Rous sarcoma viruses cause neoplasia in mammals (HANAFUSA and HANAFUSA, 1966) and it would be of interest if mammalian hematopoietic cells were found to be susceptible to AMV-D.

Host-range studies were performed with yolk-sac cultures derived from Japanese quails, ducks, and ringneck pheasants. A representative experiment is shown in Table 11.

Table 10. Host range of AMV-C and AMV-D in different chicken cell phenotypes

Cell phenotype	AMV-B	AMV-C	AMV-D
C/E	9×10^5[a]	3.5×10^4	ND[b]
C/A	8×10^3	3.8×10^4	ND
C/BE	6×10^1	ND	ND
C/ABE	2×10^1	2.0×10^4	ND
C/C	1.6×10^4	ND	ND

[a] Titers expressed as focus-forming units.

[b] ND = not done.

5 See footnote on p. 81.

Table 11. Infection of avian cells from several species by AMV-C and AMV-D

Cells (yolk sac)	Standard AMV	AMV-B	AMV-C	AMV-D
Japanese quail	R^{-}[a], T^{-}, r^{+}	R^{-}, T^{-}, r^{-}	R^{-}, T^{-}, r^{-}	R^{-}, T^{-}, r^{-}
Duck	R^{-}, T^{-}, r^{-}	R^{-}, T^{-}, r^{-}	R^{+}, T^{-}, r^{+}	R^{-}, T^{-}, r^{-}
Ringneck pheasant	R^{-}, T^{-}, r^{+}	R^{-}, T^{-}, r^{-}	R^{-}, T^{-}, r^{-}	R^{-}, T^{-}, r^{-}
Chicken (C/E)	R^{+}, T^{+}, r^{+}	R^{+}, T^{+}, r^{+}	R^{+}, T^{+}, r^{+}	ND[b]

[a] R^{-} = No replication of the transforming virus; R^{+} = replication of transforming virus; T^{-} = no transformation observed; T^{+} = transformation with appearance of myeloblasts; r^{+} = replication of associated or helper virus; r^{-} = no replication of associated or helper virus.

[b] ND = not done.

The results show that Japanese-quail cells are resistant to both AMV-C (with the coat of td-B77) and AMV-D (with the coat of RAV-50). The ringneck pheasants follow a similar pattern. Duck cells are completely resistant to AMV-D, but they permit replication of AMV-C but not induction of neoplastic transformation by this virus. This raises the interesting possibility that a transforming virus may sometimes replicate within a cell without transforming it. The situation of AMV-C in duck cells may not be unique. Similar behavior has been recently described with RSV (RAV-2) in chicken yolk-sac cells (Gazzolo et al., 1974). The overall results of the host-range studies clearly indicate that the transforming capacity of AMV remains so far confined to the order of Galliformes.

X. Colony Formation with Normal Hematopoietic Cells and with Leukemic Myeloblasts

In the late 1960's semi-solid agar culture methods were developed which support the proliferation of single mammalian hematopoietic cells (Bradley and Metcalf, 1966; Ichikawa et al., 1966; Pike and Robinson, 1970). Progenitor cells of granulocytes and monocytes proliferate in agar and generate colonies of progeny cells which can differentiate into fully mature monocytes and/or macrophages. The interest in this technique is stimulated by the assumption that the *in vitro* observation mimics events occurring *in vivo* during granulopoiesis and monocyte formation. Colonies developing in agar are clones derived from stem cells located mainly in the bone marrow although spleen and buffy coat also contain cells capable of forming agar colonies (Metcalf and Moore, 1971). These *in vitro* colony-forming cells (CFC) were shown to be the common precursors for granulocytes and macrophages (Metcalf and Moore, 1971). This relationship could explain the common observation in animals and humans that monocyte populations are often abnormal in cases of myeloid leukemia. Ichikawa (1969–1970) demonstrated that undifferentiated murine leukemic cells generate clones of mature, nondividing granulocytes and macrophages in agar cultures. Cells of acute and chronic myeloid leukemia in man are also

capable of differentiation to mature cells in semi-solid agar (METCALF, 1973). Colony formation *in vitro* by normal hematopoietic cells is dependent upon the presence of a specific glycoprotein, termed colony stimulating factor (CSF). CSF is a neuraminic acid-containing glycoprotein which can be detected in all normal mammalian sera and urine. CSF levels in mice and humans have been correlated with fluctuations in the level of granulopoiesis or monocyte formation, suggesting that CSF is a humoral regulator of granulopoiesis and monocyte formation (METCALF and MOORE, 1971).

Similar *in vitro* studies have been initiated in our laboratory to compare the proliferation and differentiation of the normal chick hematopoietic cell to the AMV-induced leukemic myeloblast (DODGE and MOSCOVICI, 1973). The results indicate that normal chicken hematopoietic cells, i.e. embryonic yolk-sac cells, bone marrow, and spleen cells can differentiate in semi-solid agar medium into macrophages. The *in vitro* colony formation and differentiation were found to be strictly dependent upon the presence of a colony stimulating factor (CSF) obtained from serum and from medium that had been conditioned by culturing chick embryo fibroblast cells for several days. Leukemic myeloblasts obtained by injecting day-old chickens with AMV were found to be capable of differentiating *in vitro* into macrophages and granulocytes. Leukemic cells were capable of forming colonies at a low efficiency in the absence of an exogenous source of CSF. However, the addition of CSF to the colony assay resulted in a marked increase in the number of colonies obtained.

A distinct morphological difference has been found between colonies obtained from normal chicken hematopoietic tissues and leukemic cells. Large and diffuse colonies were obtained from normal tissues and labeled type I. Smaller and more compact colonies derived from leukemic birds were labeled type II. This morphological differentiation may be a useful tool to study the effect of drugs on the regulation of a hematopoietic system. It has provided some interesting data on the level of CSF when the bird is normal, leukemic (progressor), or in a regressing state (regressor) (SILVA et al., 1974). When sera of normal, progressor, and regressor chickens were tested for their ability to induce type I colonies, it was found that CSF levels were significantly lower in leukemic than in normal or regressor birds. When the same sera were tested for their ability to induce type II colonies, leukemic sera demonstrated a significantly higher CSF level than either normal or regressed sera. Regressor chickens had serum CSF levels similar to normal birds (SILVA et al., 1974).

These results suggest that humoral factors may play a role in the dysfunction of normal cell differentiation. Other investigators have proposed that acute myeloblastic leukemic cells from mammals are not autonomous cancer cells in that they are still subject to regulation by humoral factors (METCALF, 1973). Altered levels of humoral regulators of hematopoiesis have been found in the chicken-leukemia system. It remains to be seen whether an imbalance of CSF levels is the principal reason for the initiation and maintenance of the leukemic state. Under the conditions of *in vitro* colony formation in the presence of varying concentrations of CSF, not all leukemic cells differentiate. Leukemic

myeloblasts can still be isolated from 3–5-week-old colonies (Moscovici, unpublished) and a similar persistence of leukemic cells must occur *in vivo*. In addition to differentiating, leukemic cells could also be removed from the blood stream by immunological mechanisms (Silva and Moscovici, 1973). The relative roles of CSF and of immune response in the development of leukemia may be subject to experimental analysis using colony formation in agar in conjunction with various methods for suppressing specific components of the immune system.

XI. General Conclusions

A review of a relatively young field of science not only helps to tabulate the established facts but quickly points out the many deficiencies which exist in our knowledge. Those areas which need further investigation will therefore now be defined and specific approaches which might prove fruitful will be suggested.

Although the morphological characteristics of the AMV-induced transforming event have been well described, there is no precise information indicating the real nature of the target cell which eventually will become a cancer cell. Recent studies (Moscovici and Moscovici, 1973) and unpublished data suggest that at least *in vitro* the target cell for leukemogenic transformation has all the characteristics of a macrophage. This conclusion derives from the fact that these target cells, besides showing typical macrophage morphology, have a high phagocytic activity as well as Fc receptors for IgG (Gordon and Cohn, 1973).

Improvements in the methods for tissue culture of embryonic and adult hematopoietic tissues should provide further confirmation on the nature of the target cell for AMV-induced transformation *in vitro*. It still remains to be seen whether the target cell in the intact animal is the same as that suggested by studies in tissue culture.

The biology of AMV and its associated agents has made steady progress. The main facts can be listed as follows:

a) MAV-1 does not transform but replicates in chick-embryo fibroblasts of C/E phenotypes. It does not replicate in the macrophage of the same phenotype (Gazzolo et al., 1974). It does not produce plaques in chick-embryo fibroblasts. It is serologically closely related to other subgroup A avian RNA tumor viruses. Because of its inability to grow in macrophages MAV-1 is ineffective as a helper virus for rescuing AMV from nonproducing transformed cells.

b) MAV-2 does not transform but replicates in chick-embryo fibroblasts of C/E phenotype as well as in macrophages. It produces plaques in chick-embryo fibroblasts. It belongs to subgroup B, but often fails to cross react in neutralization with other subgroup B viruses. Occasionally it is able to rescue transforming AMV from nonproducers.

c) Both MAV-1 and MAV-2 are oncogenic for the chicken (Smith and Moscovici, 1969), but do not cause myeloblastic leukemia.

d) Assignment of AMV to a specific subgroup still remains an open issue. Because of its defectiveness, AMV may not code for subgroup specific envelope properties. Infectious AMV has the characteristics of a subgroup B virus, but these may be supplied by MAV-2 with which infectious AMV is found associated after cloning. A helper-independent AMV has not yet been isolated nor have noninfectious particles analogous to the Bryan strain of Rous sarcoma virus without helper (RSV-) been found (HANAFUSA et al., 1970b; WEISS, 1969). However, myeloblasts have been obtained from both C/E and C/ABE cultures which continue to divide for numerous generations without releasing infectious virus. Rescue of leukemogenic activity is possible by challenging these nonproducers with leukosis helper viruses of subgroup B, C, and D. In all our studies both MAV-1 and RAV-1 of subgroup A were unable to rescue transforming AMV.

These observations suggest that AMV is defective and can be retrieved from transformed nonproducer cells by challenge with a broad range of helper viruses, although not all nonproducers can be activated to release infectious AMV. Nonproducer myeloblasts can be grown in mass quantities in shaker cultures (GAZZOLO and MOSCOVICI, unpublished). It will be interesting to use chemical and physical agents in attempts to activate AMV production in nonproducer cultures, possibly with the aid of an endogenous helper. At the same time it will be possible to resolve the important question as to whether nonproducer myeloblasts maintain their leukemogenic potential when reinjected in suitable hosts.

When nonproducers are challenged with helper leukosis viruses, we have assumed that the progeny obtained from the rescue experiments had the characteristics of a phenotypically mixed virus. In this instance, phenotypic mixing consists in the exchange of envelope components between two viruses without modification of the viral genomes. The retrieved AMVs analyzed so far follow the above criteria, i.e. they are leukemogenic for the chicken, show a host-range expansion due to the helper-derived envelope and appear to remain replication-defective. They could be considered pseudotypes, but we cannot exclude that the genomes of AMV and helper interact in such a way as to form recombinants. Recombination between helper independent RSV and leukosis viruses has recently been described (VOGT, 1971; KAWAI and HANAFUSA, 1972). Tests for the occurrence of recombinants in rescued AMV stocks are clearly indicated.

In this review we have reported that the assay for the transforming activity of AMV is reliable and reproducible. This was in part due to improvements in the growth medium but mainly to the use of yolk-sac cells derived from a particular chicken line developed by the Regional Poultry Laboratory, East Lansing, Michigan (inbred Line 6 of C/E phenotype). This assay will further advance our knowledge on the nature of the transforming property of AMV.

The uncontrolled proliferation of a cell after transformation is a problem of cell differentiation. The AMV system provides a model where hematopoiesis and its dysfunction after viral infection can be analyzed. Simple methods like

colony formation of stem cells, testing of growth factors together with products manufactured by normal or abnormal tissues have led to the exploration of new directions in viral oncogenesis. Our recent studies on spontaneous regression of leukemia (Silva and Moscovici, 1973), followed by the finding that circulating factors like CSF may play a role in the progression and in the regressoin of the disease may justify the view that although viral agents are responsible for initiation of leukemia, disturbed levels of factors regulating hematopoiesis may be necessary for the development and maintenance of the leukemic processes. It has been suggested (Metcalf, 1973) that an imbalance favoring proliferation may result in the emergence of abnormal granulopoietic populations (virus-damaged). At the same time, because of the microenvironmental alterations occurring in the marrow, the progenitors of normal granulopoietic cells are no longer in a position to compete with the prevalence of abnormal elements. The puzzling phenomenon of remission with the disappearance of leukemic populations and re-emergence of normal circulating cells may result from the restoration of a normal microenvironment as well as from killing of leukemic cells. Some of the data emerging from our studies (Dodge and Moscovici, 1973; Silva et al., 1974; Dodge et al., 1975) as well as from others (Metcalf, 1973) encourage interpretations to be made which may also be applied to similar situations observed in human leukemia.

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The Reticuloendotheliosis Viruses

H. G. PURCHASE and R. L. WITTER[1]

With 7 Figures

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I. Introduction

Reticuloendotheliosis (RE) is a predominately neoplastic disease of turkeys, ducks, chickens and other birds, caused by a group of RNA viruses. Morphologically, these RE-inducing viruses (REV) are similar to viruses of the avian leukosis/sarcoma (ALV) group (ZEIGEL et al., 1966, 1967; PURCHASE et al., 1973), but no serologic relationship between REVs and ALVs has been shown (THEILEN et al., 1966; HALPERN et al., 1913; PURCHASE et al., 1913). Similarly,

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the experimentally induced lesions of RE in chickens are distinguishable from those caused by ALVs (lymphoid leukosis) or by the DNA herpesvirus of Marek's disease (CALNEK, 1973). However, naturally occurring disease induced by REVs in any avian species remains ill defined (with the possible exception of leukosis in turkeys) and of uncertain occurrence.

The recognition of serologic relationships between several previously unclassified viral isolates from diverse origins (PURCHASE et al., 1973) suggested that REVs were far more widespread than previously realized. Considerable interest in what appears to be a third major category of avian tumor viruses (with ALVs and Marek's disease herpesviruses) has recently surfaced. Much new information has been obtained from study of those isolates presently available; furthermore, considerable efforts are being made to obtain new isolates. Because the study of REVs is a relatively new field, it was feasible and desirable to make this review as comprehensive as possible. To this end, the bibliography of this review lists all published information on REVs known to the authors at the date of this writing.

The prototype virus is considered to be REV strain T which was originally isolated from turkeys with neoplastic disease by TWIEHAUS in 1958 (ROBINSON and TWIEHAUS, 1974). This virus was studied extensively and was distributed to many laboratories during the 1960's. During passage in chickens or in cell culture, some of the properties of the virus have apparently changed so that preparations of strain T obtained from COOK (COOK, 1969), SEVOIAN (SEVOIAN et al., 1964), FISCHER (FISCHER and THOMPSON, 1967) and TWIEHAUS (ROBINSON and TWIEHAUS, 1974) are no longer of similar pathogenicity (WITTER et al., 1970).

In 1969, Cook isolated an RNA-containing virus from chickens with nerve lesions thought to be Marek's disease (COOK, 1969). The virus, designated as Chick Syncytial Virus, produced syncytia in cultures of chicken embryo cells and was serologically related to REV strain T (COOK, 1969).

Viruses have long been associated with bird passages of the malaria parasite *Plasmodium lophurae*. This parasite was isolated from a Borneo fire-backed pheasant from the New York Zoo (COGGESHALL, 1938) and was used extensively in studying the curative activity of drugs against malaria. During these studies, approximately one half of the birds died with no demonstrable malaria, and DEARBORN (1946) was able to isolate from them a filterable agent that was highly pathogenic for ducks. Although this agent is no longer available for study, it is probably a member of the REV group. TRAGER (1959) isolated a similar virus during the course of passage of *P. lophurae* in ducklings. This virus produced a rapidly fatal disease characterized especially by enlargement and necrosis of the spleen, and Trager referred to this virus as Spleen Necrosis Virus.

CORWIN and MCGHEE (1969) described an anemia of ducklings induced by malarious plasma. Ducklings that had recovered from the anemia were resistant to challenge with *Plasmodium sp.*, and the resistance could be induced

by a filterable agent (McGhee and Loftis, 1968; Ludford et al., 1969). The agent isolated from the plasma was named Duck Infectious Anemia Virus (Ludford et al., 1972). Similar or identical viruses were isolated from three other strains of the *P. lophurae*, two of duck and one of turkey origin. We do not know whether these viruses originated from the Borneo fire-backed pheasant from which *P. lophurae* was originally isolated or whether they were introduced subsequently as contaminants of the avian hosts on which this protozoon was passaged.

The afore-mentioned strains are all serologically related (Purchase et al., 1973) and are the best characterized of the REV group. However, new isolates with properties of REVs have been reported from ducks (Grimes and Purchase, 1973) and from turkeys (Paul et al., 1974; Solomon and Witter, unpublished). Because many future isolations will undoubtedly be made, we propose that all viruses of this group be designated as REV's, with strain designations appropriate to their origin or biological properties as indicated in Table 1. Thus, the Spleen Necrosis Virus, Duck Infectious Anemia Virus and Chick Syncytial Virus would be known as the SN, DIA, and CS, respectively, strains of REV. Insofar as has been determined, all REVs are similar in serological and biochemical properties and can be distinguished only by differences in pathogenicity. However, further work may likely reveal additional distinguishing characteristics.

