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Vesicular Stomatitis Virus: Structure and Function of Virion Components

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With 4 Figures

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I. Introduction

Vesicular stomatitis virus (VS) is an enveloped Rhabdovirus which is capable of productively infecting a multitude of hosts including many mammalian species, fish, and insects (HOWATSON, 1970; BUSSEREAU, 1973). Two

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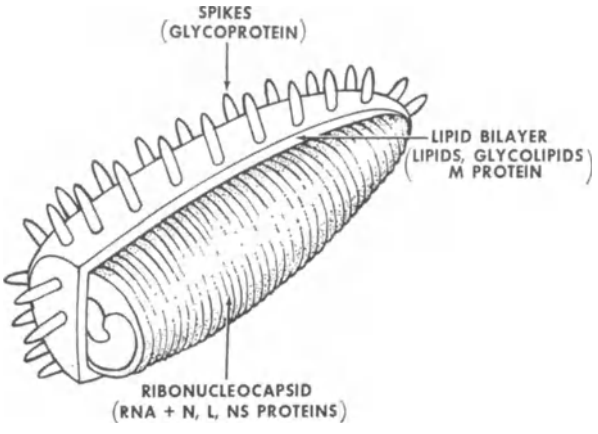

Fig. 1. Schematic representation of the vesicular stomatitis virion. Spikes (G protein) are shown attached to the planar end although there is no direct evidence that they are normally located there

serotypes of VS virus have been identified: $VS_{Indiana}$ and $VS_{New\ Jersey}$ (PRINGLE, 1975). The standard infectious virions of either serotype are rods (rhabdos = rod) of approximately 175 ×68 nm which have one hemispherical and one planar end and contain a helical nucleocapsid of approximately 30 equal turns followed by 4 or 5 diminishing coils at the rounded end (HOWATSON, 1970) (Fig. 1). Thus, virions appear bullet-shaped. Numerous "spikes" measuring 10 nm in length project externally from the entire surface of the viral envelope. Defective interfering (DI) particles which have the same morphology but are shorter and contain incomplete genomes are also produced at high multiplicities of infection. The viral envelope is acquired during maturation when nucleocapsids bud through pre-existing cell membranes; the site of budding varies according to the host species and can be on either the plasma membrane or the membranes of intracytoplasmic vesicles or on both (ZEE et al., 1970).

VS virions can best be considered as two distinct structural units: the envelope and the ribonucleocapsid (RNC). Since the RNC of VS virus is infectious (SZILÁGYI and URYVAYEV, 1973; CARTWRIGHT et al., 1970a), the nucleocapsid contains all the information required to synthesize new virions and neither the envelope nor its components are obligatory for infection. However, since the infectivity of nucleocapsids is about 3 logs lower than that of intact virions (CARTWRIGHT et al., 1970a), the presence of the envelope greatly increases the efficiency of infection. Since the envelope and nucleocapsids perform basically different functions and consist of different components, they will, for the purpose of this review, be discussed as separate entities.

VS virus has many properties which contribute to its immense popularity as an experimental system. First of all, the infective virus has a simple genome consisting of one piece of single stranded RNA which has a molecular weight of $\sim 3.5 \times 10^6$ daltons. The virion RNA serves as the template for the synthesis of smaller monocistronic mRNAs (HUANG et al., 1970; MUDD and

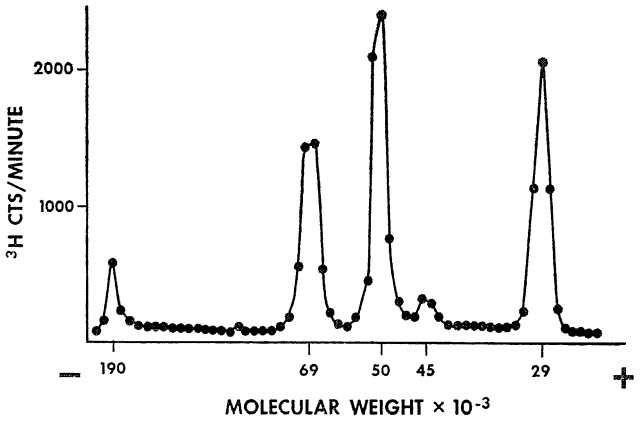

Fig. 2. Electropherogram of the VS_{Ind} virion proteins. Purified virions labeled with [^{3}H]leucine were solubilized with 8 M urea, 1 % 2-mercaptoethanol, and 1 % SDS and boiled for 2 min prior to electrophoresis on 7.5 % SDS polyacrylamide gels. A phosphate buffer was used (see EMERSON and WAGNER, 1972, for details)

SUMMERS, 1970b), hence the virus is classified as a negative strand virus (BALTIMORE, 1971; BARRY and MAHY, 1974). In 1970, BALTIMORE et al. made the exciting discovery that VS virions contained their own RNA-dependent RNA polymerase, or more specifically a transcriptase, which was able to synthesize mRNA efficiently *in vitro*. Subsequent *in vitro* experiments have led, among other things, to the partial elucidation of initiation sequences (ROY and BISHOP, 1973a), demonstration of polyadenylation (BANERJEE and RHODES, 1973; VILLARREAL and HOLLAND, 1973) and methylation (RHODES et al., 1974) of the mRNAs, and dissociation and reconstitution of the transcription complex with the resultant identification of the major protein participants (EMERSON and WAGNER, 1972, 1973; EMERSON and YU, 1975).

VS virus, because of its tremendous host range, also offers a unique system for probing cell–virus interactions. Recent studies with enucleated cells have demonstrated that VS replication is completely cytoplasmic (FOLLET et al., 1974; WIKTOR and KOPROWSKI, 1974). At high multiplicities of infection (WERTZ and YOUNGNER, 1972) or in the presence of actinomycin D, host macromolecular synthesis is inhibited, thus allowing identification of newly synthesized viral molecules by specific radioisotopic labeling. As a result it has been possible to follow the synthesis and fate of viral proteins and RNA. Significant progress has been achieved in the past few years in defining how this virus infects the cell, replicates, and matures.

Although the virions contain only five viral proteins (Fig. 2) (WAGNER et al., 1972b), these have diverse enough properties to provoke a great number of important questions. For instance, the prototype of the rhabdoviruses, $VS_{Indiana}$, which has two envelope proteins, has provided a model system for membrane structure–function relationships. The viral M or matrix envelope protein is small, only 29000 daltons, and is thus by far the least studied of the viral proteins. However, the G or glycoprotein (69000 daltons), which

forms the projections or spikes on the virion envelope, has been extensively studied both as a complex structural entity and as a major factor in infection and immunity.

The nucleocapsids of VS_{Ind} virus contain three proteins which are extremely interesting. The N or nucleocapsid protein (50000 daltons) is bound tenaciously to the RNA and probably plays a central role in transcription and replication as the major structural protein of the nucleocapsid complex which serves as the template for RNA synthesis. The remaining two proteins of the nucleocapsid are minor structural components of the virus but functionally are probably two of the most critically important proteins of the virion since both are required for RNA synthesis. The first of these, the L or large protein, accounts for almost one-half of the total genetic information of the virion and was originally considered an aggregate because of its great size (190000 daltons). However, pactamycin experiments and peptide-map analyses finally confirmed that it is a unique gene product (STAMPFER and BALTIMORE, 1973; EMERSON and WAGNER, 1973). The second, the NS protein, is a phosphoprotein. The designation NS originally meant "non-structural" protein (WAGNER et al., 1970), since large amounts accumulate in the soluble cytoplasm of infected cells but only small quantities appear in the virion (KANG and PREVEC, 1971; MUDD and SUMMERS, 1970a). The relative rate of migration of the NS protein on SDS polyacrylamide gels varies according to the buffer system used; therefore, the molecular weight estimate of 40000–45000 daltons given by WAGNER et al. (1972b) is tentative. Indeed, a protein believed to be authentic NS protein has been synthesized *in vitro* from RNA which is only large enough to code for a protein of 30000 daltons (BOTH et al., 1975b). Therefore, NS protein may be much smaller than originally presumed. The NS protein poses many perplexing problems which will be discussed later.

The sum of the molecular weights of the five proteins in VS_{Ind} virions listed above is 383000 daltons which is close to the total coding capacity of the viral RNA. Since five genetic complementation groups have been defined for VS_{Ind}, (see PRINGLE (1975) for an excellent review), these five proteins may represent the total complement of viral gene products. Given the limited number of virally coded proteins, it should be possible to define the structural and functional properties of these molecules completely. The purpose of this review is to present as integrated and complete a picture as possible of the organization and functions of the virion components as they are now understood. Most of the work reviewed here focuses on VS_{Ind}. For a comprehensive review of the entire Rhabdovirus group the reader is referred to articles by HOWATSON (1970) and WAGNER (1975).

II. Envelope

A. Lipids

VS virions contain 20% lipid by weight (61% phospholipids and 39% neutral lipids). The lipid composition of the virions reflects, but does not

exactly mimic, the lipid composition of the host cell plasma membrane (McSharry and Wagner, 1971). In addition, virions grown in BHK-21-F cells contain one glycolipid, a hematoside which is identical to the major glycolipid of the host cell (Klenk and Choppin, 1971).

The organization of the lipid and its interaction with proteins have not been fully determined. Studies using the fluorescent probe diphenylhexatrine have shown that the VS virus membrane is much more viscous than its corresponding host plasma membrane (Moore et al., 1975). When vesicles are made from the lipids extracted from the virions, the viscosity of this preparation is significantly lower than that of the whole particle. It was, therefore, postulated that the proteins have a marked effect on the viscosity of the virus membrane. The vesicles prepared from the virus lipids have a significantly higher viscosity than those made from the lipids of the host plasma membrane. Therefore, it can be concluded that an altered lipid composition in the virus is contributing to its relatively higher viscosity. However, the virus membrane is related to the host membrane to some degree since viruses grown in L and BHK cells have different viscosities which parallel the viscosity differences between the two host cell plasma membranes (Moore et al., 1975).

B. Identification of Protein Components

Two of the major viral proteins, the G (glycoprotein) and M (matrix) protein are constituents of the envelope. Both of these proteins can be separated from the nucleocapsid after dissolution of the membrane with deoxycholate (Kang and Prevec, 1969; Cartwright et al., 1970b) or digitonin (Wagner et al., 1969b) or by treatment of virions with hydrochloric acid at pH 1.5 (Mudd, 1973).

G and M proteins are also separated from the nucleocapsid during the initial stages of infection. If cells are infected with virions containing radiolabeled proteins, both the input G and M proteins can be re-isolated from the plasma membranes of the infected cells, whereas the labeled nucleocapsid is released into the cytoplasmic fraction (Heine and Schnaitman, 1969). Therefore infection by VS virions can involve fusion of viral and cellular membranes, with concomittant release of the nucleocapsid, and integration of G and M proteins into the cell membrane. Alternatively, pinocytosis or viropexis (Simpson et al., 1969) has also been observed, although in this case uncoating of the nucleocapsid, which is a prerequisite for RNA synthesis, has not been described.

Since no cellular proteins were detected in VS virions harvested from cells prelabeled with radioactive amino acids (Kang and Prevec, 1969), the VS virus envelope appears to be a rather simple structure consisting of lipids (McSharry and Wagner, 1971a), glycolipids (Klenk and Choppin, 1971), and only two major proteins, both coded for by the virus. It is still possible that some cellular proteins are incorporated into the viral envelope at levels too low to be detected. In fact, a number of enzyme activities including those

of a phosphotransferase (ROY and BISHOP, 1973), a proteinase (HOLLAND et al., 1972), a methylase (RHODES et al., 1974), and a kinase (IMBLUM and WAGNER, 1974), which are probably of cellular origin, have been detected in purified virions. HECHT and SUMMERS (1972) were also able to detect the L cell histocompatibility antigen in virions purified from these cells. At any rate, VS virus incorporates virion proteins preferentially into its envelope while excluding or limiting the incorporation of cellular proteins. The mechanism for this selectivity has not been defined although HECHT and SUMMERS (1972) have suggested that VS maturation induces turnover of at least one cell surface antigen, the L cell histocompatibility antigen.

C. Location of G and M Proteins

On account of their relative structural simplicity viruses provide model systems for analyzing membrane structure. However, since the technology of membrane chemistry is still being developed, it is not too surprising that even in such a simple system as VS virus, the exact locations of the two membrane proteins have not been strictly defined.

1. Differential Solubilities

The G and M proteins occupy quite different regions of the envelope. The G protein can be selectively released from virions by treating the membrane with the nonionic detergents Nonidet P_{40} (ARSTILA, 1973; CARTWRIGHT et al., 1970b), Triton N-101 (DIETZSCHOLD et al., 1974) or Triton X-100 (KELLEY et al., 1972). Saponin reportedly releases envelope structures which contain G but not M protein (ARSTILA, 1974). While Triton X-100 in low ionic strength solutions will solubilize only G protein, this same detergent will solubilize both G and M proteins, and leave the nucleocapsid intact, in the presence of 0.3 M NaCl (EMERSON and WAGNER, 1972).

2. Biochemical Assays

Biochemical evidence shows that the G protein is located externally to the M protein. Digestion of intact virions by a number of proteolytic enzymes including trypsin (CARTWRIGHT et al., 1970b; SCHLOEMER and WAGNER, 1974) or Pronase (MCSHARRY et al., 1971) degrades the G protein but not the M or nucleocapsid proteins if low concentrations of enzyme are used. Since enzymes cannot penetrate the envelope, only proteins on the exterior surface should be accessible to enzyme. Therefore, most of the G protein must be located on the external surface while the remaining proteins must be either embedded in, or internal to, the lipid bilayer. Proteases remove the projections or spikes concomittantly with digestion of G protein, thus the spikes are composed of G protein (CARTWRIGHT et al., 1970b; MCSHARRY et al., 1971).

Lactoperoxidase-catalyzed iodination of intact virions suggests that G protein is external to the lipid while M may be located close to the outer surface of the envelope. WALTER and MUDD (1973) and MOORE et al. (1974) found that this procedure almost completely labeled the G protein but they also detected a considerable amount of iodine-labeled M protein. The amount of labeled ^{125}I in M protein was doubled if the virions were first disrupted, and was greatly increased if the G protein was first removed by trypsinization. Therefore, a substantial portion of the M protein is not accessible to surface labeling reagents while the majority of the G protein is.

The discrepancy between the protease digestion and the lactoperoxidase labeling experiments makes it difficult to pinpoint the location of M protein. However, it would seem reasonable at this time to assume that most of the M protein molecule is within or internal to the lipid layer but that a small portion of it may be external or located just below the surface.

3. Immunological Data

Additional evidence that the G protein is external derives from immunological considerations. Antibody specific for envelope antigens neutralizes the infectivity of virions while antibody to nucleocapsids does not (KANG and PREVEC, 1970). The neutralizing antibody reacts with the G protein since antibody directed against purified G protein will neutralize viral infectivity quite efficiently (KELLEY et al., 1972; DIETZSCHOLD et al., 1974) but antibody made against purified M and N proteins does not (DIETZSCHOLD et al., 1974). Since antibody molecules will not penetrate the membrane, they can neutralize infectivity of intact virions only if the antigen is located on the outside of the lipid bilayer.

D. Attachment of G Protein to the Envelope

Evidence accumulated thus far demonstrates that the major portion of the G protein is external to the lipid bilayer. The question therefore arises as to what part of the G protein comprises the attachment site and what forces hold the spike in place. The G protein could be attached superficially, or could penetrate the lipid bilayer deeply or even cross it. Although no definitive answer is given, the available evidence suggests that the G protein does penetrate the lipid bilayer to a substantial degree.