Table 1. Known strains of REV

Strain	Species of origin	Reference	Pathogenicity		Principal lesions[a]
			host	degree	
T	turkey	Robinson and Twiehaus (1974)	chick	+++ to ±[b]	RE, nerve
			duck	++	RE, anemia
			turkey	+++	RE
SN	duck	Trager (1959)	duck	+++	RE, necrosis
			chick	++	RE, nerve
DIA	duck	Ludford et al. (1969)	duck	++	Anemia
			chick	+	Nerve
CS	chicken	Cook (1969)	duck	—	—
			chick	±	Nerve
?	duck	Grimes and Purchase (1973)	duck	++	RE, nerve
			chick	+	RE, nerve
?	turkey	Paul et al. (1974)	turkey	+++	RE, nerve
			chick	—	—

[a] RE = reticuloendothelial cell proliferation, principally in the liver; nerve = RE and lymphoid cell infiltration of peripheral nerves; necrosis = splenic necrosis.

[b] Some apparently attenuated preparations produce no reticuloendothelial proliferation and only some nerve lesions.

II. Classification

The REVs are ether sensitive, enveloped RNA viruses. The REV strain T was early recognized as being different from ALV (THEILEN et al., 1966). It did not have interfering or resistance-inducing factor (RIF) activity against Rous sarcoma virus, and it did not have the group-specific antigen of the ALV. The REV, although a C-type particle, could be distinguished electron microscopically from ALV (ZIEGEL et al., 1966). In cross neutralization tests, the REV and ALV were also unrelated (HALPERN et al., 1973; PURCHASE et al., 1973). However, the REV strains DIA, SN, CS and T are morphologically identical and serologically closely related (PURCHASE et al., 1973). Additional evidence, which will be described in more detail in sections headed Chemical Composition and Immunology, has confirmed that REVs and ALVs are unrelated. Briefly, there is no genetic or physiologic interaction (HALPERN et al., 1973), there is little or no nucleic acid sequence homology between the RNAs (KANG and TEMIN, 1973) and there is only a distant serologic relationship between the RNA-dependent DNA polmerase of REV and ALV (MIZUTANI and TEMIN, 1973, 1974). Yet the REV strains are very closely related with respect to these properties. Clearly, therefore, the REVs belong to a unique group among the avian RNA tumor viruses. Although the natural host of ALVs of subgroups A to E are chickens, that of the REVs may be turkeys or ducks. The viruses are not entirely host specific because ALVs, particularly Rous sarcoma virus, will also infect ducks and turkeys, and REVs will also infect chickens.

Another view is that there are two distinct types of oncornaviruses of avian species in a similar way that there are two types of oncornaviruses for mice (murine leukemia and mammary tumor viruses) (GROSS, 1970) and for cats (feline leukemia and a group that includes RD 114 and CCC viruses) (MCALLISTER et al., 1973). However, unlike the avian and murine viruses, the two types of feline viruses share some common antigens (SCHÄFER et al., 1973).

III. Morphology

A. Ultrastructure

A close study of electron micrographs led to an understanding of how the virus replicates (ZEIGEL et al., 1966). Virus particles appeared to proliferate from connective tissue cells and from reticular and endothelial cells budding directly from endothelial cells into capillary lumens. During the budding process, the virus nucleoid originated as an electron opaque crescent. Surrounding the nucleoid was an intermediate ring; and surrounding this ring, the envelope appeared as a unit membrane composed of two leaflets. In mature particles, the nucleoid was relatively dense and homogeneous. In contrast, the immature forms of ALV do not have a distinct intermediate membrane associated with the nucleoid area; and in mature virus particles, the nucleoid is smaller and relatively more dense and compact than that of the REV. In many respects, REV resembles the murine leukemia viruses

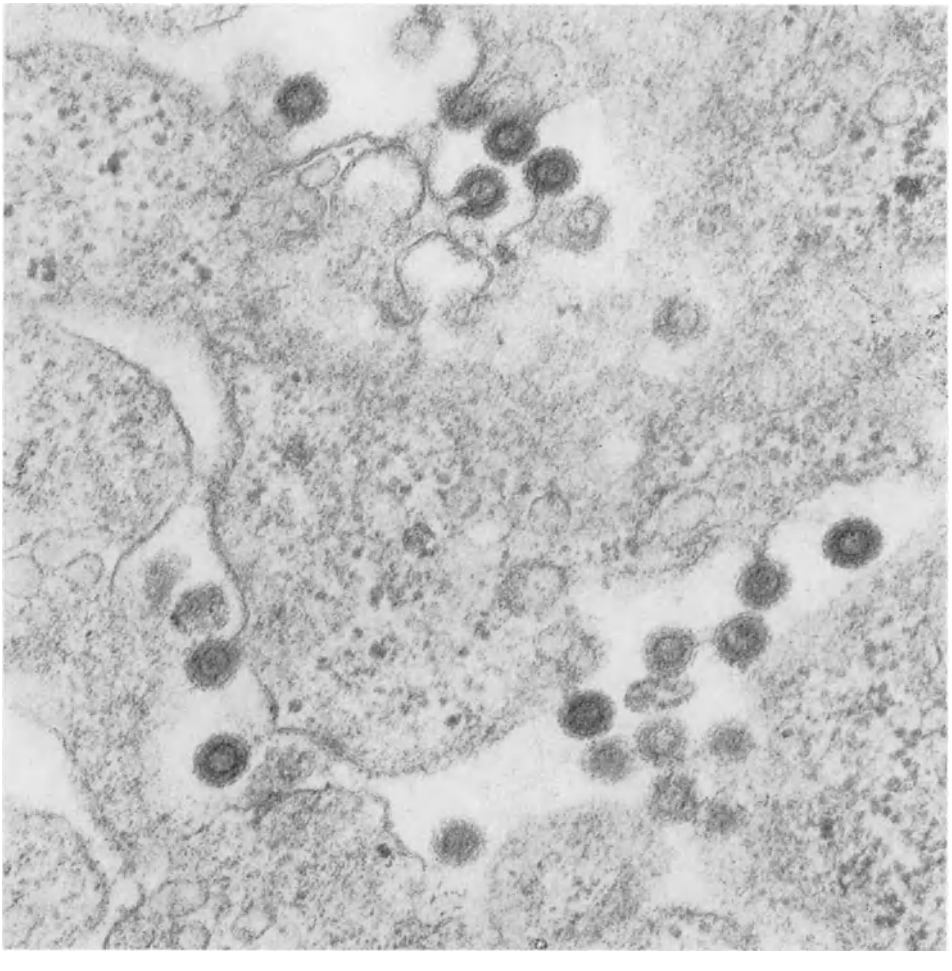

Fig. 1. A thin section of REV strain DIA in duck embryo fibroblasts. ×62,000 (Courtesy of Dr. K. NAZERIAN)

more closely than it resembles the ALVs. Although the differences between the ALVs and the REV may be difficult to detect in micrographs from different laboratories, they can be seen when the viruses are mixed before thin sectioning and staining (ZEIGEL et al., 1967). Although in some preparations the "halo" of peripheral spikes characteristic of ALV were not present on REV (ZEIGEL et al., 1966, 1967), they appear to be present in others (PURCHASE et al., 1973) particularly when negatively stained preparations are examined (PADGETT et al., 1971). During disruption of the virus, three distinct layers could be observed: a central shell, a dense homogeneous intermediate layer and an envelope bearing short projections (PADGETT et al., 1971). Like the envelope of the ALVs the envelope of REV is easily distorted into bizarre shapes during negative staining. The morphology of strain DIA in thin sections is illustrated in Fig. 1.

B. Size

In thin sections the virus has been reported to be 75 nm (PADGETT et al., 1971) to 100 nm (ZEIGEL et al., 1966; BAXTER-GABBARD et al., 1971a, 1971b) in diameter and in negatively stained preparations, 80 to 115 nm.

C. Density

In linear sucrose gradients the density is between 1.16 and 1.18 g/cc (BAXTER-GABBARD et al., 1971a, 1971b). This density is very close to that of the ALVs. However, REV can be separated from the ALVs by using formaldehyde-fixed preparations and isopycnic centrifugation in a cesium chloride gradient. The REV had a density of 1.203 g/cc and the ALV 1.242 g/cc (MALDONADO and BOSE, 1971).

IV. Chemical Composition

A. Ribonucleic Acid

Nucleic acid extracted from REV preparations with sodium dodecyl sulfate and phenol was sensitive to pancreatic RNase but not to DNase. These results indicate that the nucleic acid was single-stranded RNA. The OD ratio 280/260 of purified REV from sucrose gradient was 0.77. The estimate of BAXTER-GABBARD et al. (1971a, b) that 7 to 8% of the virus is nucleic acid may be high because of the impurity of the virus preparation and inaccuracy of the methods used since other RNA tumor viruses have about one eighth this amount.

The RNA of REV and ALV consists of two major species approximately 60 to 70S and 40S, as determined by sedimentation on sucrose gradients. The high molecular weight RNA of REV sediments faster than that of RSV (MALDONADO and BOSE, 1973). The RNAs of these two groups of viruses are indistinguishable in their rates of migration through polyacrylamide gel. However, after denaturation at 100°C, the RNA of REV sedimented slightly more rapidly than that of the ALV and migrated slightly more slowly in coelectrophoresis (HALPERN et al., 1973).

Although the different ALVs have closely related nucleotide sequences and the REVs have closely related nucleotide sequences, very little homology ($<4\%$) between the RNAs of these two groups of viruses has been found (KANG and TEMIN, 1973). However, the techniques used may not have detected homologies in short sections of the genome, which may have been infrequently represented in the complementary DNA used as a probe. In transcribing the DNA from viral RNA, KANG and TEMIN (1973) noted that the RNA of REV was as good a template-primer for the ALV enzyme as was homologous RNA. But when several different viruses in each group were used, no significant hybridization between the RNA of the REVs was obtained and the DNA produced from ALVs or *vice versa* even after 40 hours of annealing. Yet homologous RNA-DNA hybridization was between 85 and 100% complete, and the hybrid was therefore resistant to digestion with RNase. The homo-

logous hybrids had a steep melting curve that indicated the absence of extensive mismatching. Also, the C_rt values of 50% hybridization were similar for both groups of viruses; indicating a similarity in complexity of the RNAs. Interestingly the hybridization between the REV DNA product and the RNA from normal chicken cells free of group-specific antigen was 5 to 6 times above background; this indicated that normal chicken cells contained some REV-related genes. On the other hand, DNA complementary to chicken microsome fraction did not hybridize with the RNAs of ALV or REV; thus, the ALV or REV polymerases are unrelated to the chicken endogenous RNA directed DNA polymerase (KANG and TEMIN, 1972, 1973).

B. Polymerase Activity

A DNA polymerase activity can be shown in REV by using the synethic template dG:rC (BAXTER-GABBARD et al., 1971). Similarly, PETERSON et al. (1972) showed that viral DNA polymerase was RNA dependent and that the product was DNA whether the template was viral RNA or the synthetic hybrid. The polymerase has a molecular weight of 70000 to 75000 in contrast to that of the ALVs, which is 100000 to 180000 (MIZUTANI and TEMIN, 1974). In addition, it aggregated or changed in conformation or both on exposure to different salt concentrations and yielded a different sedimentation pattern in glycerol gradients. The purified polymerase of ALVs sedimented in glycerol gradients of different salt concentrations with the same band width and in the same position.

The serologic relationship among DNA polymerases of ALVs, REVs and chicken cells was examined by MIZUTANI and TEMIN (1973, 1974). Specific antibodies were prepared against the RNA dependent DNA polymerases of avian myeloblastosis virus and the small and large endogenous polymerases of normal chicken cells. In neutralization and blocking tests, the polymerases from REV strains T and SN are antigenically distinguishable from other polymerases but share some determinants with those of ALVs and the polymerases of normal chicken embryo and turkey and duck liver. They are completely unrelated to the enzymes of Hela cells and *Escherichia coli*. The polymerase of REV strain CS was closely related to that of strain T, but the polymerase of strain CS had no antigens in common with the polymerase from normal chicken cells. In this respect, strain CS is different from strain T or SN. The REV polymerase and normal chicken cells appear to share common antigens, which is consistent with the observation that normal embryonic chicken cells also contain RNA which is homologous to DNA transcribed from the REV genome (KANG and TEMIN, 1973). Thus, nucleic acid hybridization and serologic studies of polymerase demonstrate a relatedness of both ALVs and of REVs to normal chicken cells.

C. Proteins and Polypeptides

The proteins and polypeptides of REVs have been studied in detail. They were extracted with sodium dodecyl sulfate and mercaptoethanol and sub-

mitted to polyacrylamide discontinuous gel electrophoresis (HALPERN et al., 1973; MALDONADO and BOSE, 1973). The pattern of viral protein peaks produced resembled those of the ALV, but no two peaks were identical in electrophoretic mobility. The most prominent peak of the REV (29500 MW) migrated identically in REV strains T, CS and DIA and was close to the most prominent peak for the ALV. In all, five proteins with molecular weights of 12500, 15500, 19500, 22000 and 29500 and two glycoproteins with molecular weights 39000 and 105000 were detected (MALDONADO, personal communication). Lactoperoxidase-catalyzed iodination of the surface proteins of intact viruses indicated that the glycoproteins were on the surface of the virion (HALPERN et al., 1913). All proteins were iodinated when disrupted particles were labeled; this indicated that iodination of the glycoproteins was not because they contained an unusual amount of tyrosine.

V. Resistance to Chemical and Physical Agents

As mentioned previously, REVs are sensitive to lipid solvents such as ether and chloroform. They are also relatively heat labile. In general, they survive without loss of activity for long periods of time at −70°C, however, occasional stocks of virus lose titer when stored under these conditions (PURCHASE and WITTER, unpublished results). The loss of infectivity may be reduced by stabilization with glycerol (CAMPBELL et al., 1971). Homogenates of liver remained infective for 14 months at −56°C when stabilized in 50% glycerol but not when stabilized in 10% dimethylsulfoxide or in saline. Lyophilized virus retained infectivity for 4 months at −56°C (TAYLOR and OLSON, 1971). Although the virus apparently survived with little loss of infectivity after 24 hours at 4°C, 50% of the infectivity was lost in less than 20 minutes at 37°C and 99% of the virus was lost after 1 hour at 37°C. No virus was detected after incubation for 3 minutes at 56°C in stock that titered $10^{2.4}$ LD_{50} per ml before treatment (CAMPBELL et al., 1971). Similar results on thermolability and ether sensitivity were obtained by THEILEN et al. (1966). Because of the uncertainty of the stability of the virus, a cell-containing tissue homogenate to which dimethylsulfoxide has been added may be frozen slowly and the mixture stored in liquid nitrogen (WITTER and PURCHASE, unpublished observation). The optimum pH for maximum stability of purified REV was 6.0. The titer of virus decreased with time in media of a higher or lower pH (CAMPBELL et al., 1971).

VI. Laboratory Host Systems

A. Animal Inoculation

1. Host Range

Strain T is highly lethal for young chickens, quail, ducklings, goslings, turkeys, pheasants and guinea keets (TAYLOR and OLSON, 1972; THEILEN et al., 1966; RODRIGUEZ and FIELD, 1969; RODRIGUEZ, 1971). Lines of chickens

genetically resistant to Marek's disease (Regional Poultry Research Laboratory Line 6) were susceptible to REV (WITTER et al., 1970) and strains highly susceptible to Marek's disease (the Cornell S line) were as susceptible as commercial chickens (SEVOIAN et al., 1964). Mature quails (TAYLOR and OLSON, 1972) and 10-week-old chickens were susceptible to the disease; however, 1-day-old chickens were more susceptible than 10—week-old chickens (LAROSE and SEVOIAN, 1965). High levels of supplemental vitamin A did not affect the susceptibility of chicks to strain T (MITROVIC et al., 1969). Mature pigeons were refractory (TAYLOR and OLSON, 1972). Mammals, including rats, mice and hamsters, are probably not infected with the virus because the virus does not produce disease in them (TAYLOR and OLSON, 1972) and it does not grow in cultured cells of any mammalian source (CALNEK et al., 1969).

Strains CS, DIA and SN propagate in chickens and ducks (PURCHASE et al., 1973). Although they have not been tested, strains pathogenic in chickens and ducks probably have a similar host range to that of strain T in other birds and mammals.

2. Route of Inoculation

In laboratory trials, REV is usually administered by the intraabdominal route, however, there is little difference between this route and the intramuscular, subcutaneous or intracardial routes of administration. Intracranial, intranasal and oral administration were less effective (PETERSON and LEVINE, 1971; TAYLOR and OLSON, 1971). Chicks were viremic between the 4th and the 11th day after infection as determined by arthropod transmission studies (THOMPSON et al., 1968). Viremia was at its highest between the 9th and 11th day after infection (THOMPSON and FISCHER, 1970) and most tissues, including spleen, liver, skin, feather shaft and follicle, oral-nasal washings and gut washings, were infectious at 28 days (PETERSON and LEVINE, 1971). Antibody developed in most surviving chickens by 4 weeks (LAROSE and SEVOIAN, 1965).

3. Source of Virus

DEARBORN (1946) early recognized that viruses associated with avian malaria and now considered to be REV varied significantly in pathogenicity depending upon the source. Passage in cell culture appeared to result in a decrease in pathogenicity after a relatively small number of passages (SEVOIAN et al., 1964; THEILEN et al., 1966; WITTER et al., 1970; CAMPBELL et al., 1971). Thus, it is not surprising that preparations passed extensively in cell culture (that of COOK, 1969) should be less pathogenic than those passed *in vivo*, and that virulent strains should be passed only *in vivo* if virulence is to be maintained. In general, strains T and SN are highly pathogenic for both ducks and chickens and DIA and CS are less pathogenic (PURCHASE et al., 1973; PURCHASE and BURMESTER, 1973).

4. Incubation Period

High concentrations of virus cause death within three days (THEILEN et al., 1966), however, deaths more usually start at about 7 days, and often all birds

are dead by 21 days. With lower doses of virus or when the virus is injected into older animals, the incubation period is lengthened. With less pathogenic viruses and among the survivors of the acute disease, death may occur sporadically at any time thereafter.

5. Clinical Signs

In the acute disease, there are few clinical signs until a few hours before death, when birds become lethargic. If they survive the first 2 weeks or so, they are often emaciated and anemic, have poor feather development and are greatly retarded in growth. Paralysis and incoordination are rare in birds even with extensive nerve lesions. In this respect RE differs from Marek's disease (Witter et al., 1970).

6. Gross Lesions

Lesions induced by all viruses of this group are of three types: proliferative visceral, proliferative neural and necrobiotic lesions. The proportions of these types that occur with each virus vary. Thus, proliferative visceral lesions predominate with strain T, whereas necrobiotic lesions particularly of the spleen, predominate with strain SN. Proliferative neural lesions appear to take longer to develop than the proliferative visceral or necrobiotic lesions but are seen under suitable conditions in chickens with all viruses and are particularly common when viruses have been altered by passage in cell cultures. Neural lesions have also been found in ducks (Grimes and Purchase, 1973), and turkeys (Paul et al., 1974).

In the acute disease induced in chickens or ducks by strain T, proliferative or neoplastic lesions predominated. Livers and spleens were enlarged and pale, and the subcapsular surface was often covered with small, irregular, grayish foci (Fig. 2). Livers occasionally had subserosal hemorrhages. Yellowish white tumors were seen in almost any organ but were more frequently seen in the heart, kidney and intestinal lymphoid areas than in others. Bursa of Fabricius

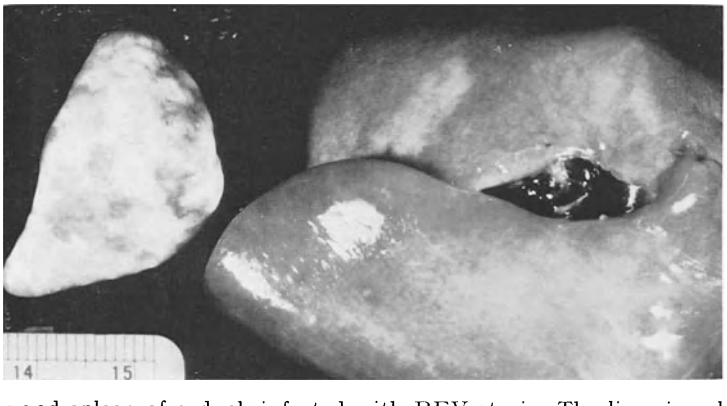

Fig. 2. Liver and spleen of a duck infected with REV strain. The liver is enlarged and has mottled whitish areas of reticuloendotheliosis. The spleen is greatly enlarged and many of the areas of reticuloendothelial proliferation are necrobiotic

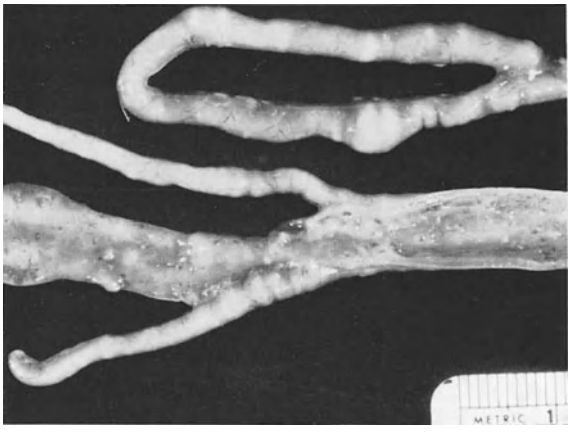

Fig. 3. Intestines of a duck infected with REV strain SN. Areas of reticuloendothelial infiltration which frequently form circumferential bands can be seen from the serosal side of the intestine and cecum. Thickening of the intestional wall and the beginning of ulcer formations are seen in the opened large intestine

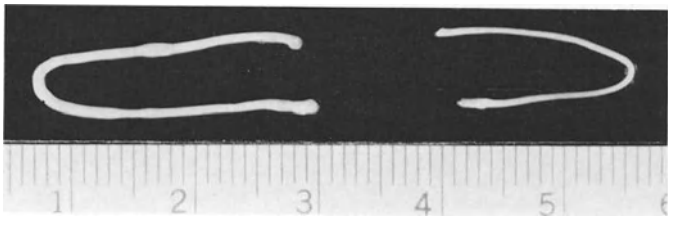

Fig. 4. Vagus nerves from a control chicken (right) and one of a similar age infected with REV strain T (left). (Printed with permission of the National Cancer Institute)

and thymus were not always spared from tumor; however, they were more frequently severly atrophied. The incidence of lesions in the various organs in any one chick was variable, though occasionally most organs were involved (SEVOIAN et al., 1964; LAROSE and SEVOIAN, 1965; THEILEN et al., 1966; OLSON, 1967; MUSSMAN and TWIEHAUS, 1971; TAYLOR and OLSON, 1973a).

The necrobiotic changes were most pronounced with strain SN in ducks (TRAGER, 1959; PURCHASE et al., 1973) although they were also seen with strain T, particularly in ducks (PURCHASE et al., 1973) and quail (THEILEN et al., 1966). Spleens were enormously enlarged and had a caseous opaque necrotic part. They frequently ruptured and hemorrhaged at the site of the rupture. Ulcers, which were sometimes hemorrhagic and had a raised border or a white necrotic center, frequently were found in the intestines in ducks though rarely in chickens (Fig. 3).

Nerve involvement developed after 4 to 6 weeks in chickens given sublethal doses of virus. Any of the nerves were affected and were up to twice the diameter of nerves of control chickens (Fig. 4). Discoloration and loss of striations were rare.

7. Hematology

In chickens inoculated with strain T, there was a decrease in packed-cell volume and white blood cell count in infected moribund chicks. In differential counts, there was a shift to the left, with an increase in immature cells, leukoblasts, and hemocytoblasts. Thrombocytes with cytoplasmic vacuoles and immature erythrocytes were commonly seen in blood smears. The sedimentation rates were increased, and clotting time was between 5 and 10 minutes (OLSON, 1967). Beginning at about 6 days post inoculation there was a marked decrease in total blood leukocytes and hetrophils but an increase in lymphocytes (TAYLOR and OLSON, 1973 b).

In ducks inoculated with strain DIA, the mean erythrocyte count fell from 2.1 to $2.5 \times 10^6/mm^3$, beginning about the 5th day, and reached its lowest value of $1 \times 10^6/mm^3$ after 14 days. Thereafter, it fluctuated slightly but generally remained low. After contact transmission in ducks, there was a more gradual drop in the erythrocyte count. This anemia was a predominent feature of the disease induced in ducks by strain DIA.

In chickens inoculated with strain T, whole serum was examined electrophoretically. The level of gamma-1-globulin (transferrin) increased greatly but that of albumin decreased. Moderate elevations in all other serum proteins, except albumin, were observed, but total serum protein did not increase significantly (TORRES-MEDINA et al., 1973).

8. Microscopic Lesions

The proliferative lesions in the liver and other visceral organs consisted of an abundant cellular infiltration and proliferation. The cells varied in size and were mononuclear and had a large vesicular nucleus, a thin chromatin network and an abundant neutrophrilic or basophilic cytoplasm. Mitochondrial alterations have been described (BALCLAVAGE et al., 1972). The cells (OLSON, 1967; SEVOIAN et al., 1964) are histiocytoid (MUSSMAN and TWIEHAUS, 1971) and are assumed to have originated from the reticuloendothelial system. Mitoses were frequent. In hepatic tissue, the proliferation was prominent around the veins and along the sinusoids (Fig. 5). Large areas of the liver were extensively invaded with the tumor cells. The same cells were involved in lesions of the other organs. In tumors, particularly in those of long standing, small and medium lymphocytes were present among the reticuloendothelial cells. Presumably, they represent the host cellular immune response to the tumor. Typically, lesions were extensive in the liver, spleen, pancreas, kidney, thymus and bursa of Fabricius, but were less extensive in many of the other organs of the body. In the intestine, the cells frequently invaded the muscularis.

In the necrobiotic lesions, cells became pyknotic, and karryorrhexis was prominent, particularly in the spleen. Eventually, large areas consisting of hemorrhages developed. In the intestine, the epithelium over areas infiltrated with reticuloendothelial cells became necrotic and sloughed off and left an

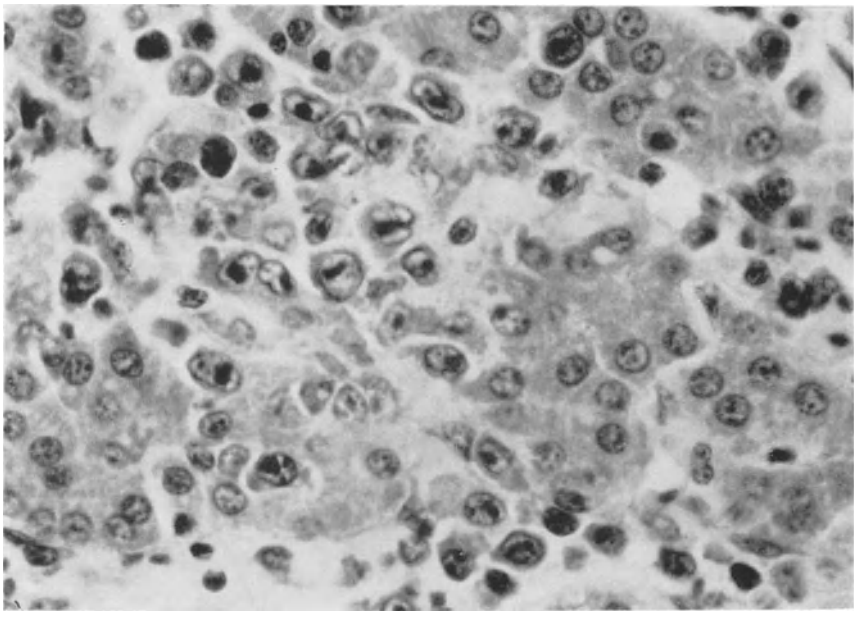

Fig. 5. Section of the liver of a chicken inoculated with REV showing reticuloendothelial cell infiltration. Occasional medium and small lymphocytes can also be seen. H and E. ×790

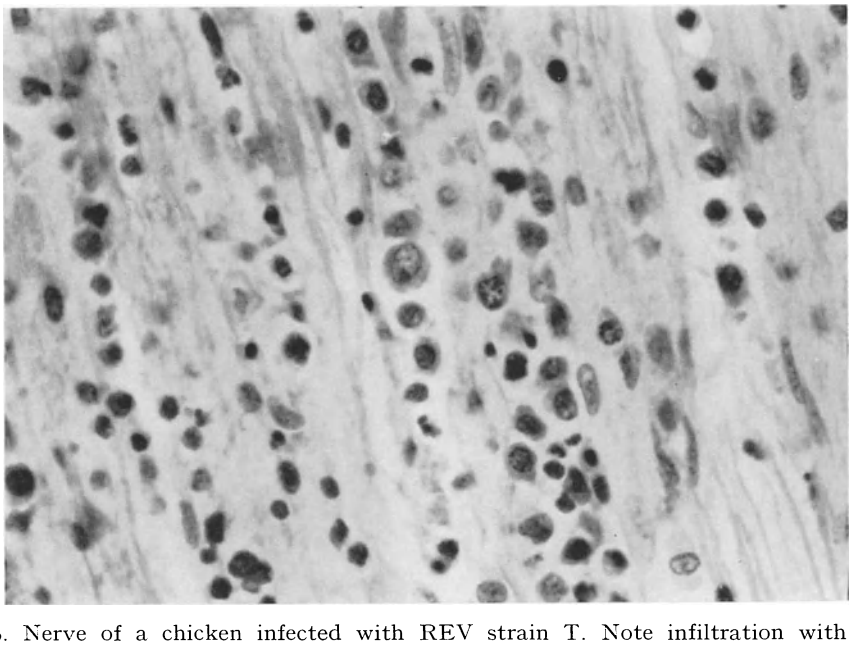

Fig. 6. Nerve of a chicken infected with REV strain T. Note infiltration with few reticuloendothelial cells and many cells of the lymphoid series. H and E. ×790

ulcer. Sometimes necrosis was extensive in the reticuloendothelial cells, and occasionally it extended into the muscularis.

The proliferative neural lesions consisted of a diffuse infiltration of the same types of cells as described above in the liver. Plasma cells also were present. Cells were often arranged linearly between the nerve fibers, and edema in some affected nerves caused separation of the fibers (Fig. 6).

Whether the proliferative lesions of RE are neoplastic is controversial Although tumors may occur at the site of inoculation (the wing web) they are more common in the visceral organs. The cells are of a primitive type and mitoses are abundant, but we do not know whether the cells can be transplanted because the virus-induced tumors have such a short latent period. However, the recent development of a cell line from the bone marrow of chicks infected with REV strain T, which produces virus that induces tumors both systemically and at the site of inoculation (FRANKLIN et al., 1974) is evidence for a true oncogenic potential of REVs.

B. Embryo Inoculation

SEVOIAN et al. (1964) inoculated 4-day-old chicken embryos via the yolk sack with graded doses of virus. Sporadic mortality was observed during the second one-half of the incubation period, however, most of the embryos survived. Affected embryos were smaller and some had extra-embryonic fluids that were thick and viscid or discolored green. Livers were enlarged and a mahogonybrown to green, and spleens were enlarged up to 20 times the normal size and occasionally had tumorous nodules. Histologically, stimulation of the primitive mesenchymal cells in the hematopoeitic tissues was found in or around the walls of the capillaries, arterioles and sinusoids of the liver and bone marrow. Similar proliferative lesions were found in many other organs, including kidneys, eye and brain. Harvested livers and spleens contained virus.

THEILEN et al. (1966) observed similar lesions in chicken embryos inoculated via the chorioallantoic membrane route. They also observed pock-like lesions on the membrane, with high concentrations of virus. The lesion was generally one large raised pock or two to three small pocks that became confluent to form a single lesion. The pocks were raised, hemorrhagic and gelatinous. Histologically, the lesions were composed of cells similar to those infiltrating the affected organs of inoculated chicks, however, the cells were pyknotic and karyorrhexis and evident. Virus particles were seen budding from cells in the lesions (ZEIGEL et al., 1966).

Chicken embryos can be used as a host for bioassay of virus infectivity or for assay of residual virus in the neutralization test (SEVOIAN et al., 1964; THEILEN et al., 1966).

C. Cell Cultures

Initial attempt at propagation of REV in cell culture was thwarted by the absence of a cytopathic effect and the lack of a rapid assay technique. SEVOIAN et al. (1964) propagated the virus through four serial passages in chicken embryo fibroblasts; but when the virus was assayed in chickens the

Fig. 7. Chicken embryo fibroblast culture infected with REV strain DIA and stained in the indirect fluorescent antibody test with antiserum to REV strain T. ×200. (Printed with permission of Experimental Parasitology)

final harvest produced less disease than tumor homogenated produced. ZEIGEL et al. (1966) and BOSE and LEVINE (1967) found that the virus would propagate in chicken embryo cell cultures, but they also found that the pathogenicity of the cultured virus was less than that of the original tumor homogenate. In neither instance were cytopathic effects observed.

The direct or indirect fluorescent antibody test has been used to show that strain T will propagate in chicken and duck embryo fibroblasts (COOK, 1969; CALNEK et al., 1969). A similar technique was used to show that strain DIA propagated better in embryo fibroblasts than in kidney cells from ducks and chickens (LUDFORD et al., 1972). Apparently, all viruses of this group replicate well in chicken or duck embryo fibroblast cell cultures, and these cell cultures may be used in combination with the fluorescent antibody test for viral assay, including residual virus assay after a neutralization test (WITTER et al., 1970; PURCHASE et al., 1973; HALPERN et al., 1973).

When the fluorescent antibody test was used, foci of infected cells could be clearly seen among the otherwise normal cells (Fig. 7). Many of the cells appeared larger in outline; but on direct examination of unstained cultures, these areas could not be identified (PURCHASE and WITTER, unpublished observations). COOK (1969) observed that the CS strain produced syncytia in chick embryo fibroblast cultures and used this property in a virus assay; however, although

others have used this procedure (WITTER et al., 1970), the appearance of syncytia is rather inconsistent, particularly with viruses other than strain CS (PURCHASE and WITTER, unpublished observations). Recently, TEMIN and KASSNER (1974) described a plaque assay for strains SN and DIA in duck embryo fibroblasts. They found that the virus replicated better in chicken, pheasant and turkey cells than in quail and duck cells. The plaque assay in duck cells worked better for strains SN and DIA than for CS and T. When the duck embryo fibroblasts were infected with these viruses, areas of approximately 10 to 20 dead cells could be seen after 3 to 4 days of incubation. However, after an additional 1 or 2 days, the dead cells sloughed off into the medium, and the plaques were difficult to see. Carrier cultures with no obvious cytopathology were established. The latent period for virus production and development of cytopathic effects was about two days. Although the normal replicative cycle of the cells is necessary for normal REV production, stationary cells could be infected. Infection of stationary cells required DNA synthesis but not protein synthesis, a finding consistent with the presence of a DNA polymerase in the virion.

VII. Immunology

A. Serologic Relatedness

In reciprocal neutralization tests, the viruses isolated from ducks (strains DIA and SN), the virus from chickens (strain CS) and the virus from turkeys (strain T) are not identical. The extent of neutralization with homologous serum is greater than that with hetrologous serum. However, strain DIA from different sources and strain SN appeared identical, and strain T from different sources appeared identical. The cell in which the virus was propagated in order to produce stocks did not seem to influence this relationship (PURCHASE et al., 1973).