Phospholipase C removes much of the lipid from VS virions yet leaves the spikes attached to the virion skeleton indicating that the G protein may interact directly with underlying protein (CARTWRIGHT et al., 1969). It is also possible to remove lipid selectively with sodium dodecyl sulfate (SDS) if the proteins are first cross-linked with formaldehyde (BROWN et al., 1974). In these experiments, virions were fixed with formaldehyde and then 90% of the lipid and phospholipid was extracted with SDS. Over 90% of the viral protein, including over 80% of the G protein, remained in the viral skeletons and spike

structures were visible by electron microscopy. Although the formaldehyde could conceivably cross-link proteins indirectly via unsaturated fatty acids, this result supports the hypothesis that the G protein is located in close proximity to one or more other proteins, and the M protein is the most likely candidate. However, since the lactoperoxidase experiments hint that some of the M protein may be located near the surface of the envelope, these experiments still do not reveal how much of the G protein lies within the lipid bilayer.

The portion of the G protein which is embedded in the lipid should be protected from digestion with proteases. Therefore, one approach to determining how much of the G protein is buried has been to digest virions with proteases and attempt to recover a residual protein fragment after dispersing the lipid. MUDD (1974) was able to isolate such a fragment with a molecular weight of 6900 ± 600 from trypsin-digested virions; a fragment of similar size was isolated after digestion of whole virions with chymotrypsin (7300 ± 200), Protease K (6900 ± 200) or Pronase (7400 ± 1600). SCHLOEMER and WAGNER (1975) degraded virion-attached G protein with thermolysin and then extracted the residual G protein with Triton X-100; a peptide of 5400 daltons which did not contain carbohydrate was recovered; this fragment is insoluble in aqueous solutions and contains a high proportion (60%) of hydrophobic amino acid residues as might be expected for a lipophilic peptide.

Lipid bilayers are about 48 Å thick (LENARD and COMPANS, 1974); a linear peptide of 3000–4000 daltons could completely span such a membrane. Therefore the G protein protease resistant fragment could conceivably pass completely through the membrane. Alternatively, the tertiary structure of the fragment could be such that only shallow penetration of the lipid occurs. Available evidence suggests that the N-terminal portion of the G protein is membrane-associated. The C-terminal portion can be isolated as a small CNBr peptide which is glycosylated and therefore presumably external to the envelope (KELLEY and EMERSON, 1975). Also, the N-terminal amino acid of both the protease-resistant fragment and the whole glycoprotein is alanine, which suggests that the protease-resistant fragment is located at the N-terminal end (SCHLOEMER and WAGNER, 1975). The above results suggest that only a small portion of the G protein molecule (10–20% maximum) is embedded in lipid and although this piece may be large enough to span the membrane and therefore contact internal proteins, there is presently no evidence that it does so.

It has been reported that the fluidity of the lipid bilayer of VS virus increases slightly after removal of the spikes with proteases and the suggestion was offered that the G protein of VS virus may penetrate the bilayer to a greater extent than the glycoproteins of certain other viruses such as influenza or Sindbis (LENARD and COMPANS, 1974). X-ray diffraction or NMR studies may provide needed information on the quantity and general arrangement of protein in the lipid bilayer. Such analyses should facilitate interpretation of the existing data. Clearly, much more information is required before the fine structure of the VS virus membrane can be resolved.

E. Immunology and Biochemical Composition of the Envelope Proteins

1. Glycoprotein

a) Immunology

The G protein is probably the most important viral protein from an immunological standpoint. Antibody to the G protein, but not to N or M proteins, will neutralize the infectivity of whole VS virions and vaccination of mice with purified G protein effectively protects them against intracerebral challenge with infective virions (DIETZSCHOLD et al., 1974). Presently, it is not known if the carbohydrate or protein portion of the G protein, or a combination of both, is responsible for the induction of neutralizing antibody. The antibody to G protein is type-specific and therefore forms the basis for division of VS virus isolates into serotypes (KANG and PREVEC, 1969). It is generally accepted that there are two major subgroups of VS virus: $VS_{Indiana}$ and $VS_{New\ Jersey}$. The VS_{Ind} subgroup presently contains four subtypes: Indiana, Argentina, Brazil, and Cocal (CRICK and BROWN, 1973; PRINGLE, 1975; CARTWRIGHT and BROWN, 1972b). The New Jersey subgroup contains only the New Jersey serotype.

b) Biochemical Analysis

The VS virus G protein provides a model system for studying glycosylation of proteins. Because VS virus has a tremendous host range, the glycosylation process can be studied in a variety of cell lines. For example, such studies could potentially involve analysis of the effect of glycosylation on glycoprotein extrusion, the cellular locations where glycosylation occurs, or the specificity of the glycosylation reactions. Elucidation of the basic structure of the G protein, which is a prerequisite for the above studies, has already begun.

Because the VS virus does not contain sufficient genetic information to code for all of the required glycosylation enzymes, it has been assumed that the oligosaccharides of the G protein are synthesized via host transferases (BURGE and HUANG, 1970). Indirect evidence for host cell transferase involvement in glycosylation of G protein was obtained by comparing the carbohydrate portions of the G protein purified from VS_{Ind} virions grown in different cell lines. Since the expression of the viral genes should be constant, regardless of the cell type, any differences in this case should reflect host cell modifications.

ETCHISON and HOLLAND (1974b) compared the overall carbohydrate composition of the G proteins derived from VS_{Ind} virions grown in four different cell lines (BHK 21, HeLa, L929, and MDCK). In all four cases the G protein was 9–10% carbohydrate by weight. Superficially the sugars of all the G proteins were similar but they were clearly not identical. N-acetyl glucosamine was the most prevalent sugar in all cases (7.62–10.2 residues per G molecule), mannose was the next most frequent (7.31–8.02), then galactose (5.25–6.26), N-acetyl neuraminic acid (4.65–4.96), and N-acetyl galactosamine (0.67–1.85);

fucose was absent in the G protein derived from L cell produced virions (ETCHISON and HOLLAND, 1974a), but low concentrations were detected in the remaining three samples (0.85–1.83). Mosquito cells reportedly do not synthesize neuraminic acid and the G protein from VS virions grown in mosquito cells do not contain this sugar (SCHLOEMER and WAGNER, 1975b). The sugar compositions determined chemically are consistent with those implied by lectin studies; VS virions grown in BHK 21 cells are agglutinated strongly by plant lectins which bind specifically to galactose or N-acetyl glucosamine, weakly by those which bind to mannose, and are not agglutinated by those which bind N-acetyl galactosamine of fucose (PENHOET et al., 1974). Although these methods do not provide information about the importance or specificity of glycosylation, the results do suggest that virions produced in different cell lines contain some non-identical oligosaccharides.

A more detailed analysis of the structure of the G protein should be forthcoming. A technique currently in vogue involves Pronase digestion and sizing of the released oligosaccharides by subsequent gel sieve chromatography on Biogel P-10. The oligosaccharides released from the G protein of L cell grown virus chromatograph as a single peak estimated to correspond to carbohydrate chains of 4000 daltons (MCSHARRY and WAGNER, 1971b). A similar peak containing carbohydrate of approximately 3750 daltons has been detected in Pronase-digested G protein from both chick and Chinese hamster ovary cells (BURGE and HUANG, 1970) and carbohydrate chains with an estimated molecular weight of 3000–3500 daltons were isolated from the G proteins of VS virions grown in BHK-21, HeLa, L929, and MDCK cells (ETCHISON and HOLLAND, 1974b). Therefore, the Biogel P-10 elution profile of G protein oligosaccharides is basically similar, regardless of the cell line the virus was grown in; that is, a peak which elutes with an assumed value of 3000–4000 daltons relative to a fetuin marker is consistently detected.

Although the oligosaccharides elute in similar positions, they may still have different compositions. MOYER and SUMMERS (1974b) used endoglycosidase digestion to compare the carbohydrate sequences from the G protein glycosylated by BHK or polyoma-transformed BHK cells. The G protein was digested with pronase and the released oligosaccharides were purified on Sephadex G-50 columns. Although the carbohydrate chains from both preparations elute from the column in the same position, different oligosaccharides were detected in the two samples after endoglycosidase digestion and high voltage electrophoresis. The investigators concluded that the internal sequences of some of the oligosaccharides were different in the two viral G proteins and that the G protein from virus grown in the transformed cells contained an increased content of sialic acid compared to that from the non-transformed. They postulate a basic sugar backbone for the BHK cell glycosylated G protein of (NANA)n-(Man)n-Fuc-GlcNac → peptide, but also state that some heterogeneity exists.

According to the measurements of ETCHISON and HOLLAND (1974b), the G protein is 10% carbohydrate by weight, or ~6700 daltons, and the oligo-

saccharides are 3000–3400 daltons each; they therefore calculate that there are two oligosaccharides per G protein. These calculations assume that all oligosaccharides will chromatograph on Biogel columns in a position which is directly proportional to their molecular weight. This assumption may not be valid, for these results do not agree with those obtained by the more direct analyses discussed below.

KELLEY and EMERSON (1975) determined the minimal number of carbohydrate chains per G protein by cyanogen bromide peptide map analysis of G protein from BHK grown virus. They were able to demonstrate the attachment of carbohydrate to four different peptides, hence there are at least four oligosaccharides present on each G protein molecule. GRUBMAN et al. (1974) isolated the G protein from HeLa cell grown virions and digested the molecule with trypsin. They were able to resolve six glycosylated tryptic peptides by high voltage electrophoresis. These reports suggest that there are at least four to six oligosaccharides per G protein, but they do not resolve the question of whether the sugar polymers are a heterogeneous or a homogeneous population. It should now be possible to sequence the individual carbohydrate chains by methods such as endoglycosidase digestion in order to determine this.

c) Composition of the G Protein from $VS_{\text{New Jersey}}$

Very little is known about the biochemical composition of the G protein from VS_{NJ}. MCSHARRY and WAGNER (1971b) compared the total sugar composition (glycoprotein plus glycolipids) of VS_{NJ} and VS_{Ind} virions grown in L cells and found them to be virtually identical. KELLEY and EMERSON (1975) compared the glycoproteins from these two serotypes by CNBr peptide mapping and presented preliminary evidence that the distribution of carbohydrate chains was similar. Much more extensive studies are required to determine whether the oligosaccharides of VS_{NJ} and VS_{Ind} glycoproteins are identical if the two serotypes are grown in the same cell line. Since the G proteins from the two serotypes are not immunologically cross-reactive, possession of identical oligosaccharides would suggest that the carbohydrate portion of the molecule is not antigenic.

2. M Protein

a) Immunology

CARTWRIGHT et al. (1970b) were the first to demonstrate that the M protein was antigenic. Antibody prepared against whole virions precipitates M protein in a standard Ouchterlony assay but the reaction is difficult to verify because the G and M precipitin bands are superimposed. Unequivocal identification of the M protein as an antigen was provided when DIETZSCHOLD et al. (1974) purified the M protein and raised monospecific antibody against it. This anti-M serum would not neutralize the infectivity of virions but would react specifically with M protein in either Ouchterlony or complement fixation

assays. Immunological comparisons of the M protein from the different VS virus serotypes have not been done.

b) Biochemistry

The M protein is the least studied of the VS virus proteins and basically nothing is known about its biochemistry.

F. Functions of the G and M Proteins

Since the G protein forms the spikes or surface projections, it has the potential to serve as the receptor by which the virus attaches to cells. High concentrations of VS virions are able to hemagglutinate goose erythrocytes under stringently controlled conditions of temperature and pH (ARSTILA, 1972; ARSTILA et al., 1969). ARSTILA (1973) released the hemagglutinin from virions with NP 40, purified it, and identified it as aggregated G protein. SCHLOEMER and WAGNER (1975a) were able to drastically decrease the hemagglutinating capacity of VS virions by selectively removing sialic acid with neuraminidase. Since hemagglutination by VS virus is specific for goose erythrocytes. this particular event is probably not of physiological importance. However, these experiments do suggest that virus–cell contact occurs via the glycoprotein.

SCHLOEMER and WAGNER (1974) have studied the relationship of the G protein to cell adsorption during the normal infectious process. They confirmed that trypsinized virions lacking G protein were not infectious (CARTWRIGHT et al., 1970b). In addition, they discovered that treatment of virions with neuraminidase decreased infectivity by 99% and inhibited virus adsorption to cell monolayers. Infectivity of these desialylated virions could be partially restored by *in vitro* resialylation (SCHLOEMER and WAGNER, 1975a). Virions lacking sialic acid can also be obtained by growing VS virus in mosquito cells which do not synthesize sialic acid; such virions are characterized by a very high ratio of particles to plaque-forming units and a very low hemagglutination titer (SCHLOEMER and WAGNER, 1975b). *In vitro* sialylation of mosquito-grown virions increased both infectivity and hemagglutination titer. Therefore, it appears that the G protein mediates adsorption of the virions to the host cell and sialic acids play a major role in attachment. The complementary adsorption site on the host cell has not been identified but since VS virus exhibits such a wide host range, this receptor must be a ubiquitous molecular species or, alternatively, many different cell molecules may be able to interact with the G protein.

ZÁVADA (1972) has constructed pseudotypes of vesicular stomatitis virus which contain the genome of VS virus and the glycoprotein of avian myeloblastosis virus (AMV) instead of the VS virus G protein. This particular pseudotype mimics the host range of AMV; that is, it will plaque in chick but not in hamster cells. Since VS virus will grow well in both of these cell types, this result implies that the G protein normally determines the host range.

The G protein is also involved in virus budding. Newly synthesized G and M proteins are inserted into the cell plasma membrane and it is likely that the cell membrane must be altered by the two viral envelope proteins before the nucleocapsids can recognize a potential envelope and bud out. Electron microscopy studies with ferritin hybrid antibody indicate that viral antigen, presumably G protein, is distributed over the entire cell surface as well as on the envelope of budding virions (WAGNER et al., 1971).

LAFAY (1974) has followed the association of membrane and the G protein of ts mutants from complementation group V (see PRINGLE, 1975). Interaction of G protein with cell membrane was monitored by labeling the viral proteins with radioactive amino acids, Dounce homogenizing the infected cells and partitioning the cell extracts on sucrose density gradients. Four bands of particulate matter can be separated by this method; the lightest band contains smooth membrane, the next band contains endoplasmic reticulum, the third band contains nucleocapsids and the heaviest band contains rough endoplasmic reticulum and free ribosomes (WAGNER et al., 1972a; CALIGUIRI and TAMM, 1970). During a pulse-chase experiment, the G protein synthesized by wild type virus appears first in the heaviest band, then is chased into the smooth membrane fraction and finally accumulates in the released virions. Ts mutants of group V at non-permissive temperature did not show this migration of G protein to the smooth membrane fraction and did not release mature virions. If the infected culture was then shifted down to the permissive temperature, the G protein synthesized at the non-permissive temperature was inserted into the smooth membrane and nucleocapsid budding occurred. These experiments provide a correlation between insertion of G protein into the smooth membrane and viral assembly.