B. Detection of Antibody

Antibody can be detected by neutralization and assay of residual virus *in vivo* (LAROSE and SEVOIAN, 1965; THEILEN et al., 1966; SEVOIAN, 1968; WITTER et al., 1970), in embryos (SEVOIAN et al., 1964; THEILEN et al., 1966) or in cell culture in which the fluorescent antibody test is to used detect virus (COOK, 1969; PURCHASE et al., 1973). Antibody can also be detected in the yolk (AULISIO and SHELOKOV, 1969) of chicken eggs and in serum from chickens and turkeys (PURCHASE et al., 1973) by the indirect fluorescent antibody test.

C. Role of Immune System in Tumor Production

The REV strain T has been reported to protect against Rous sarcomas (BAXTER-GABBARD et al., 1973). When organ extract from RE-affected birds or purified infectious virus was administered intramuscularly every 12 days for three injections, it protected against tumors induced by Schmidt-Ruppin Rous sarcoma virus (RSV) of subgroup B but not as effectively against

RSV(RAV-1). These ALVs and RAV-1 did not offer protection against strain T. From these results, we cannot clearly conclude whether REV and ALV have a common antigen or produce common antigens in birds or whether the REV used for immunizing was contaminated with infectious ALV because the tests used (for group-specific ALV antigens) were not sensitive enough to detect low levels of virus (BAXTER-GABBARD et al., 1973). Another remote possibility is that the mechanism of resistance may be similar in nature to that produced by strain DIA against *Plasmodium lophurae* (LUDFORD et al., 1969). This resistance has been postulated to be of a non-specific nature (MACKANESS, 1964).

The REV strain T has been used to study the effect of the immune system on progression and regression of tumors (HU et al., 1973; LINNA et al., 1972, 1973, 1974; TAYLOR and OLSON, 1973a; THOMPSON and LINNA, 1972, 1973a, 1973b). Groups of chicks were surgically thymectomized, surgically bursectomized or chemically bursectomized with cyclophosphamide at hatching. They were inoculated with REV intraabdominally and in the wing web, and death and development of a local tumor were studied. Thymectomy and bursectomy consistently resulted in an increase in mortality and number of local tumors and a decrease in the number that regressed. The experiments, therefore, indicated that both the thymus and the bursa had an immune surveillance function in this tumor system. The role of the bursa is further supported by the protective effect of immune serum on mortality and its enhancement of tumor regression (HU and LINNA, 1974).

VIII. Natural Disease Occurrence

AULISIO and SHELOKOV (1969), using the indirect fluorescent antibody test on yolk, showed the presence of antibody to REV in 41 of 92 chicken flocks examined. The flocks came from all parts of the United States. However, neutralizing antibody was not found by SEVOIAN et al. (1964) in four flocks, BOSE and LEVINE (1967) in three flocks, WITTER et al. (1970) in five flocks or PURCHASE et al. (1973) in 14 flocks when the indirect fluorescence antibody tests were used on serum. This discrepancy is difficult to account for, particularly because viruses of this group do not appear to spread readily horizontally in chickens. Because of the close relatedness of these viruses, particularly in the fluorescent antibody test where they are indistinguishable, the antibody detected by COOK (1969) was not likely a commonly occurring agent whose antibody would not have reacted in the systems for antibody detection used by the other investigators. However, many of the flocks examined by AULISIO and SHELOKOV (1969) possibly were back yard flocks in contact with wild water fowl and so acquired the infection from them, and the flocks examined by others were commercial flocks reared under intensive husbandry conditions where no contact with other fowl was possible. Certainly, because many of the flocks selected by WITTER et al. (1970) and PURCHASE et al. (1973) had severe losses from disease, with abundant nerve lesions that could be confused

with those induced by REV, the REV group of viruses are probably not an economic problem among chickens in the field.

Viruses of the REV group probably do not occur in domestic ducks reared under intensive commercial husbandry conditions. They appear to be natural infections of wild water fowl, particularly ducks and geese, and because the virus is readily transmitted horizontally among these birds, it is probably transmitted from them to an occasional domestic flock. We do not know whether economic losses in ducks are due to this virus, but in Australia, an isolated case of RE apparently induced by an REV-related virus has been described (GRIMES and PURCHASE, 1973).

In the turkey, REVs have been stongly associated with a naturally occurring neoplastic disease, so-called turkey leukosis. Strain T was isolated from such an outbreak (ROBINSON and TWIEHAUS, 1974). Antibodies were found in two turkey flocks with histories of losses from neoplastic disease (PURCHASE et al., 1973). Recently PAUL et al. (1974) isolated REV's from two outbreaks of turkey leukosis and reproduced the disease in turkeys by inoculation of cell-free filtrates. The experimentally induced lesions included multiple lymphoproliferative foci (lymphomas) in various visceral organs and infiltration of the peripheral nerves. Although turkey leukosis occurs only sporadically and may well include diseases of diverse etiologies, it seems to provide the best model presently available for studying naturally occurring REV infection and disease.

Very little concerning the epidemiology of REV infection in the different avian species is known. Although strain T is transmitted horizontally among chickens at only a very low rate (LAROSE and SEVOIAN, 1965; PURCHASE et al., 1973), SN strain is transmitted horizontally more frequently (PURCHASE et al., 1973). Vertical transmission of these viruses has not been investigated adequately, and the means by which these viruses maintain themselves in natural populations is not known.

THOMPSON et al. (1968, 1971) were able to show that a blood-sucking insect, *Triatoma infestans*, would readily transmit REV. These insects could harbor the virus for at least 72 hours but not 96 hours. The REV remained infectious for up to 24 hours in a variety of mosquito species and *Stomoxys calcitrans*, for at least 1 day in *Argas* ticks and for at least 1 week in *Ornithodorous* ticks. Virus did not reappear in any of these insects at a later time. Therefore, none of these would likely be biological vectors of REV although mechanical transmission was shown with *S. calcitrans*.

To further studies on naturally occurring RE infection, we need to document suspected cases of RE in various species (as was done by CARLSON et al., 1973) and to obtain specimens for virus isolation. A diagnosis of RE based on strictly pathologic criteria is virtually impossible. However, if virus can be isolated or antibodies shown in the affected bird, a provisional diagnosis can be made. Such a diagnosis can best be confirmed by isolation of the causative REV and transmission of the disease in the same species.

IX. Summary and Conclusions

Reticuloendotheliosis viruses are RNA tumor viruses similar in some respects to the ALV but different in a number of criteria. They can be distinguished morphologically, they lack common group specific antigen of ALV, they are not serologically related by neutralization tests, they do not interact genetically and the RNA-dependent DNA polymerases of the virus are different. Also, there are no detectable nucleotide sequences in common between the RNAs of members of these two groups of viruses. However, both have an RNA dependent DNA polymerase associated with virus infectivity in common with the other RNA tumor viruses of avians and mammals. The REVs can be regarded as a distinct group of viruses.

The viruses are probably worldwide in distribution, and their natural hosts are probably turkeys and wild water fowl. We do not know whether they cause significant economic loss to the poultry of any country; however, they are so pathogenic that sporadic losses seem likely. Neoplastic disease related to REV has been documented in turkeys. Considerable additional epizootiologic studies, however, are necessary to clarify the situation.

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Antigen-Binding Properties of Antibody Molecules: Time-Course Dynamics and Biological Significance

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Abbreviations

K_0	equilibrium constant
ΔF	free energy change
k·cal/mole	kilo calory·mole^{-1}
α or a	heterogeneity index
Ig	immunoglobulin
Fab	fragment of immunoglobulin bearing the antigen-combining site
DNP	dinitrophenyl
AFCP	antibody-forming cell precursor
NIP	4-hydroxy-3-iodo-5-phenylacetic acid
a.a.	aminoacid
IEF	isoelectric focusing
pI	isoelectric point
CD	circular dichroism
PCA	passive cutaneous anafylaxis
C′	complement

I. Introduction

A. Importance of the Subject and Scope of the Review

Injection of an immunogen into a susceptible, immunocompetent animal, elicits production of antibody molecules specific to the antigenic determinants

of the immunogen. If the immunogen possesses different determinants the corresponding antibody species are synthesized, thus in the case of complex immunogens the response is made up of a mixture of several antibody populations with different specificities. These antibody populations appear simultaneously or successively. Within each specificity subpopulations of molecules are often found differing in the strength with which the antigen-combining site binds to the corresponding determinant. The binding properties as well as other characteristics of antibodies, such as immunoglobulin class, allotype, and idiotype usually change with time after immunization. We shall review data on these chronological changes as a means of looking into the time-course dynamics of antibody-forming clones. Most of the information available refers to antibodies of IgG class and, to a lesser extent, to antibodies of IgM class, purposely induced by injecting experimental antigens. Data on the binding properties of other Ig classes are scarce. For IgA some information exists which was obtained using myeloma proteins; we shall consider it only marginally.

The importance of defining an antiserum, not only in terms of antibody concentration, but also with respect to its antigen-binding properties, the role of these properties in determining the biological effects of the antigen-antibody interaction, for instance neutralization of a toxin, and the modifications of these properties with time after immunization were extensively investigated and pointed out nearly a quarter of a century ago (JERNE, 1951). We shall, therefore, also review experiments and observations in pathological conditions which give clues to the understanding of the biological significance of the binding properties of antibodies, and their modifications in the course of the immune response and in certain circumstances during life.

II. Definitions and Concepts

A. Association Constant and Heterogeneity Index

The reaction between antigen and the corresponding antibody can be measured and expressed in terms of quantity. [This and related subjects have also been discussed elsewhere (MACARIO and CONWAY DE MACARIO, 1975a).] The energy of the interaction, dependent on the strength and number of the non-covalent bonds between the binding site and the determinant, may be referred to as the equilibrium constant (K_0, in liter/mole) or as the standard free energy change (ΔF, in k·cal/mole). Measurements are not possible at the level of a single site interacting with a single determinant; they are done using populations of sites and determinants allowed to interact under controlled conditions of temperature, ionic strength, etc. The final result, antibody affinity, then represents an average of all interactions in the system. The dispersion of different equilibrium constants about the mean can also be estimated, and is named heterogeneity index (a or α). When $\alpha = 1$ all interacting antigen-combining sites are considered homogeneous in terms of affinity; as heterogeneity increases, α becomes smaller than one. Usual calculations

of K assume that antibody populations with different affinities are normally distributed, which is not always the case (Pressman et al., 1970; Werblin and Siskind, 1972); therefore results may sometimes be misleading. It has to be borne in mind that $\alpha = 1$ shows restriction of antigen-combining sites, homogeneity in terms of affinity, but that it does not tell whether the antibody molecules are similar or different in other physico-chemical properties. Antibody samples have been found which have homogeneous binding activity but are heterogeneous in other properties (Pincus et al., 1968); however, homogeneity of antibody molecules according to one or more physicochemical criteria, e.g. isoelectric focusing and a.a. sequence, has been found to correlate with a heterogeneity index close to unity (Keck et al., 1973; Roholt et al., 1970).

In view of these limitations in the assessment of the degree of heterogeneity of an antibody preparation, attempts have been made to evaluate more accurately the number and distribution of antibody populations with different affinities in a given sample (Roholt et al., 1972; Steward and Petty, 1972; Werblin et al., 1973). This methodology allows the examination of sequential changes of antibody populations during the immune response. These changes can now be correlated with changes in cell populations, since methods to measure affinity of antibodies synthesized by single cells or clones have been developed (Segre et al., 1969; Andersson, 1970; Klinman, 1971; Macario et al., 1971), and applied with the purpose of quantitating the various cell populations successively synthesizing antibodies after antigenic stimulation, as we shall discuss later in this review.

B. The Role of Valence in Determining the Binding Energy of Antibody Molecules

Antibody affinity should be defined as the binding constant of a single antibody-combining site with a single determinant (Macario et al., 1975a). In this ideal condition, possible interactions of parts of the molecules outside the antigen-combining site and the corresponding determinant, are excluded. However, antigen-combining sites cannot be obtained without a supporting portion of the Ig molecule, even when Fab fragments are prepared. The same is true for the antigenic determinants: these have to be "presented" to the antigen-combining site of the antibody molecule, or fragment of molecule, in an appropriate biochemical environment, usually an aminoacid. Therefore, the measurement of the energy of interaction between a monovalent Ig fragment and a monovalent hapten-aminoacid structure may include some bonds outside the specific antigen-combining site. The probability of such an interaction occurring augments as the complexity and size of the hapten-supporting residue increases. In any event, under these conditions one can speak of an intrinsic affinity, that is, the one existing between the monovalent antibody and the complementary haptenic structure. This measurement, conceptually and experimentally very important, may tell little of the energy of interaction

between an intact antibody molecule and the corresponding antigen. Even when interactions between portions of the macromolecules (Ig and antigen) outside the antigen-combining site and the specific determinant are excluded, the "functional" affinity may be greatly influenced by the number of antigen-combining sites in the Ig molecule, the number of copies of the specific determinant on the surface of the antigen molecule and the distance between copies. Functional affinity, then, measures the whole interaction of the antigen-combining sites in one antibody molecule with the corresponding copies of the specific determinant. In practice this measurement has more probabilities of including bonds outside the antibody- and antigen-specific sites, than the measurement of intrinsic affinity has. Nevertheless, it tells more about the interaction between antibody and antigen in *in vivo* situations. Considering that viruses, bacteria and other pathogens bear multiple copies of a series of determinants on their surface, it becomes obvious that functional affinity is the one that operates in natural situations. For a review of valence and affinity see (KARUSH, 1970; CROTHERS and METZGER, 1972; HORNICK and KARUSH, 1972; GOPALAKRISHNAN and KARUSH, 1974, 1974a). In the nurse shark, which may be a good model for the study of the affinity of antibody molecules with different physicochemical properties, it has been observed (SIGEL et al., 1972; Voss and SIGEL, 1972) that 7S and 19S Ig molecules of low affinity exhibit 1 and 5 functionally binding sites respectively. As the affinity of both antibody classes augmented with time after immunization, the functional valence increased, though for unknown reasons it did not reach the expected values, 2 and 10 respectively. Furthermore, monovalent fragments showed a reduced affinity in comparison with intact antibody molecules. The affinity of the latter was shown to be higher when a divalent ligand was used, thus emphasizing from another angle the role of multivalence in the final antibody affinity. The problem has been raised, but not solved, of heterogeneity of binding sites of one single molecule. This seems open to discussion, since some workers postulate that all binding sites are homogeneous (MERLER et al., 1968; ASHMAN and METZGER, 1969) while others support the opposite view (ONOUE et al., 1968; ORIOL et al., 1971; KISHIMOTO and ONOUE, 1971; ORIOL and ROUSSET, 1974).

The role of valence in determining functional affinity and, in turn, the importance of the latter in the achievement of biological effects, is illustrated by experiments in which the neutralization of haptenated virus by purified antihapten antibodies has been investigated. It has been reported (BLANK et al., 1972) that bivalent antibodies were 1000 to 2000 times better at neutralizing haptenated T4 phage than monovalent antibodies. Molecules with 4 and 10 antigen-combining sites were better than the corresponding bivalent antibodies by only a factor of 25 to 200 times. This indicates that other factors, in addition to intrinsic affinity and number of antigen-combining sites, influence the functional binding energy finally reached in each system; for instance size, shape, and flexibility of the antibody molecule, and location of the antigen sites on the antigen macromolecule (BLANK et al., 1972;

Gopalakrishnan and Karush, 1974). Another interesting facet of this problem is the difference in intrinsic affinity shown by IgM with respect to IgG antigen-binding sites (Frank and Humphrey, 1968; Mäkelä et al., 1970; Hornick and Karush, 1972). The latter authors, working with a haptenated phage, (DNP-φX 174) measured (a) by equilibrium dialysis the intrinsic affinity of isolated antigen-binding sites (Fab fragments from purified anti-DNP-antibodies) for a monovalent ligand, and (b) by phage neutralization the functional affinity of intact IgG and IgM antibody molecules. IgG antibody was found to have a K of the order of 10^7 M^{-1}. Monovalent products showed a functional affinity 100 times lower, whereas the intrinsic affinity remained unaltered. The intrinsic affinity of IgM antibody was lower, 10^4–10^5 M^{-1} but the functional affinity was higher, 10^{11} M^{-1}, than the corresponding values of IgG antibody, thus the factor of increment of functional over intrinsic affinity was three orders of magnitude greater (10^6 in IgM rather than 10^3 in IgG antibodies).

The functional, biological and evolutionary advantages of multiple binding leading to increased affinity is obvious. As regards antigen binding by cells, for instance, it has been shown that multivalent attachment to antibody on the cell surface greatly enhances the binding constant. Thus a multivalent ligand exhibits 100–300 times greater affinity than its monovalent counterpart (Bystryn et al., 1973).

III. Quantitative Evaluation of Antibody Affinity and Heterogeneity

A. The Use of Artificial Determinants

Accurate determinations of the association constant between the antigen-combining site of antibodies and an antigenic determinant are possible only when purified antibodies and isolated haptens of known structure are used. The hapten is coupled to a carrier molecule, usually a protein, to produce an immunogen, which is injected into the appropriate animals. The same, or similar hapten, coupled to a small molecule, e.g. an aminoacid, is used to carry out affinity measurements with the corresponding antibodies. The information so obtained is likely to be valid also for the interaction of antibodies with natural determinants of macromolecules, e.g. bacterial proteins, although some distortions due to the use of artificial conjugates and haptens cannot be completely excluded. When coupled to a carrier, for instance, most haptens occupy several positions on it, building up similar but not identical determinants. The corresponding antibodies form a heterogeneous population with the same specificity but with several affinity subpopulations, which, in fact, are distinct populations with different specificities. Therefore, data on distribution of affinities of antibodies induced by polyvalent hapten-carrier conjugates may not illustrate appropriately the situation corresponding to a single, natural determinant of an antigen in its native configuration.

B. Measurement of the Association Constant of Antibodies Specific for Natural Structures

Recently, attempts have been made to measure the energy of interaction of specific antibodies with their complementary structure on proteins not altered by chemical manipulations (MACARIO et al., 1971; NOBLE et al., 1972; SACHS et al., 1972; CELADA et al., 1973). Conditions allowing such measurements are the following: a) the antigen-antibody interaction should generate a signal; b) the signal should be generated only when the antigen-combining site interacts with a particular atomic arrangement of the antigen (some sort of natural hapten); c) the interaction and the generation of the signal should not be influenced (hindered or quenched) by other atomic groups of the antigen; d) the signal should be a measurable phenomenon; e) at each antigen concentration used in the reaction in the presence of a constant amount of antibody (saturation curve) it should be possible to determine the fraction of bound and free antigen. This should be possible over a wide range of antigen concentrations; f) if measurements of the signal are to be done without isolation of specific antibodies, the binding of other antibody species to the corresponding determinants on the antigen molecule should not interfere with the interaction generating the signal and its quantitation.

The reaction of activating anti-β-D-galactosidase (E. coli) antibodies with a naturally occurring ligand, a point mutant defective enzyme (AMEF) (ROTMAN and CELADA, 1968) seems to fulfill reasonably well the conditions mentioned above. The activation of the defective mutant is a one-hit phenomenon producing enzymatic activity (the signal) whenever one activating antibody molecule reacts with the specific site, which is present only once in each antigen molecule (CELADA and STROM, 1972).

Making use of this system, a method has been developed to measure the affinity of the activating antibody and its degree of heterogeneity during the immune response (MACARIO et al., 1971; CELADA et al., 1973). The results obtained with this method will be discussed later in this review, specially in Sections V and VII. By applying the above mentioned methods it is possible at the present time, to envisage investigations on the binding properties of the antigen-combining site of antibodies which recognise determinants belonging to the bacterial and viral world.

IV. The Sequential Synthesis of the Various Antibody Specificities which Recognize Different Determinants of Complex Molecules

Following immunization with a complex molecule, several populations of antibodies should theoretically be synthetized, each one of them recognising a distinctive atomic arrangement of the immunogen (MACARIO et al., 1975 a). It could thus be expected that at any one time of the immune response only one, some or all specificities occur, namely, antibody populations recognizing only one, some or all different determinants, are present. What actually happens is that different antibody species appear in a certain sequence (MERON

et al., 1971; Timpl et al., 1972). For example, collagen does not elicit an immune response directed toward all its determinants simultaneously (Timpl et al., 1972). The sequence of appearance of the various specificities seems to depend upon the species origin of collagen. In the case of rat collagen early rabbit antisera contains only antibodies to the C-terminal sites, located in both polypeptide chains, 1 and 2. Only after several immunizing injections over a certain time interval, are antibodies to the N-terminal sites produced. It is important to emphasize that the same total dose in only one injection does not elicit antibodies to the N-terminal sites; these antibodies will only appear after a certain time period following upon several successive injections of antigen (Timpl et al., 1972). This phenomenon may occur for many other complex macromolecules. The mechanism is not known. It could be that early antibodies form complexes and modify the spatial structure of the antigen molecule creating or uncovering other antigenic sites.

Another factor of complexity, characteristic of the immune response and different from the mixture of several populations of antibodies directed to several distinctive sites, derives from the fact that within each specificity there are subpopulations of molecules differing in their affinity for the complementary structure. Furthermore, affinity tends to increase with time after immunization due to progressive predominance of subpopulations with a high association constant. This phenomenon has been named maturation of the immune response (Siskind and Benacerraf, 1969); it will be considered in detail below, as well as other features of time-course affinity changes.

V. General Features of Antibody Affinity Modifications in the Course of the Immune Response

A. Classical Picture. The Progressive Selection of High Affinity Antibody-Forming Cell Precursors by Decaying Amounts of Antigen

Years ago it was reported that the K_0 of anti-DNP antibodies isolated from rabbit sera increases progressively with time after immunization (Eisen and Siskind, 1964). The phenomenon, probably essentially similar to the gradual increase in the avidity of neutralizing antitoxin antibodies described earlier (Jerne, 1951), was explained in two possible ways: a) the cells synthesizing antibodies change with time, with a continuous progression toward higher affinity cells; b) variations in serum antibody affinity are due to a decay of circulating antigen or antigen-antibody complexes—at the beginning antigen would be abundant and only low affinity antibodies would be free; as antigen decays antibody of progressively higher affinity would be freed from the complexes. The antibody molecules to be freed last would be those of very high affinity, because they form very stable complexes which dissociate only in extreme scarcity of antigen molecules. It was soon demonstrated that the former explanation (a) was correct (Steiner and Eisen, 1966, 1967), although the second one has not yet been completely ruled out under *in vivo* conditions when measurements are done in serum samples. On these grounds

the concept of cell selection by antigen was elaborated (STEINER and EISEN, 1966; SISKIND and BENACERRAF, 1969) as a further development of the clonal selection theory of acquired immunity (BURNET, 1959, 1967; JERNE, 1967, 1971, 1972). In essence, AFCP-bearing surface, antibody-like antigen receptors are stimulated on interaction with antigen. Cells bearing receptors with higher affinity are favoured in this competitive process. Soon after immunization many AFCP are stimulated, mainly those with low affinity, because they are assumed to be more abundant, and therefore statistically favoured. Later on, antigen becomes limiting, high affinity receptors are the only ones which can bind antigen because they are thermodynamically favoured; therefore, only AFCP with high affinity receptors are triggered. A review of the experimental data supporting this view, its detailed explanation and predictions can be found in (SISKIND et al., 1966).

B. Other Patterns of Antibody Affinity Modification during the Immune Response

1. Synthesis and Predominance of Low Affinity Antibodies during the Decline of the Immune Response

Recent findings have shown that antibody affinity may change with time in a way not expected according to the concept of cell selection by antigen (MACARIO et al., 1975a). For instance a late fall in antibody affinity after the usual initial rise has been observed (DORIA et al., 1972; URBAIN et al., 1972; MACARIO and CONWAY DE MACARIO, 1974, 1974a, 1975). These findings as well as their possible significance in the mechanism of the anamnestic response will be discussed later in this review, specially in Section X.

2. Alternating Predominance of High and Low Affinity Antibody Molecules

An undulating profile has also been found; antibody affinity increases at first, then falls and rises again (KIMBALL, 1972; KIM and KARUSH, 1973; MACARIO and CONWAY DE MACARIO, 1973, 1975). Very late antibodies can be of low affinity and heterogeneous (MACARIO et al., 1973, 1974, 1974a, 1975). These findings indicate that antibody populations alternate during the course of the immune response in a fashion somewhat apart from expectation according to the concept of cell selection by antigen. In this connection, it has been observed (WERBLIN et al., 1973) that shortly after immunization antibody populations are distributed symmetrically around a low average affinity. With time the distribution becomes skewed towards high affinity. Late in the immune response, high affinity populations decay and a heterogeneous population of low affinity molecules becomes more apparent. This complicated picture is not in line with the predictions of a cell selection theory whereby high affinity AFCP are selected and triggered and low affinity AFCP are excluded. The latter are, in fact, not eliminated; they give rise to antibody forming clones all over the immune response. Moreover, sometimes, late after priming they may be predominant (MACARIO et al., 1973, 1974a, 1975).

3. Persistence of the same Antibody Affinity all over the Immune Response

It is also important to note that in several instances antibodies have been found functionally homogeneous from the first measurements (early after antigen injection) up to the last bleeding, several months later (Wu and Rockey, 1969; Haber and Stone, 1969; Howard, 1971; Montgomery et al., 1972; Gopalakrishnan et al., 1973). Similarly, antibacterial (E. coli) antibodies synthesized in the rabbit kidneys with experimental pyelonephritis do not show changes in binding affinity with time (Smith et al., 1974).

It is then obvious from the previous Sections V.B, 1 and 2 that the changes in antibody affinity do not follow a stereotyped profile and cannot easily be explained in all circumstances by the concept of cell selection by antigen at least in its simpliest version. It is perhaps difficult at present to elaborate a coherent theory capable of explaining all the facts because it should take into account a number of recent developments such as the discovery of cell interactions (Mitchison et al., 1970; Claman and Mosier, 1972; Miller, 1972; Takemori and Tada, 1974), the idea of clonal predominance (Askonas and Williamson, 1972) and other pieces of information which will be mentioned in Sections XII and XIII.

VI. The Sequential Changes of Antibody Subpopulations with Different Affinities during the Immune Response

Methods for calculating antibody affinity generally assume a normal distribution of antibody molecules about a mean (the average association constant, K_0). This does not seem to be the case (Pressman et al., 1970; Werblin and Siskind, 1972).

A first approximation of the degree of dispersion about the mean, is given by the heterogeneity index. Some authors have found a progressive decrease in heterogeneity index (Eisen and Siskind, 1964; Miller and Segre, 1972), while others have reported the reverse, or irregular patterns, i.e. periods of restriction alternating with intervals of more heterogeneity (Kitagawa et al., 1967; Roholt et al., 1970; Jaton et al., 1971; Kimball, 1972; Macario et al., 1972, 1973a; Claflin and Merchant, 1972, 1973; Claflin et al., 1973).

This sort of information is important because it helps understanding the clonal dynamics during the immune response and therefore the micro-evolutionary process which takes place within a single animal, affording information on how selective forces, cells and molecules, interplay.

At serum level, it has been observed (Werblin et al., 1973) that early after immunization antibodies are heterogeneous and symmetrically distributed about a low average association constant. During the first month a shift occurs and by days 40–60 a relatively restricted antibody population of high affinity predominates. Later on, heterogeneity increases again, as the high affinity population decreases; the average affinity is again low. Low affinity populations persist all over the immune response (Werblin et al., 1973).

At cellular level it was reported that increase in affinity is due to loss, or relative slow rate of multiplication, of low affinity cells and preservation of high affinity ones, which show a higher rate of proliferation (DAVIE and PAUL, 1972, 1973; CLAFLIN and MERCHANT, 1973; CLAFLIN et al., 1973; ROSZMAN, 1974). Antibody-forming cells synthetizing IgM antibodies were also found to follow a similar pattern, but it was noted that these cells are of shorter life span than IgG-producers (WU and CINADER, 1973).

VII. Measurement of the Temporal Modifications in Affinity and Heterogeneity of Antibodies Synthesized in Long-Lasting Micro-organic Cultures

Evolution of antibodies in terms of titer, association constant and degree of heterogeneity, was recently studied in long-lasting cultures of rabbit lymph node fragments (MACARIO et al., 1971, 1972, 1973, 1974 and 1975). The micro-organic culture has some advantages, namely the structure of the responding tissue is preserved and long-lasting responses are obtained which allow the follow-up of the antibody response by isolated cell populations, and possibly single clones over several weeks or months. Since it is an *in vitro* system, some important factors can be controlled such as antigen dose/cell, temperature, pH and duration of challenge as well as total cell number per culture and antibody concentration in the cell environment.

It was observed that the antibody response tends to be cyclic with an ascendent limb where affinity gradually increases and heterogeneity decreases (maturation) and a descendent limb whereby affinity decreases to the initial values (MACARIO et al., 1975). If the response is long enough, another cycle succeeds. It is then clear that maturation can be accomplished at the level of a restricted number of clones, in a closed system without connections with the circulating cell pool and central organs, thymus and bone marrow for example. In some cultures variations in affinity do not occur during the entire response, even over intervals that in parallel cultures would show significant variations. On the whole, the findings can be summarized in the following way: affinity maturation is, as a rule, accompanied by a progressive restriction in terms of affinity. In some cultures, affinity does not change, whereas in most of them periods of high alternate with intervals of low affinity. Very late in the response, a marked decrease in affinity takes place in almost all cultures together with increased heterogeneity, irrespective of the previous behaviour of the culture.

VIII. Determination of the Binding Properties of Antibody Molecules Released by One Cell

The association strength of antibodies released by single cells into semi-solid medium can be estimated using a modified hemolytic plaque assay (SEGRE et al., 1969; ANDERSSON, 1970, 1972; MILLER and SEGRE, 1972; WU and CINADER, 1972). The amount of antigen, or free hapten, that has to be

added to the medium to inhibit a given proportion (usually 50%) of plaques is a quantitative estimation of the strength of the bond between antibody and antigen. When the inhibitory molecule is a multivalent antigen the parameter measured is named avidity, which depends among other things on the association constant of each bond and on the valence of the antibody. Measurement of avidity is therefore a rather gross estimation of the association strength, and its significance is obscured by the fact that it depends on several uncontrolled variables. On the other hand, when the inhibition of plaques is done with a monovalent hapten, affinity can be measured. The concentration of free hapten that inhibits 50% of the plaques, for instance, is a reliable quantitative estimation of the association constant of the antibody combining site with the ligand. The principal advantages of the method are: it measures the affinity of pure antibody populations released by single cells while avoiding contamination with molecules synthesized by other cells and with antigen molecules. Such a contamination may obscure affinity determination when serum antibodies are used (as it was discussed in Section V.A.). In this case it is difficult to rule out the possibility of some antibody molecules (specially those of high affinity) being bound to antigen molecules, in the serum and/or in the tissues; moreover, when purified antibodies are obtained, these are a mixture of molecules synthesized by different cells and clones at various times. On the other hand, the method has the disadvantage that serial studies within the same cell population or clone are not feasible, since once the cells are plated, they do not proliferate any longer.

Furthermore, these studies are not even possible within a single animal because usually the donor is killed to obtain the cells, and therefore it is not possible to follow variations of antibody affinity at cellular level during the immune response. This disadvantage can to a certain extent be overcome by using animal species of which inbred strains are available.

Another difficulty with the method was recently pointed out, namely that determination of I_{50} is influenced by the size of the hemolytic plaque (North and Askonas, 1974). This fact obscures interpretation of results based on plaque counting since large plaques need more antigen to be completely inhibited than small ones, even if antibodies in both types of plaques, large and small, possess identical affinity (North and Askonas, 1974).

In early studies (Andersson, 1970) inbred strains of mice were used and time-course determinations could be performed by killing animals at different intervals after immunization. The assumption is implicit in this kind of approach that all animals respond identically, and thus the construction of a unitary profile of affinity variations using data from various individuals is allowed. It was observed that antibody avidity is determined by the antigen dose and that it increases with time after immunization. In other words, what was known to occur at serum level was confirmed at the level of single antibody-forming cells. Moreover, a new piece of evidence was added showing that the increase in antibody avidity with time after immunization is due to actual synthesis of antibody molecules with higher avidity and not merely

to increase in the number of free antibody molecules with high avidity because of progressive dissociation of immune complexes as antigen concentration decays. The use of monovalent haptens permitted the measurement of antibody affinity, and, by combining the method with a cell fractionating procedure, it was established (ANDERSSON, 1972, 1972a) that B memory cells bear surface receptors of similar or identical affinity to the antibody molecules they synthesize upon secondary challenge.

It was confirmed that the heterogeneity of antibody molecules in the serum is due to heterogeneity of antibody-forming cells; high antigen doses elicit lower affinity antibodies; escape from high-dose tolerance produces antibodies of lower affinity; administration of antibody results in the synthesis of few antibodies of low affinity; the target cell of these regulatory mechanisms would be the B-cell line.

Time-course variations in antibody affinity and heterogeneity in the serum have their counterpart at cell level, the latter being measured as cell-surface receptors or antibodies released by single cells (ANDERSSON, 1970, 1972, 1972a; DAVIE and PAUL, 1972, 1972a; MILLER and SEGRE, 1972). Progressive increase in the avidity with time after immunization was found to be paralleled by a decrease in the number of antibody-forming cells of low avidity while high avidity antibody-forming cells were preserved, which determined a gradual predominance of the latter (CLAFLIN et al., 1972, 1973; DAVIE et al., 1972, 1973; WU and CINADER, 1973; ROSZMAN, 1974). The precursors of antibody-forming cells with high affinity would proliferate at a higher rate than low affinity precursors (DAVIE and PAUL, 1973). All the precursors necessary for accomplishing maturation towards high affinity would be present before antigenic insult (CLAFLIN et al., 1973), thus diversity in terms of affinity within the B-cell line would be present by the time the first antigen contact takes place. However, further diversification during the immune response also seems to be possible (MACARIO et al., 1972, 1974; CUNNINGHAM, 1974).

Attempts at studying maturation within defined Ig classes have been made. A rapid increase in avidity of IgM antibodies has been found (CLAFLIN et al., 1972, 1973). Maturation at the level of IgM antibody was also observed in amphibian larvae (DU PASQUIER and HAIMOVICH, 1974) and in the rabbit (WU and CINADER, 1972), where it is dependent on antigen doses.

During the primary response, it was found that antihapten IgM plaque-forming cells increase in affinity with time, the rate of maturation depending somewhat on the carrier protein. The free hapten concentration needed to inhibit 20% of plaques decreased with time, i.e. the affinity of IgM plaque forming cells at the level of I_{20} increases with time and occurs at all antigen doses tested, although low and high doses produced a less marked maturation. Time-dependent changes at I_{50} and I_{70} levels only occurred in response to low antigen doses. In the secondary response maturation was also observed, but was taking place more rapidly. High affinity plaque-forming cells appeared very soon after challenge; they also disappeared rapidly, and, after that, the maturation profile acquired the shape observed during the primary response.