Evidence from experiments with pseudotypes also indicates that glycoprotein is required for maturation. ZÁVADA and ZAVODSKÁ (1973/74) tested ts mutants from all five VS_{Ind} complementation groups in combination with AMV. Only group V mutants, the group LAFAY (1974) has characterized as G mutants, were rescued by AMV at the restrictive temperature. The ts V45 (AMV) pseudotypes were neutralized by antisera to AMV but not to VS virus and displayed the host range and interference specificities of AMV. Therefore, this pseudotype incorporated glycoprotein synthesized exclusively by AMV. Since ts V45 virions do not normally mature at the non-permissive temperature and the VS virus G protein is not inserted into the membrane (LAFAY, 1974), these experiments suggest that an AMV glycoprotein can insert into the membrane and induce budding of the nucleocapsid. Other non-homologous viral glycoproteins may also perform the VS virus G protein functions. Thus, coinfection of cells with VS virus and paramyxoviruses (CHOPPIN and COMPANS, 1970), myxoviruses (ZÁVADA and ROSENBERGOVA, 1972), or murine and avian RNA tumor viruses (LOVE and WEISS, 1974; ZÁVADA, 1972) results in the production of infective pseudotypes consisting of VS virus with the glycoprotein of the co-infecting virus either partially or totally replacing the G protein. It is somewhat puzzling that combinations of VS_{Ind} and VS_{NJ} sero-

types do not complement (PRINGLE, 1975). Although a large number of non-related viral glycoproteins can replace the VS_{Ind} G protein, the more closely related glycoprotein from VS_{NJ} apparently can not. The reason for this anomaly is not all clear.

As the VS virus ts mutants in the remaining four complementation groups could not be rescued by co-infection with AMV at the non-permissive temperature, it must be that only the glycoproteins are interchangeable and the remaining four VS virus proteins are required for synthesis of infectious virions. It is especially noteworthy that the group III mutants, most probably having mutations in the M protein (LAFAY, 1974), could not be rescued with AMV. This implies that if G and M proteins do interact, as suggested earlier, either AMV glycoprotein can also recognize VS virus M protein or AMV provides both a glycoprotein and an M-like protein. In this case two or more matrix proteins should be present in the pseudotypes. Unfortunately, the protein composition of the pure pseudotypes was not determined.

MCSHARRY et al. (1971) determined the protein composition of VSV(SV5) mixed pseudotypes which contain both VS virus and SV5 glycoproteins. SV5 has two glycoproteins and both were components of the pseudotypes; however, no protein corresponding to the matrix protein of SV5 was detected. All five of the VS virus proteins were present. Thus, either the SV5 glycoprotein can interact with the VS virus M protein, or the SV5 glycoproteins are totally independent of the M protein. Alternatively, since these mixed pseudotypes still contain a significant amount of VS virus G protein, it is possible that the SV5 glycoproteins are merely passengers and the G protein, although incorporated at reduced levels, is still able to provide its required functions.

It is not yet known if the M protein can be specifically inserted into the membrane in the absence of functional G protein, but the group V ts mutants (altered G protein) might provide the system for determining this. No M protein was observed in the smooth plasma membrane fraction of ts V mutants, so perhaps M protein remains soluble if G is not inserted. The soluble fraction was not analyzed in these experiments (LAFAY, 1974), however, so alternative interpretations are possible. Binding experiments involving the M protein will require cautious interpretation because evidence has been presented that some M protein sticks non-specifically to membranes under certain conditions (COHEN et al., 1971).

The sequence of events culminating in virus assembly and maturation is not very well defined. The M protein of wild type virus is not detected in the smooth membrane fraction of infected chick embryo cell lysates, perhaps because the M protein is rapidly incorporated into released virions (LAFAY, 1974; KANG and PREVEC, 1971). During a pulse-chase experiment, 80% of the labeled M protein was recovered in released virions after 20 minutes, but labeled G protein was not detected until much later (LAFAY, 1974). In Hela cells, it takes between 30 seconds and two minutes to chase a newly synthesized G or M molecule from the ribosomes into the plasma membrane (COHEN et al.,

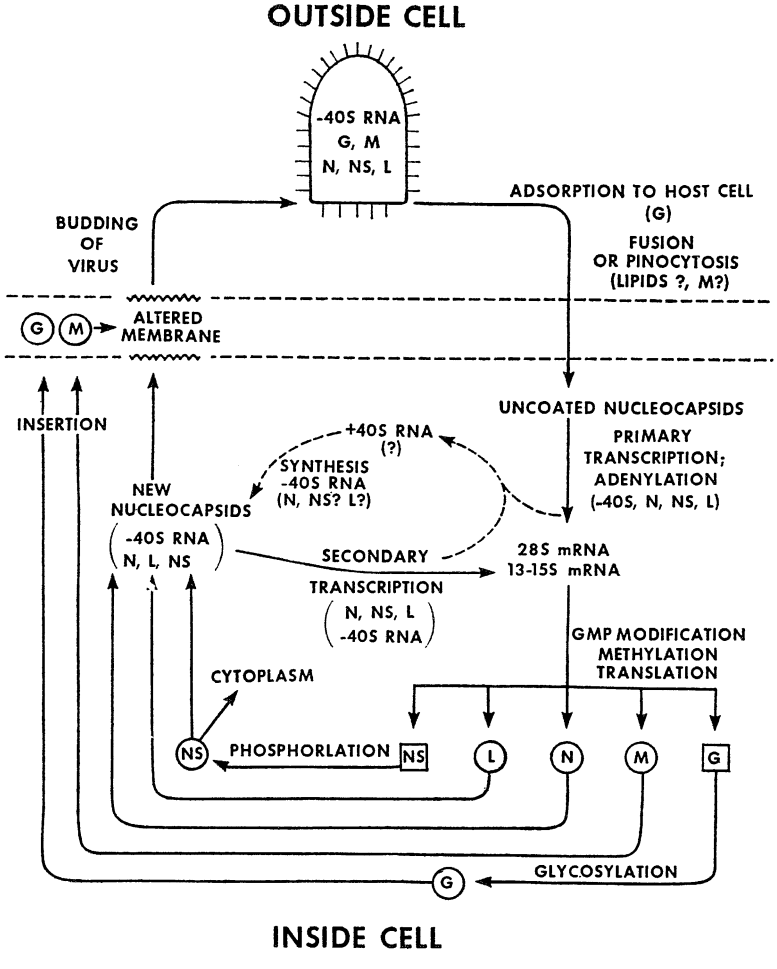

Fig. 3. Schematic representation of the probable infectious cycle of VS virus. Viral components required at each step are identified in parentheses where possible

1971; DAVID, 1973) while the amount of membrane-associated N protein gradually increases over a period between 20 to 60 minutes after labeling (COHEN et al., 1971). The concentration of M protein within an infected cell may vary according to the host cell since both ATKINSON and SUMMERS (1971) and WAGNER et al. (1972a) could detect M protein in the cytoplasm of infected HeLa and L cells respectively while CARTWRIGHT (1973) was unable to demonstrate it in infected BHK 21 cells.

CARTWRIGHT (1973) has fractionated infected cell homogenates on sucrose density gradients and assayed for the distribution of viral antigens by complement fixation. Since antibody specific for the nucleocapsid did not react with the membrane fractions, most of the nucleocapsids were not bound to membrane.

It is difficult to postulate an exact sequence for assembly of the nucleocapsid and envelope because the kinetics of maturation vary according to the host cell. However, an acceptable interpretation at this time seems to be that the nucleocapsid is assembled in the cytoplasm and remains there until the membrane is altered by the viral membrane proteins. This alteration requires the insertion of G protein into the smooth membrane and the subsequent insertion of M protein. Incorporation of M protein is exceedingly rapid and is probably the rate-limiting step in viral maturation. It is not known how or when the lipids are organized so that cellular proteins are excluded from the virion envelope. Once G and M are inserted into the membrane, the nucleocapsid recognizes the precursor envelope, or perhaps more specifically the inserted M protein, and immediately buds through the envelope complex and is released as a completed virion (Fig. 3).

III. Ribonucleocapsid

A. Virion RNA

Each VS virion contains one piece of single stranded RNA; since the viral RNA serves as the template for the synthesis of the monocistronic mRNAs, the virion RNA is complementary to the mRNAs (HUANG et al., 1970; MUDD and SUMMERS, 1970b). The RNA extracted from infectious particles has a molecular weight of approximately 3.6×10^6 daltons (estimates range between 3.0×10^6–4.4×10^6 daltons: SCHAFFER and SOERGEL, 1972; BISHOP and ROY, 1971a; HUANG and WAGNER, 1966; MUDD and SUMMERS, 1970a; NAKAI and HOWATSON, 1968; REPIK and BISHOP, 1973) while the RNA from the shorter DI particles is correspondingly smaller (HUANG and WAGNER, 1966; WEBER et al., 1974; LEAMNSON and REICHMAN, 1974; STAMMINGER and LAZZARINI, 1974) since they are in effect deletion mutants. As the plus strand of presumed replicative intermediates is also found in nucleocapsids (SORIA et al., 1974; HUANG and PALMA, 1974), it might be expected that the RNA in them could also be recovered in virions, but as a rule only the nucleocapsids containing the minus strand are packaged. One exception has been noted, however. The DI virions of the HR strain do contain a small amount of plus strand RNA but the reason for the decreased specificity of packaging is undefined (ROY et al., 1973). In contrast to the mRNA, the minus strand virion RNA is neither methylated (MOYER et al., 1975b) nor adenylated (EHRENFELD and SUMMERS, 1972; SORIA and HUANG, 1973; BANERJEE and RHODES, 1973).

Base analyses of the virion RNA indicate that the A to U and G to C ratios are approximately 1 (A $\cong$ 28%, U $\cong$ 30%, G $\cong$ 21%, C $\cong$ 21%) (MUDD and SUMMERS, 1970b; BROWN et al., 1967b). However, the equality of the A:U and G:C ratios does not reflect extensive base pairing because the virion RNA can be almost totally degraded with ribonuclease T_1 (BISHOP and ROY, 1971b). In addition, deproteinized virion RNA appears in electron micrographs as a single linear molecule about 3.1 μm long (WEBER et al., 1974); since stretched-

out nucleocapsids are 3.5 μm long (NAKAI and HOWATSON, 1968), it is clear that the RNA cannot be folded to any significant degree within the nucleocapsid.

According to hybridization analyses there is less than 10% exact sequence homology between the genomes of VS_{Ind}, VS_{Cocal}, and VS_{NJ} (REPIK et al., 1974).

B. Protein Components

Solubilization of the viral envelope by detergents such as deoxycholate (SZILÁGYI and URYVAYEV, 1973), releases the internal ribonucleoprotein complex which is composed of three viral proteins—L, N, and NS, and the viral RNA. This viral subparticle is infectious (SZILÁGYI and URYVAYEV, 1973) and synthesizes mRNA *in vitro* (BISHOP and ROY, 1972; EMERSON and WAGNER, 1972). All three of the proteins are normally protected from proteases by the envelope (CARTWRIGHT et al., 1970b; MCSHARRY et al., 1971) and they are not iodinated by lactoperoxidase to any substantial degree (MOORE et al., 1974). The RNA in the isolated ribonucleocapsids is inaccessible to ribonuclease (CARTWRIGHT et al., 1970a), apparently because the RNA is completely coated with protein (NAKAI and HOWATSON, 1968). NAKAI and HOWATSON (1968) measured the size of the subunits coating the RNA in electron micrographs and concluded that there are about 1000 protein subunits distributed along the length of the RNA. The N protein is the only protein present in the ribonucleocapsid in sufficient quantity to encapsidate the RNA. KANG and PREVEC (1969) reasoned that a protein having the dimensions described by NAKAI and HOWATSON (1968) (90 ×30 ×30 nm) would have a molecular weight of 63000; this figure is within the range of the estimate of 50000 daltons obtained for the N protein by SDS gel electrophoresis. In addition, nucleocapsids stripped of L and NS proteins by salt extraction are morphologically identical to those possessing L and NS proteins (EMERSON and WAGNER, 1972). Therefore neither L nor NS protein determines the gross structure of the ribonucleocapsid.

Ribonucleocapsids which lack envelopes can also be purified from the cytoplasm of infected cells; these intracellular ribonucleocapsids also contain L, NS, and N proteins (WAGNER et al., 1972a).

C. Biochemistry and Immunology of the Ribonucleocapsid Proteins

Technical difficulties have hindered the biochemical analysis of the ribonucleocapsid proteins; the L and NS proteins are present in such small amounts that it is difficult to obtain enough of either for biochemical studies, while the N protein is much more abundant but is extremely difficult to dissociate from the RNA. Since the N protein interacts directly with the RNA it might be expected to be a basic protein, yet the N protein is not preferentially labeled with arginine or lysine relative to the other virion proteins (WAGNER et al., 1970). Of course, this result merely indicates that the average content of

lysine and arginine is similar in all the virion proteins; the N protein may well have regions rich in these amino acids which are responsible for binding the N protein to the RNA. The N protein in ribonucleocapsids does not react with trinitrobenzenesulfonate, an amino group reagent, and a possible interpretation of this result is that the lysine residues are not available to the reagent because they are sequestered at the nucleic acid binding site (R. HUNT, personal communication).

The N protein is one of the major viral antigens (CARTWRIGHT et al., 1970b; KANG and PREVEC, 1969). Antisera prepared to N protein do not neutralize intact virions but do neutralize the infectivity of nucleocapsids (DIETZSCHOLD et al., 1974). KANG and PREVEC (1969) have shown that antibody to VS_{Ind} N protein cross-reacts with the N protein from VS_{NJ}.

The L protein has not been characterized either biochemically or immunologically. An outstanding property of the L protein is its susceptibility to denaturation; at low ionic strengths, the L protein, even in dilute solutions, precipitates (EMERSON, unpublished observation).

The NS protein is of particular interest because it is a phosphoprotein (SOKOL and CLARK, 1973). VS_{Ind}, VS_{NJ}, and VS_{Cocal} rhabdoviruses all have NS proteins which are phosphorylated during the virus replicative cycle (IMBLUM and WAGNER, 1974; MOYER and SUMMERS, 1974a; SOKOL and CLARK, 1973). In addition, virion-derived NS protein can be further phosphorylated *in vitro* by incubation of virions with ATP (IMBLUM and WAGNER, 1974; MOYER and SUMMERS, 1974a; STRAND and AUGUST, 1971), since the virions contain a kinase (STRAND and AUGUST, 1971). This kinase is of cellular rather than viral origin (IMBLUM and WAGNER, 1974) and is found in DI particles as well as in infectious particles (SOKOL and CLARK, 1973). MOYER and SUMMERS (1974a) demonstrated that the phosphates added *in vitro* differ from those added *in vivo* in that the latter are not hydrolyzed by alkaline phosphatase while the former are partially removed by the enzyme. *In vivo* only NS protein is phosphorylated while *in vitro* other viral proteins, especially M protein, are also phosphorylated (MOYER and SUMMERS, 1974a; STRAND and AUGUST, 1971). Since the specificity of the *in vitro* phosphorylation differs from that observed *in vivo*, it may be that the virion-associated kinase is not important to the virus. SOKOL et al. (1974b) performed partial acid hydrolysis of *in vivo* phosphorylated NS protein and found that both serine and threonine were phosphorylated although phosphothreonine, and a third unidentified component, which was not phosphohistidine, were synthesized. No peptide map comparisons have been done yet to determine whether cytoplasmic and virion NS proteins are phosphorylated to the same extent or at identical sites.

IMBLUM and WAGNER (1975) partially purified NS protein from the cytoplasm of L cells infected with VS_{Ind} and raised antibody against NS protein. This antiserum precipitates both cytoplasmic and virion NS proteins, indicating that the two proteins are similar. The antibody also terminates *in vitro* transcription of cytoplasmic or virion-derived nucleocapsids from VS_{Ind} but not from VS_{NJ}.

D. Functions of Ribonucleocapsid Proteins

The transcriptase of VS virus is of special interest since RNA synthesis proceeds from a single stranded RNA template rather than the double stranded DNA most viruses or other organisms employ. Nucleocapsids which lack G and M proteins can transcribe RNA *in vitro*, so the polymerase is a component of the nucleocapsid (BISHOP and ROY, 1972; SZILÁGYI and URYVAYEV, 1973; EMERSON and WAGNER, 1972; MURPHY and LAZZARINI, 1974; VILLARREAL and HOLLAND, 1974). The entire genome of VS_{Ind} infectious particles is transcribed *in vitro*, so theoretically it should be possible to study transcription of all genes as well as any transcriptional regulation that may occur (BISHOP, 1971; MOYER and BANERJEE, 1975a). Similar results have been obtained with the New Jersey serotype of VS virus (PERRAULT and HOLLAND, 1972). Originally, it was reported that the smaller DI particles did not transcribe RNA *in vitro* (EMERSON and WAGNER, 1972; BISHOP and ROY, 1971b) or *in vivo* (HUANG and MANDERS, 1972; STAMPFER et al., 1969). However, MORI and HOWATSON (1973) detected substantial levels of transcriptase activity in their preparations of purified DI particles, and BISHOP and ROY (1971b) showed that DI particles of intermediate size were also able to transcribe RNA *in vitro*. It is now evident that DI particles can contain RNA corresponding to any region of the genome although each clone contains a homogeneous species of RNA (LEAMNSON and REICHMANN, 1974). Therefore the conflicting results from different laboratories probably reflect basic differences in the type of DI particles used. For example, the HOWATSON DI particle is longer than the other DI particles tested and spans a different region of the genome (LEAMNSON and REICHMANN, 1974). Subsequently, REICHMANN et al. (1974) detected very low levels of RNA synthesis in all DI particles but the RNA produced was extremely short (5S), contained poly A, and would not hybridize to virion RNA. Since this RNA does not anneal to virion RNA and is synthesized with different kinetics than the product from infective virions, it may not reflect actual transcription. DI particles do contain an active transcriptase, but the template of the DI particles is apparently not functional (EMERSON and WAGNER, 1972). It has also been reported that VS virions contain a specific inhibitor of the transcriptase (PERRAULT and KINGSBURY, 1974). However, there is no direct evidence at present to suggest that such an inhibitor regulates RNA synthesis.

TS mutants defective in transcription have been isolated (CAIRNS et al., 1972; WONG et al., 1972; COMBARD et al., 1974; SZILÁGYI and PRINGLE, 1972), indicating that viral proteins are required for transcription. This fact explains why viral RNA is not infectious (HUANG and WAGNER, 1966), for transcription is the first biosynthetic event which occurs in the viral replicative cycle. Since nucleocapsids are infectious, whatever viral proteins are required for transcription are included in this complex. The most pertinent question about the nucleocapsid, then, is which of the three nucleocapsid proteins, L, N, and NS, are required for transcription? This question has been asked by disso-

ciating virions into a supernatant or enzyme fraction which contains L, G, NS, and M proteins, and a pellet or template fraction which contains the viral RNA and N protein. Neither fraction alone synthesizes RNA in an *in vitro* transcription assay, but the activity is reconstituted when the supernatant is recombined with template prepared from infectious particles. Since the supernatant proteins are soluble, they can be further fractionated by standard methods and individually assayed for polymerase activity *in vitro* by reconstitution with template.

1. N Protein

Addition of phenol-purified virion RNA to an *in vitro* transcription system depresses the level of RNA synthesis (BISHOP and ROY, 1971a), suggesting that naked RNA may compete for the polymerase but is unable to serve as the template. EMERSON and WAGNER (1972) were unable to detect RNA synthesis when the enzyme fraction from wild type virions was mixed with virion RNA, so soluble enzyme will not utilize RNA as a template, even if there is no other competing template available. Therefore the N protein, which is the only protein unique to the RNA-protein complex, must be required for transcription. In this case, one would expect to generate a class of ts mutants altered in transcription which display a defective template. NGAN et al. (1974) have dissociated group IV ts mutants into enzyme and template fractions and have assayed these two fractions in combination with similarly prepared fractions from wild type virus. According to their analyses, the template fraction of group IV mutants is defective. This template contained both N and NS proteins. Since NS protein was also found in the enzyme fraction which reconstituted a wild type phenotype with the wild type template, the NS is probably not defective; therefore, the N protein is most likely responsible for the ts phenotype. This particular group IV mutant is able to complement group I transcription mutants both *in vitro* and *in vivo* (CAIRNS et al., 1972) so the two complementation groups clearly have different lesions.

Since the RNA is coated with N protein to the extent that it is protected from ribonucleases, it is not at all clear how the transcriptase gains access to the RNA bases. BISHOP and ROY (1972) could not detect solubilization of N protein nor its displacement to product RNA's during *in vitro* transcription. Of course, N protein may be only momentarily displaced as the polymerase advances, and could rebind to the RNA almost immediately afterwards. In practice the N protein is extremely difficult to dissociate from the RNA and remains attached even at high ionic strengths (EMERSON and YU, 1975) which solubilize the other ribonucleocapsid proteins.

The N protein is probably involved in replication as well as transcription since the 40S plus strand, which is the presumed template for replication, is found intracellularly in ribonucleocapsids rather than as naked RNA (SORIA et al., 1974; HUANG and PALMA, 1974).

Recently evidence has been obtained which indicates that the transcription and replication processes are interrelated. However, the data are extremely difficult to interpret and as of the present no unifying hypothesis has been put forth. The evidence linking replication and transcription derives mainly from studies with the ts mutants. Originally, two complementation groups, I and IV, were classified as RNA negative while a third group, II, contained one RNA negative and one RNA positive member (CAIRNS et al., 1972; FLAMAND, 1970; PRINGLE, 1970; SZILÁGYI and PRINGLE, 1972). UNGER and REICHMANN (1973) examined certain mutants for primary transcription, that is transcription by the input virion polymerase, and concluded that only group I mutants are truly defective in this process. However, other members of group I do appear capable of primary transcription (FLAMAND and BISHOP, 1973). Primary transcription could be detected at the non-permissive temperature with members of the other two groups, II and IV, but the level of RNA synthesis was low because replication, and hence, secondary transcription did not occur. Therefore, mutants in group II and IV are actually RNA positive. Unfortunately, further studies with the ts mutant have clouded the distinction between replication and transcription defective mutants. As the experiments reviewed below forcefully demonstrate, RNA synthesis in VS virus is a complex process.

Group I ts mutants which are unable to transcribe at the non-permissive temperature are also unable to replicate their RNA under these conditions (PRINTZE-ANÉ et al., 1972). Preincubation at the permissive temperature does not overcome the block in RNA synthesis which is observed at the higher temperature. On the basis of temperature shift experiments, PRINTZE-ANÉ et al. (1972) concluded that at least one viral protein serves a dual function and is required for both transcription and replication.

The replication—transcription story was further complicated by experiments of PERLMAN and HUANG (1973). They showed that ts 114, a group I mutant, will replicate but not transcribe at the non-permissive temperature if the infected cells are first preincubated at the permissive temperature; however, if protein synthesis is inhibited by cyclohexamide at the time of shift-up replication halts and surprisingly enough transcription resumes. Therefore, replication requires concomitant protein sythesis and there appears to be a delicate balance between transcription and replication; inhibition of protein synthesis shifts this balance towards transcription even in mutants which have transcription defects. From these experiments they postulate that the same core enzyme may function both as a transcriptase and a replicase with accessory proteins determining which function is selected. Since the templates for both transcription and replication are ribonucleocapsids rather than naked RNA, it would seem reasonable to utilize the same basic enzyme for both processes.

Similarly complicated results have been obtained using non ts mutants. WERTZ and LEVINE (1973) isolated large and small plaque mutants of VSV and showed that, in these mutants also, the balance between transcription

and replication could be perturbed by cyclohexamide addition so that increased transcription resulted.

Group IV mutants which appear to have a replication defect *in vivo* are also affected in transcription (COMBARD et al., 1974; NGAN et al., 1974; UNGER and REICHMANN, 1973). One group IV mutant synthesized a defective RNA at the non-permissive temperature which was smaller than normal transcripts. Therefore the transcription defect of this mutant apparently affects the quality more than the quantity of RNA synthesized (PRINTZE-ANÉ et al., 1972). COMBARD et al. (1974) also concluded that the group IV protein is needed for accurate transcription, especially of the large 28S mRNA. They could detect RNA synthesis at the non-permissive temperature but the level was generally depressed and the 28S mRNA was synthesized very inefficiently or not at all. The mutant protein of group IV viruses is rapidly but reversibly inactivated by high temperatures. One group IV mutant is able to synthesize 38S viral RNA (i.e., replicate) at the non-permissive temperature after a shift-up while, under similar conditions, another group IV mutant does not make 38S RNA but also does not transcribe, unless cyclohexamide is included. Both of these group IV mutants appear to have decreased intracellular levels of N protein. Experimentally, ts IV 111, which does not synthesize 38S viral RNA after the temperature shift-up, has very low levels of N protein, while ts IV 100, which continues to synthesize 38S RNA at the non-permissive temperature, also produces some N protein. On the basis of this, the authors suggest that there is a correlation between synthesis of N protein and viral RNA replication. Therefore, their data are compatible with those of NGAN et al. (1974), which indicate that the group IV defect is localized to the template, and hence may involve the N protein.

Replication may be even more complicated than indicated above, for there is some evidence that host factors may also play a role. For example, in one line of human lymphoblastoid cells, transcription but not replication occurs, while in another cell line, both processes take place (NOWAKOWSKI et al., 1973). OBIJESKI and SIMPSON (1974) have isolated host-restrictive mutants of VS virus in which primary transcription and viral protein synthesis are detected; however, because these mutants synthesize very little RNA in the non-permissive cells, further studies may well show that viral RNA replication is inhibited in these cells also.

2. L Protein

The L protein is also required for transcription (EMERSON and WAGNER 1973; EMERSON and YU, 1975). EMERSON and WAGNER (1973) purified the L protein by phosphocellulose chromatography and reconstituted the transcription system by mixing purified L protein and template. Although the template preparation contained predominantly N protein complexed to RNA, a low level of endogenous transcriptase activity remained associated with it.

However, addition of purified L protein to this template markedly increased transcription.

HUNT and WAGNER (1974) performed *in vitro* reconstitution experiments by mixing wild type template and enzyme fractions with the complementary fractions derived from group I mutants. Their results indicated that the transcription lesion in group I mutants was located in the enzyme fraction. Further *in vitro* transcription studies have demonstrated that the NS proteins of certain group I mutants display the wild type phenotype (HUNT et al., 1975). Since the NS protein is not temperature sensitive in these mutants, the L protein most probably is. All the evidence accumulated thus far supports this conclusion, but definitive proof for a defective L protein has not yet been obtained.

The group I mutants form the major class of ts mutants isolated from VS virus and a number of explanations are possible to account for the abundance of these mutants. The L protein falls within the size range of a number of polymerase molecules (TRAVERS, 1969; CHAMBERLIN, 1974) and therefore it seems reasonable to assume tentatively, even in the absence of hard data, that it is the catalytic unit or subunit of the polymerase. If the L protein forms the core of the polymerase, it could well be a participant in both transcription and replication, especially since the group I mutants (L protein mutants according to HUNT et al., 1975) are affected in both transcription and replication (see section 4a). As the central component in a bifunctional enzyme, the L protein might be extremely sensitive to alterations in its primary sequence, and mutations which are normally innocuous might prove detrimental. In addition, the L protein from wild type VS_{Ind} is already extremely labile (EMERSON and WAGNER, 1973) and that from Cocal is apparently even more so (BISHOP et al., 1974). Indeed, even the wild type enzyme shows a significant temperature sensitivity when assayed *in vitro* at the moderately high temperature of 39°C (HUNT and WAGNER, 1974). Since the L protein is so labile, virtually any mutation may be enough to destabilize it further so that it is non-functional at the non-permissive temperature.

3. NS Protein

The NS protein, also, is necessary for transcription (EMERSON and YU, 1975; IMBLUM and WAGNER, 1975). In the original experiments on dissociation and reconstitution of the *in vitro* transcription system, no requirement for NS protein could be demonstrated (EMERSON and WAGNER, 1973). However, in this work the template displayed a low level of endogenous activity and, therefore, presumably was still contaminated with traces of L protein and, in retrospect, larger quantities of NS protein. After further purification of virions to remove ribonuclease and more extensive purification of the template to eliminate residual L and NS proteins, it was possible to show that both L and NS proteins were required to reconstitute the *in vitro* transcription system (EMERSON and YU, 1975) (Fig. 4). The data obtained from the most highly

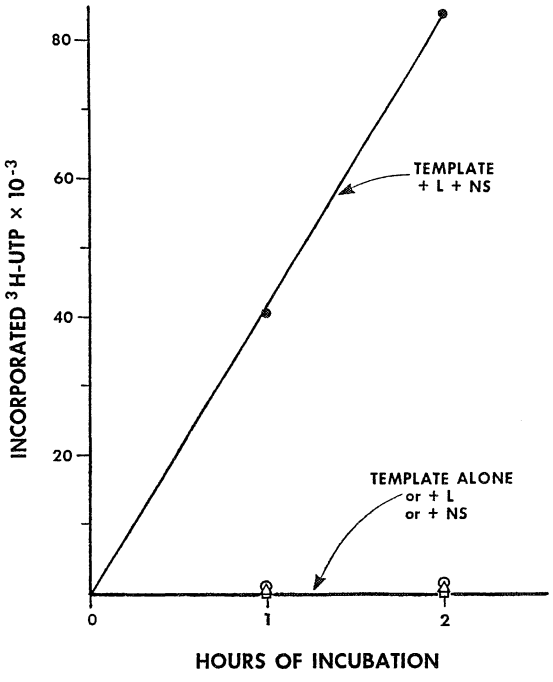

Fig. 4. Polymerase assay demonstrating that both L and NS proteins are required to reconstitute *in vitro* transcriptase activity. L and NS proteins of VS_{Ind} were solubilized and purified by column chromotography according to EMERSON and YU (1975). The template consisted of N protein complexed to virion RNA. Transcription was measured by the incorporation of [^{3}H]UTP into trichloroacetic-acid-insoluble material during incubation at 31° C. Samples assayed were template alone (▵), template plus L protein (○) template plus NS protein (□), and template plus both L and NS proteins (●)

purified preparations demonstrate that there is an absolute requirement for both the NS and L proteins.

IMBLUM and WAGNER (1975) found that antibody to NS protein terminated *in vitro* transcription immediately, regardless of when it was added. Therefore the functions NS protein performs are constantly required for transcription (i.e., elongation) or the NS protein is always associated with the polymerase, although it is not necessarily functional at all stages of transcription. Since the antibody which was raised to cytoplasmic NS protein inhibited the polymerase activity of purified virions, the cytoplasmic and virion NS proteins are antigenically similar. However, it is not known if the two classes of NS protein are identical. Cytoplasmic NS protein was able to replace virion NS protein in the *in vitro* reconstitution experiments (EMERSON and YU, 1975). However, it must be emphasized that these experiments were performed under conditions where virion NS protein was in excess. Since the specific activities of the cytoplasmic and NS proteins were not determined, it is possible that the majority of the cytoplasmic NS protein was inactive in the assay and only a subpopulation, corresponding to that present in the virion, was functional *in vitro*.