It was concluded that maturation at the level of IgM antibodies is due to constant increase in the proportion of high and medium affinity antibody-forming cells and decrease in low affinity ones (Wu and Cinader, 1972). Controversial results, some obtained with the plaque-inhibition method and some using serum IgM antibodies (Voss and Eisen, 1968; Haimovich and Sela, 1969; Sarvas and Mäkelä, 1970; Clem and Small, 1970; Baker et al., 1971; Kim and Karush, 1973; Huchet and Feldmann, 1973; Roszman, 1974) indicate that during the primary response there is no, or little, maturation of IgM antibodies. However, maturation may be observed after secondary challenge (Voss and Eisen, 1968) or by analyzing separately the two populations of sites which would be present in the the IgM molecules, one with high affinity and the other with nearly 100-fold lower affinity (Oriol and Rousset, 1974, 1974a); the former show increase in the average equilibrium constant with time after antigenic stimulation whereas the K_0 of the low affinity population remains constant (Oriol and Rousset, 1974).

The affinity of antibodies released by single cells can change with time in parallel to the variations of the number of antibody-forming cells (Doria et al., 1972); thus increase, peak and decline can be observed.

IX. Determination of the Binding Properties of Antigen Receptors on the Cell Surface

In the foregoing Sections methods and results were described with regard to determination of affinity of antibodies secreted into the serum and liquid tissue culture media by many, few, or only one clone; and into semisolid medium by single cells. Attempts have also been made to measure affinity of cell-bound antibodies and antibody-like cell surface receptors (Davie et al., 1972, 1972a; Bystryn et al., 1973; Möller et al., 1973; Revoltella et al., 1973; Wilson and Feldmann, 1973; Claflin et al., 1974). An increase with time after immunization was observed in the avidity of antigen-binding cells, which were considered antibody-forming cell precursors (Davie et al., 1972, 1972a) but it is not clear whether receptors or cell-bound antibodies were being measured. Other authors (Revoltella et al., 1973a) have found sequential changes in the avidity of antigen-binding cells very early (a few hours) after antigenic stimulation. Changes at cellular level seem to correlate with the time-course increase in avidity, or affinity, of serum antibodies (Davie et al., 1972, 1972a; Revoltella et al., 1973a). A distinction between B and T antigen-binding cells has also been attempted (Möller et al., 1973; Wilson et al., 1973; Möller, 1974; Smith et al., 1974) and it has been reported that B-cell receptors show increasing affinity with time after immunization, whereas T-cell receptors do not change. However, by studying the antigen dose needed to elicit macrophage migration inhibition, in a system supposed to involve T-cells, it was observed that with time after immunization the amount of antigen needed to bring about a certain percentage of inhibition decreased. Although indirect, this was taken as evidence of time-course

increase in the functional affinity of T-lymphocyte antigen receptors (JOKIPII and JOKIPII, 1974).

It should be emphasized that measurements of affinity of cell-receptors involve a series of variables (e.g. number of receptors per unit area at any given time, and movement of the receptors in the plane of the cell membrane constantly altering density and distribution), which makes the system more complex than in the case of measurements with antibodies in solution, and not subjected to the same thermodynamic rules. Therefore, interpretation of these data has to be cautious (see also Section XVI).

X. Time-Course Restriction of High-Rate Antibody-Forming Clones Towards High Affinity and Expansion of the Memory Potential

Gradual increase in antibody affinity with time implies a shift of antibody forming clones towards high affinity. It may be pertinent to ask whether or not memory cells are also only capable of initiating synthesis of antibodies with high affinity. This is suggested by some reports (PAUL et al., 1967; STEINER and EISEN, 1967a; BULLOCK and RITTENBERG, 1970; FELDBUSH and GOWANS, 1971), where it was claimed that memory cells are restricted to high affinity (MACARIO et al., 1975). However, recent data obtained *in vivo* (CELADA et al., 1969) and *in vitro* (MACARIO et al., 1973a, 1974) indicate the contrary. Maturation of the immune response does not as a rule lead to a restriction of the memory potential. During the primary immune response, the spectrum of high-rate antibody-forming clones becomes restricted, with predominance of high affinity ones, but the same does not hold true for memory cells. After a long maturation process it is possible to detect a wide range of memory cells. They can be stimulated under conditions avoiding competition with other cells and circulating antibodies, and start a secondary immune response which shows an affinity progression similar to the one occurring during the primary response. There is therefore evidence for a great heterogeneity of memory cells, which would have a selective advantage, since it will provide the organism with the capacity to react adequately against a wide range of doses upon secondary antigenic aggression. It has been suggested that low affinity heterogeneous antibodies, present late in the immune response, may be the product of the heterogeneous memory cell populations existing at that moment. Immunological memory would thus be built up by cells and circulating molecules, both distributed over a wide range of affinities (MACARIO et al., 1973, 1974a, 1975).

XI. The "Original Antigenic Sin" and Degeneracy of the Immune Response from the Viewpoint of Antibody Affinity

Late after immunization antibodies are usually of high affinity for the homologous and also for cross-reacting ligands, a phenomenon not observed shortly after priming (EISEN et al., 1969; LITTLE and EISEN, 1969) which means that, as time goes by, antibody specificity becomes less restricted.

Moreover, a secondary response, which has been called "original antigenic sin" or degeneracy of the immune response can be elicited by cross-reacting antigens and may be another way of looking at affinity maturation with expansion of the memory potential (Fazekas de St. Groth and Webster, 1966, 1966a; Eisen et al., 1969; Gershon and Kondo, 1972; Deutsch et al., 1972, 1973). Whatever the mechanism may be, we would like to draw attention to one viewpoint which seems to account for the needs of positive selection in evolution. In this respect, it would appear tenable that with time after immunization the organism learns how to produce antibodies capable of reacting strongly with the initially offending antigen and of putting into action high-rate antibody-forming clones producing antibodies with high affinity which become predominant; these antibodies are also capable of reacting against related structures, as if this learning were enabling the immune system to cope with any antigenic variant of the attacking immunogen that might eventually appear. Furthermore, memory cells also become capable of being stimulated by cross-reacting antigens, i.e. not only are circulating antibodies efficient toward a wide spectrum of cross-reacting specificities, but the immune machinery can also be started by any of these specificities, if circulating antibodies are not enough. However, memory cells with low affinity, less stimulable by cross-reacting antigens, are still present, in case a second attack should occur with a high dose of the priming antigen. A high antigen dose may paralyse high affinity cells but not low affinity ones (Davie et al., 1972) (see also Section XIII); the latter remain functional and can give rise to an efficient immune response. The evolutionary advantage of such an adaptable immune system is obvious (Macario et al., 1975).

XII. The Role of Interactions between Cells and Antigenic Determinants in the Regulation of Antibody Affinity

A. Cell Cooperation and Carrier Pre-Immunization

It has been reported that under experimental situations in which a deficit in T-cell functions is established, antibody affinity tends to be low (Gershon et al., 1971, 1972, 1972a). Experimental data in thymectomized animals, whether reconstituted with thymus cells or not, tend to show that antihapten (anti-DNP) antibodies are of higher affinity in sham-operated and thymectomized mice reconstituted with thymus cells than in non-reconstituted mice. Furthermore, in reconstituted mice, affinity increases in parallel with the dose of reconstitutive thymus cells (Gershon et al., 1971, 1972, 1972a). The affinity of antihapten antibody depends also on the carrier molecule; challenge with a heterologous conjugate induces antibodies with affinity higher than antibodies elicited by secondary challenge with the homologous conjugate (Siskind et al., 1966).

Furthermore, pre-immunization with the carrier protein (hen egg albumin for example) increases the rate of appearance of anti-hapten (NIP:4-hydroxy-3-iodo-5-phenylacetic acid) antibodies with high affinity, therefore the peak

affinity is reached sooner in carrier pre-immunized than in non-pre-immunized animals (HURME et al., 1973). Also indirect evidence strongly suggests that cooperation of cells and of antigenic determinants takes place throughout the immune response in the sequential triggering of antibody-forming clones that proceed after antigenic challenge (MACARIO and CONWAY DE MACARIO, 1974b); this mechanism may be responsible for stimulation of low affinity precursors even when the antigen is scarce (MACARIO and CONWAY DE MACARIO, 1973). All these sets of data indicate that the antibody affinity is influenced by cooperation between cells and antigenic determinants. However, the intimate mechanism of this cooperation remains unknown.

B. Suppressor Cell Mechanisms

This topic is nowadays under active investigation and since new data are constantly coming out, it is difficult to draw definitive conclusions or even to make a comprehensive synthesis of all the findings reported (TAKEMORI and TADA, 1974; OKUMURA and TADA, 1974; BAKER et al., 1974). Nevertheless, it is already clear that suppressor-cell mechanisms can be considered as a form of cell-cell interaction closely related, at least formally, to the phenomenon of T–B cell cooperation in the response to certain hapten-carrier conjugates. These regulatory mechanisms seem also to be connected with tolerance induction inasmuch as in both phenomena high affinity AFCP are the most affected (TAKEMORI and TADA, 1974; see also Section XIII. A). The effect of suppressor T cells, i.e. thymocytes or spleen cells from carrier pre-immunized donors, on the avidity of antihapten antibodies produced in recipient mice shortly after primary or secondary immunization was studied, with the hapten coupled to the same carrier used to immunize the donors of the suppressor cells (TAKEMORI and TADA, 1974).

The results indicate that the latter cells provoke a certain diminution of the avidity of primary antibodies, as shown by inhibition of direct and indirect PFC with free hapten and determination of I_{50} (see Section VIII). The same picture was observed in the secondary response, accompanied by low avidity antibodies in the serum, according to estimations of K_0, made by applying a modified Farr's test. The overall diminution of antibody avidity was apparently due to preferential suppression of B cells capable of producing high affinity antibodies whereas B cells synthesizing low affinity antibodies were almost unaffected.

More experimentation is needed before these findings can be adequately interpreted in terms of mechanism and physiological role.

XIII. The Affinity of Antibodies Produced under Conditions of Immunodepression

A. Immunological Tolerance

It has been reported that antibodies synthesized by partially tolerant animals and after tolerance has been broken, are of low affinity (LINSCOTT

and Weigle, 1965; Theis and Siskind, 1968; Anderson and Wigzell, 1971; Pickard and Havas, 1972; Davie et al., 1972; Heller and Siskind, 1973; Weksler et al., 1973; Seppälä, 1974). Rabbits rendered tolerant at birth by injection of DNP horse serum albumin in large doses during the first 12 days of life produce antihapten antibodies with lower affinity upon challenge at 5–6 weeks of age than normal controls (Theis and Siskind, 1968). The same was reported to occur in mice made tolerant either at birth or in adulthood (Pickard and Havas, 1972). Despite the fact that antibodies synthesized by these animals made tolerant before immunization, are of low affinity, the binding constant increases with time after immunization in a fashion similar to the one observed in normal animals after priming.

Synthesis of low affinity antibodies by low responder strains of mice has also been reported (Steward et al., 1974). Low responsiveness would be a form of tolerance due to elimination of high affinity precursors by antigen doses which are immunogenic for other strains: i.e. a certain antigen dose may be immunogenic for a given animal and tolerogenic for another individual of the same species. To explain these findings a greater susceptibility of high affinity precursors to tolerance induction in low responder strains has been postulated (Steward et al., 1974). Although this remains to be established it is clear that a thorough understanding of diseases involving immune-complex deposition and elimination requires examination of antibody affinity and its role in pathogenesis (see also Section XVI, A.).

In this connection, it is pertinent to mention that the rate of increase in affinity is faster after administration of a low antigen dose than in the case of a large dose. The latter induces longer responses and the affinity progression, which starts from very low initial values, continues over relatively long periods until the association constant of antibodies is as high, or even higher, than antibodies elicited by a low antigen dose at their peak affinity. These observations were made by studying the response *in vitro* toward a natural determinant of a bacterial protein (Macario et al., 1972, 1974b) and recently confirmed by using DNP-carrier conjugates *in vivo* (Kim and Siskind, 1974). These findings have been interpreted in terms of cell selection by antigen, on the assumption that AFCP-bearing surface receptors with high affinity are made tolerant more easily than cells with low affinity receptors.

B. Antigenic Competition

Administration of two antigens into the same animal, either simultaneously or within a short interval, results in a depression of antibody synthesis to both antigens as compared with the response to each one of them injected separately in different animals (Kim et al., 1974a). Usually the test antigen is administered first and the competing one a few minutes, hours or days later. The larger the difference between the doses of the two antigens (low for the test and high for the competing one), the greater the depression of the antibody response to the test antigen. If the dose of both antigens is similar or identical, com-

petition, i.e. immunodepression may be negligible (HAREL et al., 1970; KIM et al., 1974a). The mechanism of antigenic competition is not known although several hypotheses have been formulated (KIM et al., 1974a). Some clues for its understanding may be given by the alterations of antibody affinity which have been observed accompanying antigenic competition.

The small amounts of antibodies produced in cases of strong competition are of very low affinity; high affinity molecules are apparently not synthesized. When competition is weak, i.e. when antibody levels are only slightly diminished, no significant alterations in the distribution of affinities are seen (HAREL et al., 1970; KIM et al., 1974a). It appears then that under conditions of strong competition, AFCP with high affinity are the most affected.

C. Non-Specific Immunodepression

The magnitude of the antibody response can be reduced by administering cytotoxic drugs, methotrexate or 6-mercaptopurine for example, but this does not necessarily mean that antibody affinity is equally affected (HAREL et al., 1970; MOND et al., 1974). Antibody levels can be reduced more than 70% in treated animals with respect to controls which have not received the drug. This high degree of depression is observed only when treatment is started before the first immunizing injection and maintained throughout the observation period (several weeks). In spite of the great diminution in the magnitude of the response, modification of antibody affinity is slight, with a tendency to average low affinity. High affinity molecules tend to diminish. It would seem then that treatment with cytotoxic drugs preferentially affects the production of high affinity antibodies, provided the drug is administered in relatively high amounts over long periods to provoke a marked diminution in antibody levels; otherwise average affinity and maturation are not affected. The lack of maturation characterized by a failure to produce high affinity molecules observed in strongly depressed animals has been interpreted as being due to reduction in cell proliferation and consequent diminution of the frequency of AFCP. Under these circumstances the probability of selection of high affinity precursors by decreasing concentration of antigen with time after immunization would be very low (MOND et al., 1974).

XIV. Studies on the Persistence and Sequential Changes in Antibody Populations Identified by Molecular Markers other than the Binding Properties of the Antigen-Combining Site

A. Idiotype

Idiotype is an antigenic marker located in the variable region of the Ig molecule. It seems closely related with the antigen-combining site of the antibody molecule (MACARIO et al., 1975); it has been postulated that all antibody molecules with identical specificity possess the same idiotype, not shared with antibodies with other specificities. Thus idiotype would be a marker of a given specificity within a single Ig class. It has also been postulated

that each idiotype is a marker of the product of one antibody-forming clone (Winfield et al., 1972; Annales d'Immunologie, 1974). However, exceptions to these general assumptions have been reported; for example in some antigen-antibody systems the reaction idiotype-anti-idiotype cannot be totally inhibited by the corresponding antigen (complementary to the site supposed to be the idiotypic determinant). This finding strongly suggests that the idiotypic determinant does not exactly overlap with the antigen-combining site (Brient et al., 1970, 1971; Sher and Cohn, 1972a; Carson and Weigert, 1973). Moreover, it was found that a given idiotype can be shared by different Ig classes (IgG and IgM) as well as by antibody molecules with different specificities (Oudin et al., 1969, 1971; Cazenave, 1973, 1973a; Cazenave and Oudin, 1973; Annales d'Immunologie, 1974; Schreiber and Reichlin, 1974), by Ig molecules with and without a given specificity (Oudin et al., 1971) and by antibody molecules having different allotypes in the same animal (Kindt et al., 1973). It appears then that idiotypes cannot be taken as clonal markers, unless it is accepted that switches in Ig class and/or specificity can occur within a clone. If that were the case, the original clone and the subclones derived from it would have the same idiotype.

Another possibility is that different clones possess identical or very similar idiotypes, as suggested by the above mentioned idiotypic cross-reactions of antibody molecules differing in class, allotype, and specificity and between antibodies produced in different individuals of the same species (Sher and Cohn, 1972a; Eichmann, 1972, 1973; Pawlak and Nisonoff, 1973; Kindt et al., 1973; Klapper and Kindt, 1974).

With these clarifications in mind we can now discuss the sequential changes of antibody populations, as measured by idiotypic determinations, without drawing definitive conclusions as to whether or not these changes reflect intra or interclonal variations. Several idiotypes may appear shortly after immunization (Oudin et al., 1969, 1969a); some of them can persist several months, disappear and then reappear upon antigenic challenge, together with new idiotypes, not present earlier (Oudin et al., 1969, 1969a, 1969b, 1971; Macdonald and Nisonoff, 1970; Spring et al., 1971; Winfield et al., 1972, 1973). Attempts at defining the factors that may influence idiotype replacement have shown that idiotypic populations gradually change in rabbits challenged several times, the more marked changes taking place at about 2–4 months after the initial injection. Some idiotypes persist during long resting periods as well as during long intervals of repeated antigenic challenges. In late bleedings new idiotypes appear which were not present earlier. The rate of these gradual changes is not affected by antigen dose and only slightly accelerated by increasing the number of antigen injections. In BALB/c mice responding to phosphorylcholine reestimulation after a resting period always recalls the same idiotype (Lee and Kohler, 1974). Thus, replacement of idiotypic populations does not seem to be significantly influenced by antigen variables. However, idiotypic specificities may reappear upon challenge after a long resting period causing their temporal disappearance. No definitive

explanation of these phenomena is so far available especially for the long persistence of some idiotypes and for the relative insensitivity of the rate of idiotypic changes to antigenic challenge.