NS protein is very heat stable so it may be difficult to isolate ts mutants in which this protein is affected (EMERSON and YU, 1975). Even if such mutants exist, it may not be possible to design *in vitro* experiments which demonstrate the heat sensitivity of NS protein since the L protein, which is also needed, is extremely heat labile. Alternatively, the effects of a ts NS protein may be too subtle to detect by current methods. If group I contains L protein mutants and group IV contains N protein mutants, the group II mutants might be expected to have a defective NS protein. Unfortunately, only two mutants have been identified which belong to this group and neither has been studied.

One of the more interesting questions that can now be asked concerns whether or not phosphorylation affects NS protein functions. NS protein is partitioned within the cell into a soluble and a nucleocapsid-bound fraction. This compartmentalization could represent different functional roles of the two NS populations, active and inactive forms of the NS protein, or excess production of NS protein resulting in saturation of available nucleocapsid binding sites. It will be critical to compare cytoplasmic and virion-derived NS proteins by specific activities and parameters of phosphorylation to see first of all if they differ, and secondly, if the extent or sites of phosphorylation affect activity in the transcription assay.

As stated earlier, VS virions do contain a kinase which is capable of phosphorylating NS protein *in vitro*, although the specificity of the reaction differs from that observed *in vivo* (SOKOL et al., 1974b). WATANABE et al. (1974) have suggested that this virion kinase activates viral transcription. According to their kinetic measurements, the maximum rate of transcription was preceeded by a lag period during which phosphorylation occurred. They utilized two ATP analogues, dATP which is a substrate for the kinase but not the RNA polymerase, and AMPPcP which has the opposite substrate specificities. Only a small quantity of RNA synthesis was detected if only one of the analogues was present alone but transcription increased 5–6 fold if both analogues were included. From these results they suggest that the transcription rate is not maximal until the kinase has phosphorylated the proper component of the transcription system. Unfortunately, huge amounts of either analogue were required and the level of RNA synthesis was extremely low even under optimal conditions. Also, alternative interpretations of the lag period were not discussed. However, these results are very intriguing and it is hoped that they will soon be confirmed and extended.

E. The Nucleocapsid Complex

All three viral proteins associated with the nucleocapsid are obligatory components of the viral transcription system. However, since at least some non-transcribing DI particles contain all three proteins (KANG and PREVEC, 1971), clearly the mere presence of these is not sufficient to ensure that transcription will occur. A transcriptase can be demonstrated in DI particles if the enzyme fraction is assayed with the template from infectious rather than

DI particles (EMERSON and WAGNER, 1972). Therefore, either the proteins are incorrectly arranged in such DI nucleocapsids or the DI RNA itself can not be transcribed. For instance, the DI RNA could lack specific initiation sequences or other signals required for polymerase attachment. This hypothesis implies that the replication initiation site is distinct from that for transcription since DI particles replicate efficiently.

Genetic complementation studies and *in vitro* transcription experiments suggest that only proteins from very closely related viruses are interchangeable so the proteins comprising the nucleocapsid complex must interact in a very specific manner. Thus, there is no genetic complementation between VS_{NJ} and VS_{Ind} ts mutants while that between VS_{Ind} and the closely related VS_{Cocal} virus is limited to groups III and V which probably represent the envelope proteins (PRINGLE et al., 1971; PRINGLE and WUNNER, 1973; LAFAY, 1974).

Even though VS_{Cocal} is considered to form a subgroup of VS_{Ind}, it is unable to rescue group I ts mutants which probably contain a defective L protein (EMERSON and WAGNER, 1973; HUNT and WAGNER, 1974; HUNT et al., 1975). Contrary to this result, BISHOP et al. (1974) demonstrated *in vitro* complementation between VS_{Cocal} enzyme and VS_{Ind} template and vice versa in the reconstituted transcription system. However, the VS_{Ind} enzyme was much more active than that from VS_{Cocal}. No reconstitution could be demonstrated with the reciprocal combinations of enzymes and templates from VS_{NJ} and VS_{Ind}. In addition, BISHOP et al. (1974) were able to dissociate VS viruses into enzyme and template fractions and reconstitute infectious nucleocapsids. Although homologous combinations reconstituted infectious particles of VS_{Ind}, VS_{NJ}, and VS_{Cocal}, the only heterologous mixture which displayed infectivity was that of between VS_{Cocal} and VS_{Ind} and even in these cases the heterologous reconstitution was not as efficient as the homologous. These data predict that VS_{Cocal} should be able to complement transcription mutants of VS_{Ind}. Failure to detect *in vivo* complementation might reflect insensitivity of the assay or could arise from the inability of the heterologous enzyme to compete with the homologous enzyme for the template. For example, although VS_{Cocal} enzyme will utilize a VS_{Ind} template *in vitro* if that is the only option it has, it may have a much higher affinity for its own template; if this is so, the VS_{Cocal} enzyme might bind exclusively to its own template and thus would not show complementation under *in vivo* conditions. Alternatively, an active enzyme might result only if both the L and NS protein were from the same source. In this case, two individual proteins might have to associate independently with the heterologous template and the probability of this occurring at the right time and in the proper proportions might be low. It should be possible to isolate L and NS proteins from both the VS_{Cocal} and VS_{Ind} viruses and test each protein separately for the ability to reconstitute transcription in combination with the complementary proteins from the heterologous system. Such experiments should facilitate interpretation of the genetic data and might provide evidence as to how the proteins interact.

F. Polyadenylate Synthesis

The VS virus mRNAs isolated from polysomes of HeLa cells contain 13–20% of the adenosines in poly A tracts (EHRENFELD and SUMMERS, 1972). The 28S species of mRNA as well as the 13–15S contain poly A (SORIA and HUANG, 1973). In contrast, no poly A sequences of significant length could be detected in the virion RNA (EHRENFELD and SUMMERS, 1972; SORIA and HUANG, 1973; BANERJEE and RHODES, 1973). Poly A synthesis also occurs *in vitro* (BANERJEE and RHODES, 1973; VILLARREAL and HOLLAND, 1973; GALET and PREVEC, 1973). Viral nucleocapsids, cores, or purified virions synthesize RNA *in vitro* which contains up to 35% of the adenosines in poly A tracts which range from 50–200 nucleotides long. This poly A is covalently connected to the 3′ terminus of the mRNA. Poly A synthesis requires all four nucleoside triphosphates and is not inhibited by cordeycepin triphosphate (BANERJEE et al., 1974; EHRENFELD, 1974), an analogue which inhibits adenylation of cellular mRNAs. DI particles also synthesize poly A which is attached to short segments of RNA (REICHMANN et al., 1974). Exogenous viral RNA, transcripts, transfer RNA, poly (U) or oligo (A) could not serve as primers for poly A synthesis *in vitro* (BANERJEE et al., 1974). Along this same line it was shown that oligo (I), (G), (A), (C), and (U) are not adenylated in the presence of either Mg^{++} or Mn^{++} (MURPHY and LAZZARINI, 1974).

It appears that nucleocapsids purified from infected cells or derived by partial degradation of virions are virtually identical to each other and to intact virions in their transcribing and poly A synthesizing capabilities. In no case has it been possible to uncouple poly A synthesis and transcription. It is not known whether adenylation is a viral or host-specified function. Even if viral proteins were responsible for poly A synthesis, it is not obvious that ts mutants deficient in this process could be selected for since the function of poly A sequences, if indeed there is one, is not known.

The mechanism of poly A synthesis is undefined. No long tracts of poly U can be detected in the virion RNA (EHRENFELD, 1974; GALET and PREVEC, 1973), so it is unlikely that poly A is transcribed directly from corresponding poly U tracts in the template. Since poly A synthesis requires concomitant transcription, adenylation may be a function of the transcriptase itself. BANERJEE et al. (1974) have suggested that short tracts of poly U in the template signal the termination of transcription and extensive stretches of poly A are synthesized from short poly U sequences by a slippage mechanism. This explanation would obviously require that the replicase react differently to the putative poly U sequences than the transcriptase does. It is tempting to speculate that poly A synthesis is a means of regulating whether transcription or replication occurs. If poly A synthesis terminated transcription, inhibition of polyadenylation might allow synthesis of a single piece of RNA complementary to the entire genome, which could then serve as template for production of more negative strands. However, until more data are accumulated which define how adenylation occurs and which proteins are responsible, such a model cannot be tested.

G. Methylation of Transcripts

Nucleocapsids also contain enzymes which modify the transcripts by methylation (RHODES et al., 1974). Methylation requires de novo RNA synthesis but RNA synthesis can occur in the absence of methylation; that is, transcription proceeds even if the methyl donor; S-adenosyl-L-methionine is omitted from the *in vitro* reaction. Two different bases are methylated both *in vivo* or *in vitro* to generate a blocked 5′ terminal structure (ABRAHAM et al., 1975; MOYER et al., 1975b). One methyl group is attached to a guanosine which is linked through a 5′–5′ pyrophosphate bond to the original 5′ adenosine which begins the transcripts; the second methyl group is found on this adenosine. The negative strand of RNA is not methylated, however.

BOTH et al. (1975a) suggest that methylation of viral RNA normally occurs during the initiation step of translation and is required for *in vitro* translation. Since uninfected cells also contain methylase activity, the enzyme is probably a cellular constituent.

IV. Conclusion

The approximate locations of the virion components have been defined and certain functions have been assigned to various proteins. However, in spite of the monumental amount of data which has been collected recently, more questions remained unanswered than answered. For instance, the exact location of the M protein and its contribution to the integrity of the lipid bilayer has to be ascertained. The transcriptase molecule has yet to be defined. Are both L and NS proteins components of the polymerase? How many subunits constitute the polymerase? Which protein actually catalyzes the polymerization of nucleotides? What defines the initiation sites for transcription and replication? These and numerous other questions insure that the study of VS virus will be actively pursued for many years to come.

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Integration of DNA Tumor Virus Genomes

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With 5 Figures

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It has been known for some time that certain bacteriophage genomes, such as lambda, can be covalently integrated into the host bacterial chromosome. Of particular interest is the observation that this integration leads to lysogeny, a condition which affects the fate of both the phage and the host bacterium. In the lysogenic state, the integrated phage does not replicate independently but can express a certain portion of its genetic information. The host cell is also modified, becoming resistant to superinfection by the same bacteriophage strain; in the absence of phage replication, the bacteria does not undergo lysis but can continue to grow and replicate (HERSHEY, 1970).

Since the discovery of DNA tumor viruses, investigators have attempted to obtain experimental evidence for the analogy between lysogenic conversion and animal cell transformation. Many parallels have in fact been substantiated. It is clear that after inoculation with a tumor virus, the transformed host cells are significantly altered. They acquire a resistance to superinfection by

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intact virions and undergo a number of metabolic and morphologic changes. The most prominent of these are an increased growth rate, loss of contact inhibition, and the ability to persist indefinitely in serial subcultures (ENDERS, 1965; KOPROWSKI, 1966). For a number of reasons, however, it has been particularly difficult to demonstrate the covalent integration and evaluate the expression of integrated viral genomes in eukaryotic cells. Many of these problems have been purely technical. For example, while the *Escherichia coli* genome is about 200 times as large as that of phage lambda, the human cell contains approximately 2×10^6 times as much DNA as some transforming animal viruses. The task became one of developing methods sensitive enough to detect the "needle in the haystack".

I. Biological Considerations

The most direct approach for demonstrating the integration of viral DNA sequences within the host genome requires the use of carefully controlled hybridization analyses (see below). But even before such techniques were available, a number of biological observations suggested the presence and expression of certain viral genes in transformed cells. During the early phases of a productive infection of monkey kidney cells by SV40, one can detect the presence of a virus-specific intranuclear antigen (RAPP et al., 1964). This antigen appears to react specifically (in complement fixation or immunofluorescence tests) with the serum from animals bearing SV40-induced tumors and has been called tumor or T-antigen. The same antigen is also present in the nuclei of all SV40 transformed cells regardless of species, despite the absence of viral DNA replication or virus production in these cells (BLACK et al., 1963). Similar findings have been reported for polyoma and adenovirus infected and transformed cells (HABEL, 1965; HUEBNER et al., 1963). While there is no proof at present that T-antigen is virus-coded (that is directly specified by a viral mRNA), it is clear that the various T-antigens are virus-specific. The SV40, polyoma, and adenovirus T-antigens are found in normal cells and do not cross-react with one another. The presence of a transplantation antigen, presumably located on the surface of transformed cells (HABEL, 1961; COGGIN et al., 1969), is another example of the continuing effect of a virus on a cell which it transforms. While these antigens are suggestive of viral gene activity, they do not necessarily provide evidence for the stable association of the viral genome with the transformed cell DNA.

Convincing evidence for the continued presence of viral DNA in SV40 transformed cells was first obtained in a series of virus-rescue experiments. When virus-free, SV40 transformed cells were co-cultivated with permissive monkey kidney cells, small amounts of virus were released (GERBER, 1966). The virus thus obtained was propagated in green monkey cells and identified by its typical cytopathic effect, host range. antigenicity, and its sensitivity to neutralizing antisera. The induction of SV40 by co-cultivation was shown to

be greatly facilitated by the addition of inactivated Sendai virus, which causes the cells to fuse into heterokaryons (KOPROWSKI et al., 1967; WATKINS and DULBECCO, 1967). This strongly suggests that the mechanism of induction requires fusion of the virus-free transformed cell with the permissive cell. One might further speculate that a factor from the permissive cell activates the covert genome in the transformed cell. An analogous approach has recently been developed by BOYD and BUTEL (1972) in which high molecular weight DNA is isolated from transformed cells and inoculated into permissive monkey kidney cultures. The subsequent amplification of the induced virus by either of these methods in the permissive cell requires, of course, that it be non-defective and capable of replication. This is particularly important in view of the fact that certain SV40 transformed cell lines (MELNICK et al., 1964) and virtually all polyoma and adenovirus transformed cell lines are noninducible.

II. Methods for Detecting and Quantitating Integrated Viral Genomes

The earliest evidence that viral DNA was present in transformed mammalian cells was indirect at best. Several laboratories reported the existence of viral specific RNA in papovavirus transformed rodent cells (see below). The major obstacle hindering the detection and quantitation of integrated viral DNA was the large difference between the physical size of mammalian cell DNA and most animal viral genomes. If one assumes that a diploid quantity of mammalian DNA is 3.9×10^{12} daltons in size (SOBER, 1968), the ratio of cellular DNA to SV40 DNA (3.6×10^6 daltons) and adenovirus 2 DNA (22.9×10^6 daltons) is 1.08×10^6 and 1.70×10^5, respectively. Thus a 100 μg sample of transformed mouse DNA would contain 9.26×10^{-5} μg of SV40 DNA, assuming that only a single copy of viral DNA was present in each transformed cell. A technique with a sensitivity of nearly one part in 10^6 was clearly needed for the direct detection and quantitation of integrated viral DNA.

In 1968, WESTPHAL and DULBECCO, exploiting the filter hybridization technique described by GILLESPIE and SPIEGELMAN (1965), quantitated the amount of SV40 and polyoma DNA in a series of transformed cell lines. Their procedure was based on the hybridization of radiolabeled viral RNA (cRNA), synthesized in vitro with *E. coli* RNA polymerase, with large amounts of transformed cell DNA immobilized on nitrocellulose filters. The analysis consisted of three steps. In the first, radiolabeled cRNA was hybridized with uninfected, nontransformed cellular DNA. In the second, the labeled RNA was annealed to nontransformed cellular DNA and known amounts of unlabeled, purified, viral DNA. From such "reconstruction experiments", the amount of labeled RNA, capable of reacting with known amounts of viral DNA, could be determined. In the third step, the radiolabeled RNA was reacted with transformed cell DNA and, from the number of counts hybridized, the authors were able to determine the number of viral DNA equivalents in each line tested. Their results indicated that 3.5–29 copies of SV40 DNA per diploid mass of

cellular DNA were present in a series of mouse and hamster transformed cells[2]; 2.5–5 polyoma DNA equivalents per cell were also detected.

This procedure has been used extensively to monitor oncogenic viral genomes in transformed cells. There are, however, several inherent drawbacks to this method of analysis. First, a reconstruction or calibration experiment *must* be simultaneously carried out so that the hybridization of radiolabeled cRNA and transformed cell DNA can be "normalized" to a reaction with known amounts of viral DNA. Secondly, the labeled cRNA used in such studies must be a faithful and representative copy of the viral genome. Third, it has been suggested that the DNA component of fully duplexed DNA-RNA hybrid molecules can become detached from nitrocellulose filters under the conditions generally used for such experiments (greather than 60°C) (HAAS et al., 1972). As a consequence, the reaction of radiolabeled cRNA with purified viral DNA in the calibration experiment may not reflect the amount of RNA needed to react with a given quantity of DNA since a portion of the viral DNA falls off the filter subsequent to the formation of fully duplexed DNA-RNA hybrids. DNA-RNA hybrid molecules formed with cRNA and integrated viral DNA, on the other hand, would remain bound to the filter since adjacent host cell DNA sequences remain single-stranded throughout the course of the reaction. This may explain the significantly higher values of viral DNA equivalents per cell reported by those who have used this method rather than reassociation kinetics (see below) to quantitate integrated viral genetic information. An exception to this are the studies of LEVINE et al. (1970) who reported the presence of 2–9 copies of SV40 DNA in a series of transformed cell lines using a modification of the nitrocellulose filter procedure and the report of BOTCHAN et al. (1973).

A second approach used to quantitate the small amounts of viral DNA present in transformed cells involves an analysis of DNA reassociation kinetics. Reannealing of double-stranded DNA is a collision-dependent reaction that is directly proportional to the initial DNA concentration (C_0) and the time (t) of reassociation (BRITTEN and KOHNE, 1968; WETMUR and DAVIDSON, 1968). Assuming that a given DNA will reassociate twice as fast at a concentration of 2 μg/ml than at 1 μg/ml, GELB et al. (1971) demonstrated that the reassociation of highly radiolabeled SV40 DNA was exquisitely sensitive to the addition of unlabeled viral DNA. They showed that the concentration of viral DNA sequences (labeled and unlabeled) in a reaction mixture could be calculated directly by measuring the influence of known amounts of unlabeled SV40 DNA on the reassociation of a labeled DNA probe. SV40 transformed cell DNA likewise accelerated the reassociation ^{32}P-labeled SV40 DNA by an amount equivalent to 1–4 copies of viral DNA per diploid mass of cellular DNA (GELB et al., 1971) (Fig. 1). This procedure offered several important advan-

2 It has been customary to report the amount of viral DNA present in transformed cells per diploid mass (3.9×10^{12} daltons) of cellular DNA. The number of "copies per cell" reported in this review is based on this amount of host cell DNA even though some transformed and many revertant cell lines may contain significantly more DNA/cell.

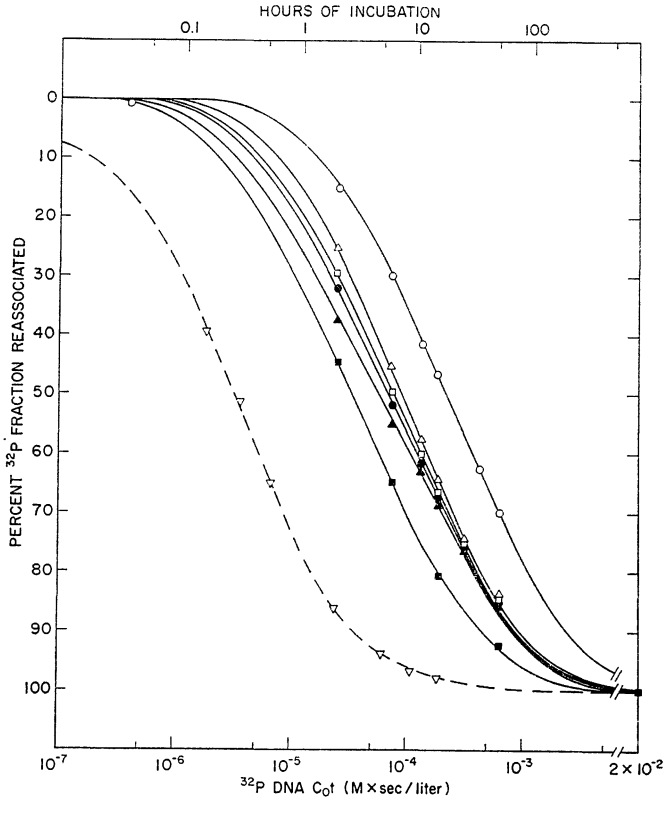

Fig. 1. Reassociation of ^{32}-P-labeled SV40 DNA in the presence of unlabeled virus transformed cell DNA. Reaction mixtures containing 2.5×10^{-5} O.D./ml. ^{32}P-labeled fragmented SV40 DNA (9.8×10^{5} cts/min/μg), 0.0025 M EDTA, 0.6 M sodium phosphate buffer and 46.6 0. D./ml. Sheared salmon sperm DNA (0), sheared SV-UV-15 clone 1 DNA (▵), sheared SV PY 3T3-11 DNA (▫), sheared SVT2 DNA (•), sheared SV40 hamster tumor DNA (▪) or sheared SV-UV-15 clone 5 DNA (▴), were heat-denatured and incubated at 68° C. Percentage of ^{32}P-labeled SV40 DNA in duplex molecules was determined on hydroxyapatite and the results plotted as a function of the ^{32}P-labeled SV40 DNA C_0t. Effect of 35 copies of SV40 DNA per diploid mass of cellular DNA (▿) was determined from a reconstruction experiment done in the presence of unlabeled green monkey DNA

tages over the DNA-RNA filter hybridization procedure described above. It not only dispensed with the need for an in vitro transcript of the viral genome, but eliminated the requirement for calibration or reconstruction experiments. The concentration of viral DNA sequences could be calculated directly provided the initial concentration of the radiolabeled probe was known. A major shortcoming of this method, however, becomes evident when only a portion of the viral genome is present in a transformed cell and the reassociation of radiolabeled viral DNA, representative of the entire viral genome, is used to quantitate viral DNA equivalents. This will be described in greater detail below (III. B.).

A third method that has been used to quantitate viral genetic material in transformed cells utilizes the separated strands of viral DNA (Khoury et al.,

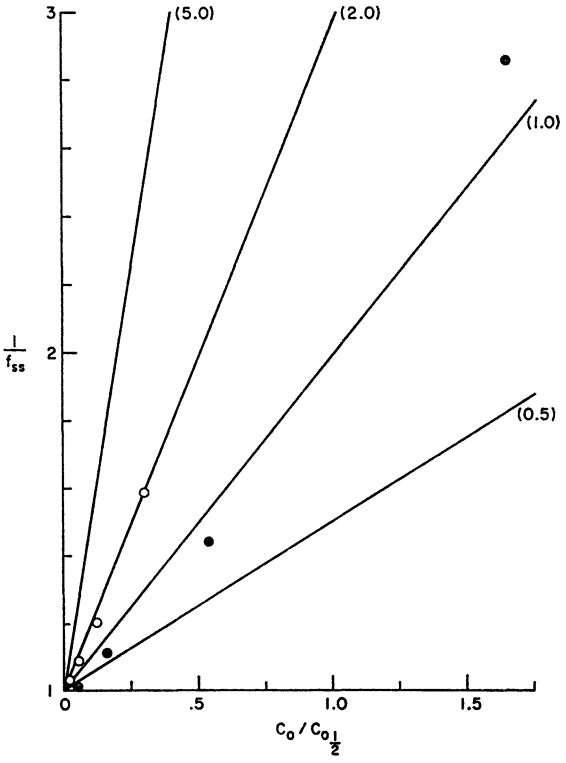

Fig. 2. A determination of copy number using the separated single strands of SV40 DNA as a probe. Reassociation of the ^{32}P-labeled plus strand fragments of SV40 DNA was examined in the presence of denatured unlabeled SV40 DNA or transformed cell DNA. Reaction mixtures containing 1 M NaCl, ^{32}P-labeled plus strands of SV40 DNA (2×10^{-3} μg/ml), and (1) unlabeled, fragmented SV40 DNA (6.4×10^{-5} to 2.1×10^{-1} μg/ml) plus unlabeled, fragmented salmon sperm DNA (•), or (2) unlabeled 11A8 SV40 transformed mouse cell DNA (0) (4.0×10^{-2} to 1.5 mg/ml) were incubated at 68° C for 40 hrs. This would be equivalent to 5 times the $C_0t^1/_2$, for the ^{32}P-labeled SV40 DNA employed. The fraction of the radiolabeled DNA remaining single-stranded (f_{ss}) was determined by hydroxyapatite chromatography. In the figure, $1/f_{ss}$ is plotted against $C_0/C_0{}^1/_2$, where C_0 is the concentration of viral-specific DNA in the unlabeled preparation and $C_0{}^1/_2$ is that amount of unlabeled viral DNA required for half the labeled single strand SV40 DNA probe to to be bound in duplex molecules at saturation (here, at $5 \times C_0t^1/_2$). Theoretical curves for 0.5, 1.0, 2.0, and 5.0 copies of SV40 DNA per diploid cell are shown. The experimental points shown by the open circles (0—0) are those obtained by adding increasing amounts of denatured, unlabeled 11A8 DNA to the ^{32}P-SV40 plus strands. The data fall on the theoretical line for 2 SV40 copies, in good agreement with previous results of 1.96 copies using reassociation kinetics (SMITH et al., 1972). For further details of this technique see KHOURY et al. (1974)

1974a). This approach is obviously limited to those viral DNAs from which separated DNA strands can be readily prepared, such as SV40. An advantage of this method over the DNA-RNA filter hybridization procedure is that the radiolabeled, separated DNA strands contain sequences as they are represented in the viral genome; the vagaries associated with in vitro RNA synthesis utilizing a bacterial polymerase are eliminated. At high ratios of unlabeled

viral or transformed cellular DNA to radiolabeled separated-strand DNA, one can also ascertain whether a portion, or the complete viral genome, is present in an unknown preparation. The quantitation of viral DNA equivalents in SV40 transformed cells by this method agrees quite well with values obtained by reassociation kinetics of a double-stranded DNA probe (Fig. 2).

III. Quantitation and Representation of Integrated Viral DNAs

Integrated viral genetic information can be detected by the methods outlined in the preceding section. Since infectious SV40 can be recovered from virus-free transformed cells by co-cultivation or fusion with permissive African green monkey cells (GERBER, 1966; KOPROWSKI et al., 1967; WATKINS and DULBECCO, 1967), it can be assumed that the entire viral genome is present in a latent form. Although it has been reported that polyoma can be rescued from a transformed rat cell line (FOGEL and SACHS, 1969), this appears to be the exception to the rule since all other attempts to recover this agent from hamster and mouse cells have been unsuccessful. Likewise, there has been no reported rescue of adenovirus from adenovirus-transformed cells. Whether the failure to recover virus reflects a lack of technical expertise or indicates that only a segment of the viral DNA is present in the transformed cell is currently under study in several laboratories. As will be point out below, it now appears that only a portion of adenovirus 2 is present in some transformed rat cell lines.

A. Papovaviruses

WESTPHAL and DULBECCO (1968), employing a DNA-RNA filter hybridization method, reported the presence of 3.5–22 SV40 genome equivalents per diploid mass of cellular DNA in three transformed mouse cell lines and 29 copies of viral DNA per cell in one hamster tumor (H-50) cell line (Table 1). LEVINE et al. (1970), using a similar analytic approach, found 2–5 copies of SV40 DNA per cell in three transformed hamster cell lines, 4 copies per cell in a virus-yielding, transformed mouse cell line and 9 copies per cell in the H-50 hamster tumor cell line examined by WESTPHAL and DULBECCO (1968) (Table 1). Low numbers of viral DNA equivalents were reported by GELB et al. (1971), who analyzed the influence of unlabeled transformed cell DNA on the reassociation of highly radiolabeled SV40 DNA. They found 1.5 copies of SV40 DNA per diploid mass cell DNA in two transformed mouse lines which yielded virus following fusion and 1 and 4 copies per cell in two lines from which no virus could be rescued (Table 1). Approximately two copies per cell were detected in a SV40 induced hamster tumor. OZANNE et al. (1973) have confirmed the low values for the two virus-yielding, transformed mouse lines using reassociation kinetics to quantitate viral genetic information and reported that two other transformed mouse cell lines contained about eight copies of SV40 DNA per diploid mass of cellular DNA (Table 1). OZANNE et al. (1973) examined four revertant lines for the presence of SV40 DNA and found similar number of

Table 1. Quantitation of SV40 genome equivalents in transformed cells

Cell line	Species	Phenotype	Virus rescued	*Copies* / Cell	Method[a] used	Reference
SV3T3-47	Mouse	Transformed	+	10	1	Westphal and Dulbecco (1968)
SV3T3-56	Mouse	Transformed	—	3.5	1	Westphal and Dulbecco (1968)
SVPy-3T3-11	Mouse	Transformed	+	22	1	Westphal and Dulbecco (1968)
SVPy-3T3-11	Mouse	Transformed	+	1.4	2	Gelb et al. (1971)
SVPy-3T3-11	Mouse	Transformed	+	1.3	2	Ozanne et al. (1973)
SVT2	Mouse	Transformed	+	1.6	2	Gelb et al. (1971)
SVT2	Mouse	Transformed	+	2.2	2	Ozanne et al. (1973)
SVT2	Mouse	Transformed	+	1.6	1	Botchan et al. (1973)
11A8	Mouse	Transformed	+	2.0	2	Smith et al. (1972)
11A8	Mouse	Transformed	+	2	3	Khoury et al. (1974)
SV3T3-3479	Mouse	Transformed	+	2	1	Levine et al. (1970)
SV101	Mouse	Transformed	+	8–9	2	Ozanne et al. (1973)
SV B30	Mouse	Transformed	+	6.1	2	Ozanne et al. (1973)
SV3T3-9	Mouse	Transformed	+	8–9	2	Ozanne et al. (1973)
SV3T3-9	Mouse	Transformed	+	9.5	1	Botchan et al. (1973)
SV UV15–1	Mouse	Transformed	—	1.0	2	Gelb et al. (1971)
SV UV15–5	Mouse	Transformed	—	3.9	2	Gelb et al. (1971)
H-50	Hamster	Transformed	—	29	1	Westphal and Dulbecco (1968)
H-50	Hamster	Transformed	—	4.5	1	Levine et al. (1970)
T-H-7	Hamster	Transformed	?	32	1	Tai and O'Brien (1969)
2675	Hamster	Transformed	+	2.5	1	Levine et al. (1970)
1808	Hamster	Transformed	—	1	1	Levine et al. (1970)
2673	Hamster	Transformed	—	1.5	1	Levine et al. (1970)
C1 21	Ch. Hamster	Transformed	?	10–15	1	Hirai and Defendi (1971)
C1 71	Ch. Hamster	Transformed	?	6	1	Haas et al. (1972)
F1SV101	Mouse	Revertant	+	8–9	2	Ozanne et al. (1973)
CA^{r}30.4	Mouse	Revertant	+	8–9	2	Ozanne et al. (1973)
CA^{r}32.6	Mouse	Revertant	+	8–9	2	Ozanne et al. (1973)
CA^{r}41.6	Mouse	Revertant	+	8–9	2	Ozanne et al. (1973)

[a] Quantitation of SV40 DNA was determined by DNA-RNA hybridization employing nitrocellulose filters (Method 1), reassociation kinetics with radiolabeled double-stranded viral DNA (Method 2), or reassociation with separated strands of viral DNA (Method 3).

copies of viral DNA per diploid mass of mammalian DNA as were detected in the parental transformed lines (Table 1).