B. Allotype

Allotype is an antigenic marker on the Ig molecules (MACARIO et al., 1975 a). Allotypic systems have been defined at the genotype and phenotype levels in several animal species, and methods for quantitating the number of molecules and cells bearing a given allotype have been developed (MAGE, 1971; ROPARTZ, 1971; DUBISKI, 1972; TOSI and LANDUCCI-TOSI, 1973; Annales d'Immunologie, 1974). It is then possible to control variations in cell and antibody populations during the immune response by following changes in the allotype distributions. Rabbit antibodies of restricted heterogeneity of b5 allotype, with a given a.a. sequence at the N-terminal end of L-chains, were found in one animal as long as 28 months apart (MAGE et al., 1973). Therefore, by looking at allotypic markers, the information obtained with idiotypes (see above, Section XIV.A) concerning the long persistence and reappearence of a given antibody population, was confirmed. The increase in the proportion of antibody molecules with L_k-chains of b4 allotype paralleling an increase in affinity with time after immunization was reported (FONTANA et al., 1973). This suggests that antibodies with k chains are better suited to bind antigen more strongly than antibodies with λ-type chains (see below, Section XIV.D). In heterozygous rabbits (a1/a2) antibodies of high affinity are more often a2, whereas molecules with the allotype a1 are of low affinity (WERBLIN et al., 1973). *In vitro*, it has been observed by following allotype markers (CONWAY DE MACARIO et al., in preparation) that L_k b4 allotype predominates as affinity increases with time, whereas the proportion of non L_k-chains augments very late in the response, after the bulk of high affinity antibodies has waned (see also Section, XIV.D). This finding is in line with the reported preference of antibodies to group C streptococci for the b4 allotype over the b9 in heterozygous rabbits (KINDT, 1974) and suggests that b4 molecules are better suited to react with the antigen probably because their combining site exhibits more affinity for the relevant determinants.

No definitive explanation for these findings is available. Nevertheless the use of allotypic markers to follow clonal dynamics during the immune response is a promising approach and should provide useful data.

C. L-Chain Type

Affinity of anti-DNP antibody increases with time after immunization in guineapigs. This increase in affinity is paralleled by a progressive predominance of antibodies with L-chains of k-type (NUSSENWEIG and BENACERRAF, 1967). Antibodies with high affinity ($K_0 > 10^7$) are 100% of k-type. Shortly after immunization, there are antibody molecules with L-chains of λ-type and these antibodies are of low affinity. Similar findings have been reported to

occur in the rabbit (Fontana et al., 1973). Antigen injection (DNP-keyhole limpet hemocyanin) was followed by production of antibodies with increasing affinity; in parallel, augmentation in the absolute number of antibody molecules with L-chain, type k, and diminution of L-chains, type λ, (or not k-type) was observed. It was postulated that L_k-chains are better suited for antibody function than L λ-chains, and that among L_k-chains there are discrete subpopulations capable of building up an antigen-combining site with different affinities. For instance, L_k-chains of subtype B (Fontana et al., 1973) with an additional intrachain disulfide bridge would form antigen-combining sites with higher affinity than do L_k-chains of A subtype which lack the above mentioned additional disulfide bond. Preliminary results suggest that antibodies with low affinity, with L_λ-chains (or with L_k-chain, subtype A) persist all over the immune response (Fontana et al., 1973). *In vitro* it was observed that by the end of the immune response antibody affinity falls and molecules with L_k-chains tend to disappear. The very low titers at this moment seem to be due to antibody molecules of L_λ-type, or at least not k; these antibodies are of very low affinity (Conway de Macario et al., in preparation). Is has been postulated (Macario and Conway de Macario, 1973, 1974, 1974a) that these molecules may be a T-cell product and/or the product of memory cells.

These molecules are of average low affinity and heterogeneous in terms of affinity and therefore highly specific; they could react with antigens to form multivalent complexes suitable for B-cell triggering. Immunological memory would then be built up not only by memory cells but also by circulating molecules whose function would be to recognize and make immunogenic even small amounts of antigen that attack a primed animal. Facilitation of meeting and interaction of cooperating cells would also be mediated by these molecules through formation of antigenic complexes.

D. Reconstitution of the Antigen-Combining Site by Recombination of Ig Chains

Isolated Ig chains can recombine and form a functional molecule (Macario et al., 1975a). Isolated chains possess much less antibody specificity than the intact molecule but antibody activity can be recovered up to 100% upon reassociation of the chains, provided the pools of H- and L-chains used belong to a homogenous antibody population with a given specificity. If the two pools of chains mixed together belong to antibodies with different specificities the binding activity for either antigen after recombination may not even reach 50% of the initial activities. Therefore, regain of a high percentage of antibody binding capacity after reassociation of H- and L-chains is taken as an indication of great similarity between the antibody populations from which the two chains were derived (Ghose and Karush, 1974). When these criteria were applied, antibody molecules were found with very similar or identical properties in serum samples taken 6 months apart after several antigen injections (MacDonald et al., 1969). This was explained as persistence of a clone through

continuous stimulation of memory cells derived from the proliferating high-rate antibody-forming clone. The role of antigen in this continuous recruitment appears obvious at first glance but it does not seem to be proved.

For instance, in pathological conditions in which a clone produces a homogenous Ig, such as the myelomas, not only within a single animal but also after transplantation into several successive hosts, or *in vitro* (POTTER, 1972) the role of antigen in the maintenance of clonal proliferation and function is not at all clear.

E. L-Chain Electrophoretic Pattern

Electrophoresis in polyacrylamide gels of isolate L-chains give discrete banding patterns that in the case of restricted populations of antibodies are quite simple and easy to identify (MACARIO et al., 1975 a). It is then possible to follow changes in banding patterns of L-chains from antibody isolated at various times during the immune response, after one or several antigen injections. If the patterns change but retain simplicity, one may conclude that antibody-forming clones have also been replaced; when the patterns become complicated by addition of bands, the interpretation is made that several clones have been stimulated to synthesize antibodies. Along these lines investigations have been reported on persistence and replacement of restricted populations of antibodies with specificity for various antigens. However, it should be emphasized that L-chains of a single mobility do not necessarily mean molecular homogeneity of the antibody sample. In order to assess molecular homogeneity other criteria have to be satisfied simultaneously, such as homogeneity in binding constant, a.a. composition of the chains, and sequence of a.a. residues at the N-terminal end (PINCUS et al., 1968; ROHOLT et al., 1970). The appearance and disappearance of restricted antibody components in rabbits immunized with Type III pneumococci, was observed (KIMBALL et al., 1971); a restricted pattern in cellulose acetate electrophoresis was accompanied by a simple, discrete pattern in polyacrylamide gel electrophoresis of L-chains. Temporal variations of these patterns were observed, but no clear-cut relationship could be found with immunization schedule and antibody titers. In some rabbits, the same pattern persisted all over the immune response (several months) until the animal died or was killed.

F. Primary Structure of Antibody Molecules

This is a reliable criterium for determining homogeneity of an antibody sample (MACARIO et al., 1975 a). However, it can not always be applied, the main practical limitation being the amounts of purified antibody needed to do a.a. sequence. This determination is limited to some stretches of the molecule, whose participation in the antigen-combining site is still under investigation. In a rabbit producing antibodies of restricted heterogeneity against pneumococcus Type VIII the same a.a. sequence of the N-terminus

of L-chains was found after three successive courses of immunization over a period of about 6 months (Jaton et al., 1971). After the first and second course of immunization other peptides appeared in addition to the persistent one, which was highly predominant after the second immunization course. Anti-idiotype antibodies raised against the homogeneous antibodies which predominated after the second course of immunization were found to recognize antibodies present after the first and third courses. It is concluded that a given antibody species, identified serologically by anti-idiotype antiserum and biochemically by the sequence of a.a. at the N-terminus of L-chains, persisted all over the immune response, in variable concentrations. This antibody was accompanied by other antibody species which appeared after antigenic challenge in an unpredictable fashion, i.e. apparently not related to antigenic challenge in a straightforward manner. In another report (Seon et al., 1972), the first eleven residues at the N-terminal end of L-chains of rabbit anti-azobenzoate antibodies were described. Two major sequences were found during about half a year and only one of them persisted for an additional interval of half a year. In spite of continuous antigen administration a very restricted response was elicited, which became still more restricted with time. The two major antibody species persisted over long periods; apparently the immune response once established was insensitive to antigenic challenge in respect to broadening the spectrum of antibody species. A similar finding was reported (Chen et al., 1973) i.e. persistence of a single a.a. sequence at the N-terminal end of L-chains during an immune response toward pneumococcal polysaccharides yielding homogeneous antibodies over a period of 6 months. This was seen in one rabbit out of 70 immunized in the same manner. Sixty-seven animals showed restricted patterns at one time or another during a long period of observation with periodical antigenic challenges with the pneumococcal vaccine. All of them exhibited complex patterns of alternation and overlapping of antibody species in a random fashion. It was concluded that in view of the diversity of the responses obtained by a single immunization schedule it is not possible to propose a simple hypothesis to explain the mechanism governing restriction of the immune response and clonal dynamics. More recently the persistence of a predominant sequence of a.a. at the N-terminal of L-chains was found to go up to 28 months (Mage et al., 1973); together with this sequence two or three others were found to appear and disappear during the period of observation. This last observation reemphasizes the fact that the immune response seems to be taken over by very few antibody-forming clones, if not only one, which may be accompanied at intervals by other clones, of shorter life span.

G. Isoelectric Focusing (IEF) Spectrum

Isoelectric focusing is a high resolution method allowing separation of antibody molecules differing in pI (Macario et al., 1975a). A homogeneous population of Ig molecules, e.g. the product of a plasmocytoma, exhibits a

distinctive spectrum of a few bands on polyacrylamide gel (ASKONAS et al., 1970). The particular spectrum of each myeloma protein is maintained indefinitely which makes it possible to follow the product of a given clone during a period of time in a single animal, after transfer to appropriate recipients and during cultivation *in vitro*. The method has been applied to perform time-course studies of the product of antibody-forming clones induced by artificial immunization with a variety of antigens. In rabbits, several courses of immunization with Type VIII pneumococcal polysaccharide alternating with resting periods induced restricted responses, with synthesis of one, or very few, molecular antibody species (PINCUS et al., 1970, 1970a). The restricted response was permanent or only temporal. The same type of responses against DNP were observed upon immunization with ε-DNP-lysine coupled to Type III pneumococci (MONTGOMERY and PINCUS, 1973). There were periods of restriction alternating with periods of more heterogeneous antibody populations; usually one antibody species which was highly predominant during the intervals of restriction persisted all over the response, being present even when other antibody species came into play. A restricted anti-DNP response was also obtained in neonatal rabbits immunized with DNP-bovine gamma globulin; one antibody species in each individual predominated and persisted at least 92 days, in spite of continuous antigenic challenge, which slowly led to appearance of other antibody molecules (MONTGOMERY and WILLIAMSON, 1970). Antihapten antibody responses of restricted heterogeneity in the rabbit are not exclusively induced by DNP. Immunization with p-azobenzoate coupled to a carrier protein (bovine gamma globulin or ovalbumin) (KITAWA et al., 1967; ROHOLT et al., 1970) elicited restricted antibodies. The homogeneity of these antihapten antibodies was assessed by several methods: light chain disc electrophoresis, a.a. composition of the N-terminal end of L-chains, tryptic peptic maps and specificity of recombination of H- and L-chains (ROHOLT et al., 1965). Isoelectric focusing provided further and probably definitive evidence for such restriction in some animals, for a very simple banding pattern of IgG antibodies, and L-chains isolated therefrom was found, together with a heterogeneity index of binding affinity equal to unity (HOFFMAN et al., 1971, 1972).

Altogether these results show that a substantial proportion of rabbits respond in a restricted manner; few antihapten antibody species are synthetized even after repeated challenge with hapten-protein conjugates which possess the haptenic structure in a variety of molecular microenvironments. That is, heterogeneity of haptenic determinants, number of antigen injections and total dose are rather ineffective in generating very heterogeneous responses. Furthermore, with time after immunization the tendency is to more restriction, one antibody species may persist over months, even more than one year, whereas other antibody species that may appear tend to disappear soon. These patterns were also found in guinea-pigs (ROELANTS and GOODMAN, 1974) and mice. In the latter species an antibody-producing clone was followed for about one year through serial passages in syngeneic mice (8 generations

of transplanted recipients) (Askonas and Williamson, 1972; Williamson and Askonas, 1972). One of the striking facts is that in spite of the periodical antigenic challenges (at each transplant) which were needed to elicit antibody synthesis, only one clone, always the same, was stimulated during six months. Other clones appeared thereafter, in coincidence with decay and final disappearance of the dominant clone (Askonas et al., 1972c). It was also established that memory cells are long-living cells which may be the basis for clonal persistence (Askonas et al., 1972a, b). In conclusion, the role of antigen in stimulating a given clone to continue antibody production appears to be determinant, but its role in modifying the clonal spectrum during the immune response is much less clear, since in all animal species investigated it was not possible to derive rules concerning manipulation of antigen variables that could allow elicitation of a given type of response, more or less restricted, and its progression in one sense or another. Genetic factors may also be involved (Sher et al., 1972; Eichmann, 1972, 1973; Braun et al., 1973; Kindt et al., 1973; Pawlak et al., 1973; Annales d'Immunologie, 1974) together with some other, not yet defined, regulatory factors.

H. Circular Dichroism (CD)

Antigen-binding sites complementary to chromophoric haptens can be characterized by the extrinsic circular dichroism induced by the antibody-hapten complex (for references see Gollogly and Cathou, 1974). The optical activity of the bound hapten depends on the architecture of the antigen-binding site: therefore, different sites interacting with the same chromophoric hapten induce different CD spectra. This principle has recently been applied to the study of the sequential changes in antibody populations (Gollogly et al., 1974). Three antibody types named I, II and III were found to appear in this order in time in rabbits hyperimmunized with fluorescein-keyhole limpet hemocyanin. Each type persisted a few weeks. Usually only one type was detected at any given time but some transitional mixtures were also found. The same sequence was repeated after each booster. The remark should be made that the association constant of the antibodies appearing last, namely type III, was lower than that of type I and II molecules, which appeared sooner after boosting. No satisfactory explanation is available for these findings.

XV. Factors Involved in Determination of Affinity Levels and Rate of Maturation

A. The Triad Antigen-Cell-Antibody

All factors controlling affinity are in some way or another included in the triad antigen–antibody–cell. Most of them were discussed in the previous sections of this review. On the antigenic side it is important to remember molecular nature and structure, dose, number of injections of one or more antigens, route of administration and use of adjuvants. In relation to the

latter two parameters, recent information (MOND et al., 1974) indicates that administration of the antigen emulsified in Freund's complete adjuvant, subcutaneously or via foot-pad, induces responses of greater magnitude with production of antibodies of higher affinity than when incomplete adjuvant is used. Similar effects can be obtained with E. coli endotoxin instead of complete adjuvant. Antigen alone given intravenously tends to elicit production of lower amounts of antibodies with low affinity; however, by repeating the injections a considerable increase in K_0 can occur, provided that the antigen doses are not too high.

The regulatory effect of circulating antibodies which compete with cell-receptors for the antigen available would also contribute to accelerate the process of maturation by augmenting the selective pressure in favour of precursors with higher affinity (for reference see SISKIND et al., 1969; ANDERSSON and WIGZELL, 1971; ANDERSSON, 1972a).

High initial cell number and subsequent high rate of proliferation as well as cell-cell interactions favour progression in affinity (see previous Sections V, VI, VIII, IX, X, XII, XIII and XIV).

The information available on genetic control of antibody affinity as well as its modifications in pathological conditions is very scarce. Nonetheless, we shall consider it in the following two paragraphs because it deals with very promising areas of research.

B. Genetic Control

Immunogenetics is an expanding field of research at the present time. However, little is known yet on the genetic regulation of antibody affinity and its dynamics during the immune response. It has been reported that the rate of increase in affinity with time is more or less distinctive of each rat strain (LAMELIN and PAUL, 1971). The capacity to produce antibodies with high affinity is not equally manifested in all strains of rats (RUSCETTI et al., 1974) and mice (SOOTHILL and STEWARD, 1971; PETTY et al., 1972). In the latter species production of high affinity antibodies is related to highly efficient macrophage phagocytosis (PASSWELL et al., 1974), a function whose genetic control is now under scrutiny.

Since maturation in affinity seems to depend mainly on macrophage functions, initial number of precursors with the same specificity, rate of lymphocyte proliferation, magnitude of clonal expansion and clonal instability, it is likely that genetic control of antibody affinity will turn out to be exercised through these cellular properties.

C. Pathological Conditions

The role of affinity in the pathological effects eventually brought about by antigen–antibody interactions will be discussed below (Section XVI.A). We examine here data showing that some pathological conditions preclude synthesis of high affinity antibodies regardless of the time elapsed after immunization. Malaria infection strongly interferes with production of high

affinity antibodies (Steward and Voller, 1973) and the same alteration is provoked by malnutrition (Passwell et al., 1974a). Mice fed with a diet poor in proteins, exhibit impaired macrophage function and synthesize only low affinity antibodies. Since in this case absence of high affinity molecules correlates with poor macrophage function and the same correlation was found in the strains of mice which are genetically limited to the synthesis of low affinity antibodies (see Section XV.A) it was postulated that impaired affinity maturation in malaria infection is due to macrophage blockade by the parasites (Passwell et al., 1974a).

Regardless of whether or not ageing is a pathological condition it is worth mentioning that investigations on the performance of the immune system in terms of antibody affinity during involution should begin. It is hoped that these studies will yield information on the role of antibody molecules in self recognition and destruction as the tissues change with age.