Quantitation of polyoma viral DNA in transformed cells has been less extensive. Analysis has been mainly by the DNA-RNA filter hybridization method. Between 5 and 10 polyoma genome equivalents per diploid cell have been detected in transformed mouse and hamster cells (Table 2). As was observed with SV40, approximately similar amounts of viral DNA were found in revertants of polyoma transformed cells.

An examination of the data presented in Table 1 indicates that the number of copies of viral genetic information detected in a transformed cell is a function

Table 2. Quantitation of polyoma genome equivalents in transformed cells

Cell line	Species	Phenotype	*Polyoma DNA copies* / Diploid mass of cell DNA	Reference
Py-3T3-6	Mouse	Transformed	2.5	Westphal and Dulbecco (1968)
SVPy-3T3-47	Mouse	Transformed	5	Westphal and Dulbecco (1968)
Py8	Mouse	Transformed	3.5	Westphal and Dulbecco (1968)
H-polyoma	Hamster	Transformed	3–4	Shani et al. (1972)
LPT	Rat	Transformed (Inducible)	15–17	Manor et al. (1973)
Variant 1	Hamster	Revertant	3–4	Shani et al. (1972)
Variant 18	Hamster	Revertant	2	Shani et al. (1972)
Variant 19	Hamster	Revertant	2	Shani et al. (1972)

The number of viral DNA equivalents was determined by DNA-RNA hybridization employing radiolabeled complementary RNA synthesized in vitro.

of the method of analysis. In general, higher values were observed when the analysis was carried out by DNA-RNA hybridization. This is particularly striking with line SVPy-3T3-11. Twenty two viral DNA equivalents per diploid cell were detected by DNA-RNA hybridization while two different groups reported 1–2 copies of SV40 DNA by reassociation kinetics. The studies reported by LEVINE et al. (1970) and BOTCHAN et al. (1973), however, provide strong evidence that low values of viral DNA equivalents per cell can be obtained with the DNA-RNA hybridization method.

The findings summarized in Table 1 suggest that a single copy of SV40 DNA is sufficient to transform a cell. The presence of multiple copies of viral DNA in some virus-yielding transformed cell lines cannot be correlated with any phenotypic difference or with the amount or the type of virus-specific RNA present (KHOURY et al., 1973a; OZANNE et al., 1973). Cell lines which failed to yield virus following co-cultivation or cell fusion appear to contain amounts of SV40 DNA similar to those from which virus can be rescued. Finally, the data presented in Tables 1 and 2 indicate that revertants, which have many of the growth properties of nontransformed cells, have similar numbers of integrated viral genomes per diploid mass of cellular DNA as transformed cells. It should be pointed out, however, that many of these revertant lines contain subtetraploid amounts of cellular DNA so that the absolute number of viral DNA equivalents per cell is higher (OZANNE et al., 1973).

Since SV40 virions can be recovered from a majority of transformed cells, it is clear that all viral DNA sequences must be present. It had also been assumed until recently that all segments of the SV40 genome were represented equally in virus transformed cell lines. BOTCHAN et al. (1974), employing radiolabeled *Hpa* I cleavage fragments for reassociation experiments and the analytic approach described by SHARP et al. (1974) (which will be described in detail), found that specific portions of SV40 DNA are amplified in one trans-

Table 3. Quantitation of adenovirus genome equivalents in transformed cells

Adeno-virus	Cell line	Species	Method Used	Results	Reference
12	5524	Hamster	DNA-RNA hybridization	53–60 copies/cell	Green et al. (1970)
12	Tumor	Hamster	DNA-RNA hybridization	22 copies/cell	Green et al. (1970)
12	Trans-formed	Hamster	DNA-DNA reassociation kinetics	22 copies/cell	Green et al. (1972)
7	Tumor	Hamster	DNA-RNA hybridization	86–97 copies/cell	Green et al. (1970)
7	Tumor	Hamster	DNA-DNA reassociation kinetics	25–27 copies/cell	Green et al. (1972)
2	8625	Rat	DNA-RNA hybridization	37 copies/cell	Green et al. (1970)
2	8629	Rat	DNA-RNA hybridization	29 copies/cell	Green et al. (1970)
2	8638	Rat	DNA-RNA hybridization	14 copies/cell	Green et al. (1970)
2	8617	Rat	DNA-RNA hybridization	22–30 copies/cell	Green et al. (1970)
2	8617	Rat	DNA-DNA reassociation kinetics	5–7 copies/cell	Green et al. (1972)
2	8617	Rat	DNA-DNA reassociation kinetics	1 copy/cell	Pettersson et al. (1973)
2	8617	Rat	DNA-DNA reassociation kinetics employing R·*Eco*RI fragments of Ad-2 DNA	1.65 copies of R·*Eco* RI fragments C, D, E, and 40% of A/cell	Sharp et al. (1974)
2	F-17	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	3.5 copies of R·*Hpa* I fragments E and 50% of C/cell	Gallimore et al. (1974)
2	F-18	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	2.9 copies of R·*Hpa* I fragments E and 50% of C/cell	Gallimore et al. (1974)
2	F-19	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	4.9 copies of R·*Hpa* I fragments E and 50% of C/cell	Gallimore et al. (1974)
2	B1	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	6.0 copies of R·*Hpa* I fragments E and 50% of C/cell	Gallimore et al. (1974)
2	2T8	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	4.5 copies of R·*Hpa* I fragments E and 50% of C/cell	Gallimore et al. (1974)

Table 3. (continued)

Adeno-virus	Cell line	Species	Method Used	Results	Reference
2	2T4	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	6.9 copies of R·*Hpa* I fragments E and 50% of C/cell	Gallimore et al. (1974)
2	REM	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	0.3 to 6.37 copies/cell of all fragments ex cept *Hpa* I-A and *Eco* RI-F	Gallimore et al. (1974)
2	F4	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	3.0 to 16.3 copies/cell of sequences corresponding to *Eco* RI fragments A, B, and C only	Gallimore et al. (1974)
2	T2C4	Rat	DNA-DNA reassociation kinetics employing R·*Eco* RI and R·*Hpa* I fragments of Ad-2 DNA	0.4 to 6.9 copies/cell of sequences corresponding to the various *Hpa* I and *Eco* RI fragments of Ad-22 DNA	Gallimore et al. (1974)

formed cell line (SVT2). Specifically, they reported DNA segments encompassing predominantly the early region of the viral genome (*Hpa* I fragments C and A; 0.17 map units to 0.76 SV40 map units) are represented 5 to 8 times per diploid quantity of cell DNA; they found that sequences corresponding to *Hpa* I fragments D and B were present approximately 2 and 0.8 times. Previous reports have indicated that SV-T2 cells contain between 1.56 and 2.2 genome equivalents of SV40 DNA per diploid mass of cell DNA. The significance of the amplification of early viral genes is difficult to evaluate at present. However, it is known that the major species of stable, SV40 specific RNA in transformed cells is complementary to sequences on the minus strand of SV40 DNA extending from 0.17–0.66 map units (KHOURY et al., 1975). Why amplification of this portion of the SV40 genome occurs and whether a duplication of early viral genetic information is a common feature of all transformed cell lines is currently being evaluated.

B. Adenoviruses

GREEN et al. (1970) initially determined the number of viral DNA copies in adenovirus-transformed cells and virus-induced tumors by DNA-RNA filter hybridization. As shown in Table 3, they detected 14 to 30 copies per diploid mass of cellular DNA in Ad-2-transformed rat cells and significantly more in Ad-7 and Ad-12 transformed cells and tumors. By reassociation kinetics, GREEN (1972) subsequently found 5–7 Ad-2 DNA equivalents per cell in a transformed rat line (8617), 25–27 Ad-7 DNA equivalents per cell in a hamster

tumor and 22 Ad-12 DNA equivalents per cell in a transformed hamster cell (Table 3). PETTERSSON and SAMBROOK (1973) also examined two sublines of Ad-2 transformed line 8617 by reassociation kinetics and detected 1.00 viral DNA equivalent per diploid quantity of cell DNA (Table 3).

The advent of bacterial restriction endonucleases and the resultant specific cleavage products of viral DNA provided new tools to probe virus transformed cells. Since at least one entire SV 40 genome was present in SV40 transformed cells, it was generally assumed that the same was true for other virus-transformed cells. The failure to rescue adenovirus from transformed cells following cell fusion, for example, was ascribed to inadequate procedures for virus induction. SHARP et al. (1974) proved the fallacy in this notion when they showed that only 46 percent of Ad-2 DNA was present in the transformed rat line 8617. This study pointed out a major problem associated with the use of reassociation kinetics to quantitate viral DNA in transformed cells. When radiolabeled double-stranded DNA is allowed to reassociate in the presence of unlabeled DNA, which is homologous to only a portion of the probe, deviation from second-order kinetics occurs. The reassociation of a portion of the labeled DNA will be accelerated while the annealing of the remainder will be unaffected. The detection of such deviation from second order-kinetics is frequently difficult, particularly when less than half of the labeled probe is unaffected by the unlabeled viral DNA sequences and when insufficient molar excesses (less than 200 fold) of unlabeled DNA are employed (which usually is the case when transformed cell DNA is added). SHARP et al. (1974) first demonstrated that the reassociation of each of the six *Eco R1* fragments of Ad-2 DNA was predictably influenced by unlabeled Ad-2 DNA and then examined the effect of unlabeled transformed rat cell DNA (line 8617) on the reanealing of each DNA fragment. They found that the reassociation of *Eco* RI fragments B and F was not accelerated. The results of this study were consistent with the presence of 1.65 copies of *Eco* RI fragments C, D, E and approximately 40 percent of A per diploid mass of cell DNA. GALLIMORE et al. (1974), employing radiolabeled *Eco* R1 and *Hpa* I cleavage products of Ad-2 DNA, quantitated the viral DNA sequences present in 9 other transformed rat cell lines. Six of the transformed lines contained 3–7 copies of Ad-2 DNA sequences per cell, corresponding to *Hpa* I fragments E and about 50 percent of C (or about 14 percent of *Eco* RI fragment A). These sequences are topographically located at the left-hand end of the physical map of the Ad-2 genome. An analysis of the other three Ad-2 transformed cell lines indicated that varying amounts of other DNA segments, in addition to those located at the left end of the physical map, were present. It was concluded that no Ad-2 transformed cell line contained a complete set of viral DNA sequences. No correlation could be made between either the multiplicity of virus initially used to transform the rat cells or the tumorogenecity of resultant transformed cell and the amount and type of integrated Ad-2 DNA sequences. In Ad-2 transformed cells at least, as little as 14 percent of the viral genome (reiterated three times) is sufficient to maintain cells in the transformed state.

C. Herpesviruses

Experiments quantitating herpesvirus genetic information in mammalian cells have been difficult to interpret because the relationship between resident viral genomes and cellular DNA has still not been resolved. Most of the published reports in this area have dealt with the detection of EB viral DNA in biopsy material and in the Raji line of Burkitt lymphoma cells (PULVERTAFT, 1965). A major stumbling block has been the small amounts of EBV DNA that can be readily prepared. ZUR HAUSEN and SCHULTE-HOLTHAUSEN (1970), nonetheless, using DNA-DNA filter hybridization and DNA labeled in vivo, reported that Raji cells, which are negative for EBV virions and EBV antigens, contained 6 copies of EBV DNA per cell (Table 4). NONOYAMA and PAGANO (1971) also examined the DNA of the Raji cells by DNA-RNA hybridization with an RNA probe labeled in vitro and found 65 copies of EBV DNA per cell. Using the same approach, ZUR HAUSEN et al. (1972) later reported that Raji cells contained 50 EBV DNA equivalents per diploid amount of cellular DNA; a similar value (51 copies) was also obtained by an analysis of DNA reassociation kinetics (NONOYAMA and PAGANO, 1973). The low value (6 copies/cell), originally reported by ZUR HAUSEN and SCHULTE-HOLTHAUSEN (1970), most likely reflected the use of a DNA probe with a low specific activity and thefailure to completely saturate the EBV DNA sequences in the Raji cell DNA preparations.

EBV genetic information has also been detected in biopsies of patients with Burkitt lymphoma and carcinoma of the nasopharynx (Table 4). Since most of this material is positive for EBV capsid and early viral antigens, the

Table 4. Quantitation of herpesvirus DNA equivalents in transformed cells

Cell line	Source	Presence of Virus or Viral antigens	Method used	*DNA equivalents* / Diploid mass of cell DNA	Reference
Raji	Burkitt lymphoma	Negative for virus by EM and immunofluorescence	1	6	ZUR HAUSEN and SCHULTE-HOLTHAUSEN (1970)
Raji	Burkitt lymphoma	Negative for virus by EM and immunofluorescence	2	65	NONOYAMA and PAGANO (1971)
Raji	Burkitt lymphoma	Negative for virus by EM and immunofluorescence	2	50	ZUR HAUSEN et al. (1972)
Raji	Burkitt lymphoma	Negative for virus by EM and immunofluorescence	3	51	NONOYAMA and PAGANO (1973)
Biopsy	Burkitt lymphoma	Positive for virus	3	49	NONOYAMA and PAGANO (1973)
Biopsy	Burkitt lymphoma	Positive for virus	4	8.2	KAWAI et al. (1973)

Table 4 (continued)

Cell line	Source	Presence of Virus or Viral Antigens	Method used	*DNA equivalents* Diploid mass of cell DNA	Reference
Biopsy	Nasopharyngeal Ca	Positive for virus	3	19	NONOYAMA and PAGANO (1973)
Biopsy	Nasopharyngeal Ca	Positive for virus	4	13.5	KAWAI et al. (1973)
HKLY-1	Nasopharyngeal Ca	Negative for virus by EM and immunofluorescence	2	26	zur HAUSEN et al. (1972)
HKLY-2	Nasopharyngeal Ca	Negative for virus by EM and immunofluorescence	2	20	zur HAUSEN et al. (1972)
RPMI 6410	Chronic myeloblastic leukemia	Negative for virus by EM and immunofluorescence	2	45	zur HAUSEN et al. (1972)
RPMI 6470	Chronic myeloblastic leukemia	Negative for virus by EM and immunofluorescence	2	23	zur HAUSEN et al. (1972)
SK-L1	Monocytic leukemia	Negative for virus by EM and immunofluorescence	2	3–4	zur HAUSEN et al. (1972)
584	Hodgkin's disease	Negative for virus by EM and immunofluorescence	2	31	zur HAUSEN et al. (1972)
595	Hodgkin's disease	Negative for virus by EM and immunofluorescence	2	23	zur HAUSEN et al. (1972)
Leukocyte	Infectious mononucleosis	Negative for virus by EM and immunofluorescence	4	5.5–7.1	zur HAUSEN et al. (1972)
Leukocyte	Healthy donor	Negative for virus by EM and immunofluorescence	2	121	zur HAUSEN et al. (1972)
Tumor	Cervical Ca	Negative for virus by EM and immunofluorescence	5	3.5 copies of 39% of herpes simplex 2 DNA	FRENKEL et al. (1972)

The number of viral DNA equivalents was determined by DNA-DNA hybridization using nitrocellulose filters and DNA labeled *in vivo* (Method 1), DNA-RNA hybridization using nitrocellulose filters and RNA labeled *in vitro* (Method 2), DNA-DNA reassociation assayed on hydroxyapatite using labeled *in vitro* (Method 3), DNA-DNA reassociation assayed with S_1 nuclease using DNA labeled *in vitro* (Method 4), and DNA-DNA reassociation assayed with *N. crassa* nuclease using DNA labeled *in vitro* (Method 5).

biological significance of these findings is unclear. NONOYAMA and PAGANO (1973) reported 49 copies of EBV DNA per cell in one Burkitt lymphoma biopsy by DNA-DNA reassociation, monitored by hydroxylapatite chromatography; when DNA-DNA reassociation was assayed with the single-strand specific nuclease S1, 8.2 copies of EBV DNA per cell were found (KAWAI et al., 1973). Two lines of nasopharyngeal carcinoma cells, which were negative weor virus by electron microscopy and immunofluorescence (ZUR HAUSEN et al., 1972), were shown to contain 26 and 20 EBV DNA equivalents per cell, respectively. EBV DNA has also been detected in cells from patients with chronic myelocytic leukemia, chronic monocytic leukemia, Hodgkin's disease, infectious mononucleosis, and at least one healthy donor (ZUR HAUSEN et al., 1972). An analysis of one cervical carcinoma by DNA-DNA reassociation kinetics with a DNA probe labeled in vitro, indicated the presence of 3.5 copies of approximately 39 percent of herpes simplex virus type 2 (FRENKEL et al., 1972a). The relationship of this observation to cervical carcinoma in humans is not presently understood.