XVI. The Biological and Practical Significance of Antibody Affinity and its Time-Course Variations

A. Role of Affinity in the Determination of the Biopathological Effects of Antigen–Antibody Interactions

A progressive increase in affinity during the immune response seems justified if one thinks that this will increase the strength of the reaction against a given antigen on a second aggression, and will allow reaction with related structures (Macario et al., 1975a). In this manner, the organism would learn with time how to improve the reaction against the same agent and expand its reactive potential to other related antigens. But, at the molecular level, what are the biological consequences of a stronger bond? One would like to think that it contributes to a more efficient and extensive modification of the tridimensional structure of the molecules involved, thus facilitating the exposure of hidden sites, or even creating new sites by spatial rearrangements of different stretches of the molecules. The sites thus exposed may have affinity for other molecules, that on binding to the complex can start a chain of reactions, for instance, activation of enzymes leading to fission and fusion products with biological functions at the cell surface, or inside them. This reasoning seems justified since it has been shown that complement fixation by rabbit IgG anti-DNP antibody is more and more efficient as antibody affinity increases (Fauci et al., 1970). This was confirmed (Warner and Ovary, 1970) using IgG 2a with a very low affinity for DNP. It fixes C′ but it does not produce a PCA (passive cutaneous anaphylaxis) reaction on guinea-pig's skin, after interaction with DNP-aminoacid, although these two effects can be produced by an IgG 2a-antibody with higher affinity for DNP. Similar observations were made by studying the reaction of a mouse myeloma macroglobulin with anti-dextran activity (Leon et al., 1970). A minimal energy of binding was needed for the C′ fixation to occur; above this threshold,

the efficiency of C′ fixation increased as the affinity between the macroglobulin and a series of dextrans increased. More recently the role of antibody affinity in complement-dependant damage of biological membranes was investigated using a liposomal model in which the membrane was sensitized with dinitrophenylated phospholipids (SIX et al., 1973). IgG antibodies with high affinity were shown to be more efficient in causing membrane damage than low affinity antibodies of the same Ig class. Comparison of low affinity antibodies of IgG and IgM classes showed that the latter are more efficient, which indicates that multivalent antibodies produce marked biological effects even if they have low affinity for the antigen (see Section II.B.).

Other biological phenomena involving antigen–antibody interactions have been found to be influenced by the association constant of the antibody; for example, inactivation of trypsin by antibodies raised against the enzyme is more efficiently achieved by antibodies with high affinity (ERICKSON, 1974). Furthermore, it was observed several years ago that the neutralization of toxins (JERNE, 1951) and the degree of hemagglutination *in vitro* (LEVINE and LEVITSKA, 1967) correlates with the affinity of the antibody (see following Section XVI.B). More recently the role of antibody affinity in some immunopathological phenomena, such as immune-complex diseases ("autoimmune" nephritis for example) has been investigated. High affinity antibodies would facilitate immune-complex elimination whereas low affinity antibodies would contribute to a failure of immune-elimination leading to persistence of antigen and formation of pathogeneic complexes (SOOTHILL et al., 1971; ALPERS et al., 1972; PETTY and STEWARD, 1972; PETTY et al., 1972; STEWARD and VOLLER, 1973; STEWARD et al., 1973, 1974).

It is remarkable that studies on the correlation between affinity and "protective" capacity of antibodies are scarce, although it would appear an obvious issue for investigation. A recent report indicates that indeed such a correlation exists. Antibodies against *Escherichia coli* antigen 0, produced in rabbits, protected mice infected with the bacterium. The higher the affinity of the antibodies the higher the number of infected animals that survived (AHLSTED et al., 1974).

These studies will most probably help understanding mechanisms of tumor rejection and enhancing, and of persistent virus and parasitic infections.

B. Remarks on the Fact that Methods for Quantitating Antibodies are Affinity-Dependant

The interdependence of affinity and measurement of antibody levels can be viewed from four angles: Firstly, definition of an antiserum should include both parameters, namely antibody concentration and quality, as was pointed out many years ago (JERNE, 1951); a statement which often seems to be ignored. Secondly, the efficiency of antibodies utilized as tools for detection and/or quantitation of a series of substances (serum proteins, hormones, tissue

antigens, etc.) is dependent on the association constant of the antibody preparation used. For example, the automated nephelometric immunoassay is more efficient in protein determination if high affinity antibodies are used (Cambiasso et al., 1974). The reason would be that a stronger bond between antibodies and antigen (the molecules which are being sought) provokes more intense light scattering and in so doing it increases the efficiency of the method which is based on the registration of that signal. Thirdly, analysis of low affinity antibodies is difficult or even impossible in mixtures where high affinity subpopulations are also present. Low affinity molecules can be studied only by previous isolation from high affinity subpopulations in serum samples (Kim et al., 1974b). Another way is to establish a series of cultures with limiting numbers of cells to reduce the probability of multiclonal responses and thereby increasing the frequency of production of low and high affinity antibodies in separate tubes (Macario et al., 1974a). Lastly, a number of methods for determining antibody titer are affinity-dependent; we have already mentioned (see Section XVI.A) as an example that hemagglutination *in vitro* correlates with antibody affinity (Levine et al., 1967). It is probable that a good proportion of techniques detect only high affinity molecules. Interpretation of results must be cautious because it could very well happen that some negatives (animals, cultures, patients) may not in fact be non-antibody producers but producers of antibodies with low affinity undetectable by the method used. It is thus obvious that standardization of tests must be established for the range of affinities which they are capable of measuring.

XVII. The Various Levels at which the Temporal Changes in Antibody Affinity Can be Analyzed in the Search for their Causing Mechanism and Biological Significance

A. Serum Level

In sera and tissue culture media one sees changes in antibody populations, namely: various populations replace each other as time goes by, the ones appearing later are constituted by molecules with higher affinity. After some time, high affinity molecules are largely predominant. However, populations of molecules with low affinity do not disappear; they can be found all over the immune response; sometimes they can even take over the response during relatively long intervals, and they can persist also after the bulk of the antibody response has waned.

B. Cellular Level

The above mentioned changes in antibody populations most probably reflect modifications in the populations of antibody-forming cells. There is supporting evidence that high affinity antibodies are produced by cells dividing at a higher rate than cells synthesizing antibodies with low affinity (Davie

et al., 1972, 1973; CLAFLIN et al., 1973). The assumption can then be made that high-rate dividing cells are high-rate antibody-forming cells. The spectrum of these cells (clones) tends to narrow from a rather wide dispersion of affinities towards a restricted range of high affinity. Low-rate dividing cells can be considered low-rate antibody producers. They persist over long intervals, and synthesize antibodies with an average low affinity. These molecules have been proposed as memory molecules; irrespective of whether this proposition is correct or not, it is evident that memory cells do not tend to be predominantly those with high affinity (MACARIO et al., 1973, 1973a, 1974, 1974a). All these cell population changes have their origin at the level of single cell precursors. The questions raised are where, when and how the precursors arise. Are all present before or does the great majority of them arise after antigenic stimulation? If so, does this diversity originate within the antibody-forming clones (intraclonal generation of diversity) or not (interclonal generation of diversity)? The problem is then to distinguish whether all possible precursors are present before first contact with the corresponding antigen, or if some, or the majority of them, arise during proliferation of a few specific (perhaps polyfunctional) precursors present at the time of primary antigenic stimulation. We are not dealing here with the problem of how the array of precursors arises, before antigen contact. In this respect, germ-line theories (HOOD and TALMAGE, 1970; SMITH et al., 1971; PRENKUMAR et al., 1974; BARSTAD et al., 1974) postulate that the whole spectrum of precursors is present, because it has developed through phylogenetic evolution. On the other hand, somatic theories support the view that the entire repertoire develops during ontogeny starting from a few germ-line encoded precursors, by modifications either at the DNA level (COHN, 1967, 1970; GALLY and EDELMAN, 1970, 1972; JERNE, 1971, 1972; CAPRA and KEHOE, 1974; TONEGAWA et al., 1974), or at the level of RNA (immunodifferentiation) (HAUROWITZ, 1973). A common idea is that before antigenic stimulation the whole spectrum of precursors is present. However, generation of further diversity driven by antigen has also been postulated (CUNNINGHAM, 1974). Some data suggest that diversification may occur during expansion of a clone (STORB et al., 1972; MACARIO et al., 1972; FRIEDENSON et al., 1973; HAIMOVICH and DU PASQUIER, 1973; LITVIN et al., 1973; PRESS and KLINMAN, 1973; CONWAY DE MACARIO et al., 1973; CUNNINGHAM, 1974; CUNNINGHAM and FORDHAM, 1974; CUNNINGHAM and PILARSKI, 1974) (see also Section XIV). However, even in case a certain degree of diversification were found, it should still be established how frequent the phenomenon is, to what extent, if any, antigen plays a determinant role in it, and what its magnitude and importance are in the *in vivo* immune response.

C. Molecular Level

What is the meaning, at molecular level, of antibody with high affinity? It can be postulated that high affinity molecules possess larger antigen-

combining sites than low affinity molecules. The large site would permit interaction with larger portions of the immunogen than in the case of low affinity antibodies; they could be polyfunctional, i.e. capable of recognizing and interacting with more than one determinant or subsites. This idea is supported by at least three lines of arguments (Macario et al., 1975 a), namely: (a) Antibodies synthesized late in the immune response possess a larger antigen-combining site than antibodies synthesized shortly after immunization (Eisen et al., 1964; Fujio and Karush, 1966; Atsumi et al., 1968; Little and Counts, 1969; Murphy and Sage, 1970). (b) Antibodies elicited by immunization with a big haptenic structure, which is likely to fill up the largest possible combining-site completely do not show any increase in affinity with time after immunization (Gopalakrishnan et al., 1973). (c) Late antibodies with high affinity are highly cross-reactive (Gershon et al., 1972; see also Section XI). Assuming that the antigen-combining site is polyfunctional on the basis of recent work (Rosenstein et al., 1972; Richards and Konigsberg, 1973; Varga et al., 1973; Hirschfeld, 1974) maturation in affinity would lead to predominance of those sites capable of recognizing larger stretches of the immunogen molecule, or greater numbers of adjacent copies of the same atomic arrangement, just because cells bearing such sites would be selectively stimulated. Of course, the last statement assumes that the precursors bear surface receptors with similar or identical properties to the combining site of the antibody molecule released by the antibody-forming cells derived from them; this assumption seems to be well substantiated (Andersson, 1972, 1972a; Claflin et al., 1974, 1974a). In summary, this hypothesis postulates that antigen-combining sites are polyfunctional, some molecules having a wider range of specificities than others because their combining-site is bigger; they would be high affinity antibodies, or receptors. It follows that precursors could also be polyfunctional and they could be triggered by a variety of determinants sharing one or more subsites. Even low affinity interactions at the level of individual determinants or subsites would be stimulatory if they occur simultaneously to bring about a multiple bonding between immunogen and cell surface (see Section II.B). The biological advantages of polyfunctional sites have already been emphasized (Rosenstein et al., 1972; Richards et al., 1973; Varga et al., 1973; Hirschfeld, 1974, 1974a) as well as the energetic consequences and biopathological implications of multiple bonding (see Sections II.B. and XVI.A.), therefore the importance of the tendency to increase the concentration of antibody molecules with larger antigen-combining site and the number of precursors bearing bigger receptor sites seems obvious. To what extent if any, interaction of larger cell surface areas with bigger stretches of the immunogen molecule could enhance modulation of gene expression is not known. It could be possible that through more extensive and stronger interactions at the cell surface some influence could be exerted on the genome, for instance at the levels of the redundant genes (which would code for slightly different polypeptide chains), to further increase the size of the antigen-combining site.

XVIII. Summary, Conclusions and Perspectives

Antibody responses have usually been defined only by titrating antibody levels. In some instances the respective concentrations of IgM and IgG-antibody have also been determined. However, it is clear at present time that appropriate characterization of an immune response requires also definition of other properties of the antibody molecules, including Ig class, which are known to play a role in the causation of biological effects. In this regard, the association constant with the relevant ligand(s) ranks first. This is due to the fact that some important biological effects brought about by the interaction between antibody (free in solution—serum—, cell bound or as cell receptor) and the corresponding antigen (in solution or attached to a cell surface) depend on the energy of that interaction.

The immune response should then be defined at different time-points in terms of antibody populations with regard to antigen-binding properties and also to Ig class, idiotype, allotype, capacity to bind C′, and any other property that can have biopathological significance. Otherwise, the risk of misinterpretation of data because of incompleteness is serious.

Definition of antibody populations can nowadays be done at serum level and also at clonal and cellular levels since methods have recently been worked out which permit measurements of antibodies released *in vitro* by small cell numbers, single clones and even single cells.

Antibody properties were classically defined for molecules in solution, in the serum, and more recently, in liquid and semisolid media. Furthermore, some properties of antibody-like receptors on the cell surface can now also be studied, such as binding constant, valence, idiotype, allotype, heavy-chain class and light-chain type. Thus, it is possible to study gene products directly on the cell membrane, its physiology and physiopathology *in situ*, in mammalian systems.

Most of the knowledge on the interaction between antibody and antigen has been acquired by using artificial haptens and conjugates. A step forward has recently been accomplished by the development of methods that permit the measurement of the interaction of the antigen-combining site with natural atomic arrangements on macromolecules in their native configuration. This will probably lead to a more complete understanding of the interaction of antibodies and antigens in natural situations, for example in anti-bacterial immunity.

A thorough characterization of the immune response at different time-points, as described in the foregoing paragraphs, is necessary not only in experimental situations but also in clinical investigations. The two facets of this topic related to antibody affinity are: a) The role of antibody affinity in determining the biopathological effects eventually produced by the antigen-antibody interaction; and, b) The influence of certain diseases and functional anomalies that interfere with production of antibodies with high affinity. Studies in this direction are now beginning and others should follow with the

purpose of defining the antibody properties in pathological conditions, whereby these molecules can play a defensive and/or pathogenetic role, as effector, regulatory or modulating agents. Some types of cancer, "slow" virus and persistent parasitic infections, and autoimmune diseases are good candidates for such studies. In this respect, investigations on the binding properties of Ig other than IgG and IgM should also be carried out.

Another important property of the antibody molecule that must be considered is valence. The number of bonds between antibody and antigen contributes to the energy of the interaction, and may therefore, greatly influence the outcome of the interaction, for instance, conformational changes of the molecules; regulation of cell function by antibodies delivering signals at the cell membrane level; cell triggering; target-cell killing with participation of antibodies; etc. The valence and physicochemical status of the antigen (in solution or bound to a cell surface) are important, too, since they also contribute to the total number of bonds, and in so doing to the final energy changes.

The facts mentioned above regarding valence have to be considered together with the mobility of the antibody-like receptors in the plane of the lymphocyte membrane if a description of the mechanism causing cell triggering is sought for. Receptors become redistributed upon interaction with a ligand (antibody or antigen) and come closer to each other, which would favor multiple bonding. Thus establishment of multiple bonding would proceed "catalitically" after the threshold interaction starts the chain of reactions leading to microredistribution of receptors. Under these lights some of the conclusions derived from the precommitment principle (for Review see Lefkovitz, 1974) may need revision, if the phenomena described above do indeed play a role in antigenic stimulation. Immunocytes are highly restricted because they can synthesize only one, or extremely few, antibody species, that is only one V-region. These antigen-combining sites would possess low affinity for the relevant determinant and no detectable affinity for other determinants, otherwise they would be less specific since it is conceivable that sites with high affinity for one determinant can show a gradient of affinities for other determinants. Considering the surface of a restricted immunocyte, with many copies of identical receptors restricted to only one specificity, distributed all over, it would appear possible that the functional affinity for some other seterminant(s) could reach the functional threshold due to multiple bonding. Thus, any antigen molecule bearing several determinants which separately are not enough affine with individual receptors could react with a group of them crowded in a microarea and become actually active in cell triggering. It follows that despite strict molecular precommitment (receptors and antibodies released) precursors may operationally be less restricted. Cell triggering could then be accomplished by a variety of determinants, provided they are adequately presented to fit the multiple copies of receptors on the cell surface.

A thorough examination of the antibody response over long periods of time after antigenic stimulation has recently demonstrated that the association constant of the antibodies does not change with time following a stereotyped

profile. Rather, several patterns have been found, namely: increase up to a peak or plateau; late fall; not significant changes; complex patterns of alternation of low and high affinity molecules more or less superimposed. High affinity antibodies show often a clear tendency to take over the response, but this is only temporary; after sometime high affinity populations decay while low affinity molecules persist or emerge. In spite of the periodical predominance of high affinity clones, low affinity ones are not eliminated, nor are eliminated memory cell capable to initiate synthesis of antibodies with low affinity on secondary challenge. It has been demonstrated by looking at antibody markers other than affinity that certain clones can predominate and persist a long time; replacement of clones is not very much influenced by administration of antigen, even repeatedely; some clones can switch from the synthesis of IgM to IgG molecules. Changes in specificity within a cell lineage have also been observed. In conclusion the mechanism of clonal persistence and replacement is not yet well understood as it is not yet established to what extent new antigen-combining sites (with different specificities or affinities) can be generated during expansion of a clone. Nevertheless, some data indicate that this can occur with a frequency that justify the prediction that it is not a rare event, more or less artifactual, but a significant mechanism in adaptive immunity.

It should be borne in mind that tests for antibody titrations may detect only high affinity antibodies; therefore, whenever antibodies are synthesized but they possess low affinity, below the sensitivity threshold of the assay, they will not be detected. It is then obvious that standardization of methods for quantitating antibodies must include a description of the range of affinities they are capable to measure.

Attempts at making an unifying hypothesis to explain affinity changes and clonal dynamics during the immune response are hampered because information concerning the mechanism of several basic phenomena is incomplete at present time, for example tolerance induction at cellular level and the molecular mechanisms of lymphocyte stimulation including cell to cell interactions (cooperation and regulation).

Data reviewed in this article indicate that molecular evolution proceeds during the immune response. We hope that examination of the latter response will also afford material for understanding ageing and involution.

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