IV. Physical State of Cellular Associated Viral Genomes

While it is clear that viral DNA can be detected in several virus-free transformed cells (see preceding section), the physical relationship of the viral genome to host cell DNA has not been fully resolved. The viral DNA could exist as a separate genetic entity in the nucleus of transformed cells replicating in a controlled, yet independent manner, analogous to the bacteriophage P1-*E. coli* system. A second alternative, which is based on the well-studied relationship between bacteriophage lambda and *E. coli*, involvens the covalent attachment of viral DNA sequences to chromosomal DNA. While most published reports concerning the relationship of papova- and adenovirus DNAs to cellular DNA suggest that viral DNA is integrated into the cell genome, the definitive experiment (viz. one demonstrating the linkage of specific viral DNA sequences to sequences that are unequivocably cellular) remains to be performed.

A. Papovaviruses

The principal strategy employed in most studies examining the relationship between cellular and viral DNAs in transformed cells has been to demonstrate that viral DNA sequences are associated with high molecular weight DNA species when sedimendation behavior is analyzed under denaturating conditions. SAMBROOK et al. (1968) employed such a technique to examine the physical state of the SV40 genome in transformed mouse cells. With few exceptions, others have used the same approach to evaluate systems involving the same or different viral DNAs and their corresponding transformed cell DNA. SAMBROOK et al. (1968) were unable to detect significant amounts of SV40 DNA in transformed mouse cells which (a) existed in the supercoiled configuration, or (b) could be recovered in the Hirt supernatant. Viral DNA

was present in the Hirt pellet (HIRT, 1967) and was associated with the chromosomes of SV 3T3 cells. In a final experiment, SAMBROOK et al. (1968) gently lysed transformed cells on the top of an alkaline sucrose gradient and then, following centrifugation, detected SV40 DNA sequences associated with cellular DNA which sedimented at 110S. Under the conditions employed, the free supercoiled form of SV40 DNA sedimented at 53S. These experiments were interpreted as indicating that SV40 DNA is linked to cell DNA by alkali-stable covalent bands. SHANI et al. (1972), using similar methodology, reported that 97–98 percent of polyoma DNA, in one transformed hamster cell and in two revertant cell lines, was present in the Hirt pellet. Additionally, they detected polyoma DNA sequences in the band of cellular DNA (which sedimented greater than 100S) following centrifugation through alkaline sucrose. One polyoma transformed rat line (LPT), which spontaneously produces infectious virus, was examined in a similar fashion (MANOR et al., 1973). Approximately one quarter (6–9 genome equivalents) of the polyoma DNA detected in these cells was present in the high molecular weight cellular DNA fraction of an alkaline glycerol gradient and the remainder sedimented with free viral DNA.

Several groups have attempted to examine the association of papovavirus DNA with cellular DNA at various times following infection. HIRAI et al. (1971) reported an accumulation of SV40 DNA sequences in the Hirt pellet 10–15 hours after infection of Chinese hamster cells and a concomitant decrease in the amount detected in the Hirt supernatant. It should be pointed out that SV40 does not replicate in Chinese hamster cells and, therefore, progeny viral genomes would not interfere with this analysis. The amount of SV40 DNA appearing in the Hirt pellet following infection was not strikingly affected by the presence of cytosine arabinoside. SV40 DNA was also detected in the rapidly sedimenting fraction of cellular DNA 30 hours after infection.

The detection of viral DNA sequences associated with cellular DNA in productively infected cells is fraught with the added complication of discriminating between the relatively large amount of nonintegrated progeny viral genomes and viral DNA molecules covalently linked to cellular DNA. HIRAI and DEFENDI (1972) approached this problem by examining green monkey kidney cells infected with a low multiplicity (0.5 PFU/cell) of SV40. They reported the appearance of SV40 DNA in both the Hirt pellet and rapidly sedimenting (alkaline sucrose) fractions of infected cellular DNA commencing 20 hours post infection. HÖLZEL and SOKOL (1974) subsequently demonstrated that the methodology employed to assay integration of papovavirus genomes into cellular DNA, particularly during productive infection, is woefully inadequate. They showed that differential salt precipitation (HIRT, 1967) and alkaline sucrose sedimentation, alone or together, failed to discriminate between free and integrated viral DNA molecules. The purification of high molecular weight cell DNA, free of contaminating unintegrated viral DNA involved at least four steps: (1) Hirt fractionation; (2) velocity sedimentation through alkaline sucrose; (3) equilibrium centrifugation in CsCl-ethidium bromide; and (4) an additional velocity sedimentation in alkaline sucrose

(HÖLZEL and SOKOL, 1974). Using this multistep protocol, "integration" of SV40 during the lytic cycle was detected between 30 and 50 hours post infection; by 72 hours, more than 20000 copies per diploid amount of cell DNA were found.

RALPH and COLTER (1972), exploiting an approach originally described by DOERFLER (1970) for adenovirus, infected mouse embryo cells, previously grown in the presence of BUdR, with 40–50 PFU/cell of polyoma virus. Following fractionation of "heavy" and "light" DNA in CsCl equilibrium density gradients, 8–15 polyoma genome equivalents per cell were detected in the BUdR containing DNA, 14 hours following infection.

The results of these studies suggest that in established transformed cells, at least, papovavirus DNA is covalently linked to cellular DNA. The methods employed are crude at best and depend to a large degree on "reconstruction experiments" which demonstrate that added virus or virus DNA is separable from high molecular weight cellular DNA. In no case has a specific portion of a papovavirus genome been shown to be *chemically linked* to DNA sequences known to be cellular in a manner similar to that described for adeno-SV40 hybrid viral-genomes. The reported integration of viral genetic information in acutely infected cells is even more difficult to evaluate particularly in cells productively infected with virus. In most cases, the entrapment of free viral DNA molecules in the high molecular weight cellular DNA fraction has not been convincingly ruled out. The one study in which a rigorous, multistep procedure was used to purify large cellular DNA molecules indicated that integration occurred late in the lytic cycle near the time of maximal cytopathic effect (HÖLZEL and SOKOL, 1974). The published literature thus indicates that a class of papovavirus DNA sediments more rapidly than monomeric viral genomes following virus infection of both permissive and nonpermissive cells. Whether covalent linkage to cellular DNA has occurred or whether a hitherto undescribed "free" form of viral DNA has been synthesized remains to be determined.

B. Adenoviruses

The methods employed to demonstrate integration of adenovirus DNA into chromosomal DNA have, in general, not relied on centrifugation through alkaline gradients. It has been reported that similar amounts of highly radiolabeled adenovirus-specific complementary RNA, synthesized in vitro with *E. coli* RNA polymerase, react with the chromosomes and nuclei of Ad-12 transformed hamster cells (GREEN et al., 1970). The DNAs isolated from four different size classes of chromosomes appear to contain similar amounts of Ad-12 specific DNA sequences (GREEN et al., 1970).

The association of Ad-12 DNA with cell DNA was evaluated during the abortive infection of hamster cells by DOERFLER (1970). BHK-21 cells, propagated in the presence of 5-BUdR, were infected with Ad-12, labeled with ^{3}H-thymidine. Forty-two hours postinfection, a significant portion of the

^{3}H-radioactivity banded in the "heavy" region of a CsCl gradient and hybridized to both Ad-12 and cellular DNAs. Sonication of the preparation prior to isopycnic centrifugation resulted in a shift of the "heavy" DNA into bands of "intermediate and light" density. This was interpreted as indicating covalent linkage of Ad-12 DNA sequences to cellular DNA (DOERFLER, 1970). Inhibition of DNA synthesis with cytosine arabinsoide prior to or during infection did not appreciably affect the association of Ad-12 DNA with cellular DNA. An analysis of the high molecular weight cellular DNA by DNA-RNA hybridization suggested that nearly 300 Ad-12 genome equivalents per cell were integrated 28–53 hours after infection.

C. Herpesviruses

The demonstration of the covalent integration of herpesvirus DNA into cellular DNA has proven to be an elusive undertaking. Unlike the situation with the small papovavirus and adenovirus genomes, the high molecular weight of most herpesvirus DNAs (1×10^8 daltons) has made analysis of covalent linkage by centrifugation through alkaline gradients that much harder. Not only is it difficult to prepare cellular DNA which is significantly larger in size than the herpesvirus genome (which is a sine qua non of this approach) but the suggestion that some herpesvirus DNAs contain alkali labile bonds (KIEFF et al., 1971; FRENKEL and ROIZMAN, 1972) raises the serious question of whether this approach will yield meaningful information. While EBV DNA in nonproducer Raji cells has been shown to be associated with cell chromosomes, most of the detectable viral DNA had sedimentation properties, in alkaline sucrose, characteristic of nonintegrated viral genomes (NONOYAMA and PAGANO, 1972; ADAMS et al., 1973). Raji cell DNA has also been fractionated by several cycles of isopynic centrifugation in neutral CsCl, taking advantage of the buoyant density differences of EBV (1.718 g/cm^3) and cellular (1.700 g/cm^3) DNAs. EBV DNA in Raji cells has been shown to be associated with DNA having a density characteristic of cell DNA by DNA-RNA hybridization (ADAMS et al., 1973). The amount of viral DNA banding with cellular DNA is reduced if the preparation is mechanically fragmented prior to isopycnic centrifugation. (ADAMS et al., 1973). A recent analysis of the physical state of EBV DNA in nonproductively infected Raji cells indicated that viral DNA sediments separately from high molecular weight cellular DNA in neutral glycerol gradients (TANAKA and NONOYAMA, 1974). The EBV DNA isolated in such a manner was shown to have a density of 1.717 g/cm^3. The results of these studies, while suggesting that EBV DNA is not covalently linked to cellular DNA by alkali stable bonds, may require further evaluation. If, for example, discontinuities or labile regions are present in herpesvirus genomes, integration into the cell genome could never be demonstrated by the methods used for the papova and adenoviruses. Secondly, it must be kept in mind that large numbers of viral DNA equivalents have been reported to be present in Raji cells (see Table 4). If only 1 or 2 copies of the EBV genomes were integrated into cellular DNA in

the classical fashion, it is doubtful whether they would be detected using current technology. It is interesting to note that FRENKEL et al. (1972) were able to demonstrate an enrichment for herpes simplex virus-2 DNA when reiterated human cervical tumor DNA was examined. DNA, fractionated in such a manner, should contain no free viral genomes. This result, therefore, suggests that a portion of herpesvirus and cellular DNAs are covalently linked.

V. Transcription of the Integrated Viral Genome

While the presence of an integrated viral genome in a transformed cell provides no information concerning the role of the genome in the transformation process, recent studies with temperature-sensitive mutants of SV40 suggest that a viral gene product is necessary for the continued maintainance of the transformed state (MARTIN and CHOU, 1975; TEGTMEYER, 1975; BRUGGE and BUTEL, 1975; OSBORN and WEBER, 1975). If this is the case, one should be able to detect and characterize the virus-specified mRNA in transformed cells which codes for the necessary gene product.

A. Papovaviruses

In 1966 BENJAMIN demonstrated the presence of virus-specific RNA in SV40 and polyoma transformed cells. A number of subsequent studies confirmed and extended these findings. Competition hybridization experiments (ALONI et al., 1968; ODA and DULBECCO, 1968; SAUER and KIDWAI, 1968), summation hybridization studies (KHOURY et al., 1973a), and eventually, direct mapping experiments (KHOURY et al., 1973b, 1975; OZANNE et al., 1973; KAMEN et al., 1974) all indicated that the major RNA species synthesized in SV40 and polyoma transformed cell lines was essentially identical to the early virus-specific RNA. This observation suggests that if transcription occurs from an integrated template, the early gene sequences remain intact (i.e., the site for integration within the viral genome may occur in the late genes). Another set of virus specific RNA sequences present in lower concentrations were shown to map at the 5′ RNA end of the early RNA (KHOURY et al., 1975). Since RNA is synthesized in a 5′ to 3′ direction, this finding suggests that the recombinaiton site involved during integration of SV40 into host DNA may be located in the late viral genes, adjacent to the 3′ DNA end of the early gene region (0.66 map units). These results also suggest a "read through" from anti-late to early viral DNA sequences during transcription of the integrated viral genome (Fig. 3). However, until there is a better understanding of the primary transcription product and the extent of processing of mRNA, such conclusions are premature.

A number of studies of transcription in SV40 transformed cells have focused on the size of the virus-specific RNA (LINDBERG and DARNELL, 1970; TONEGAWA et al., 1970; MARTIN, 1970; WEINBERG, 1974). There seems to be general agreement that the SV40-specific nuclear RNA is heterogeneous, a portion of

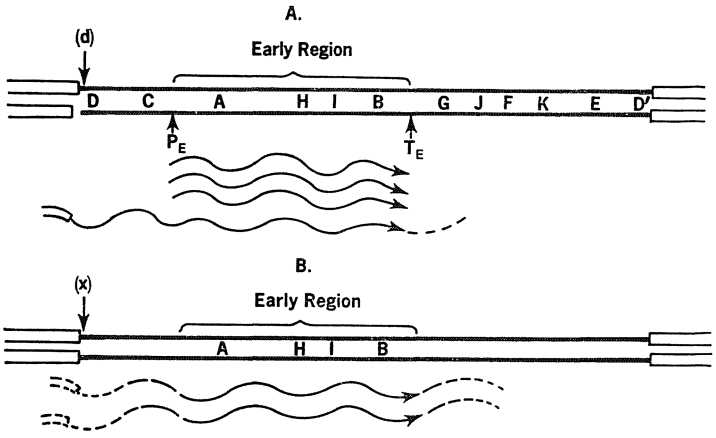

Fig. 3. Possible models for the transcription of SV40-specific sequences in transformed cells. Both models rely on the fact that the minus strand of SV40 DNA is expressed in transformed cells and that the direction of transcription on this strand is counterclockwise on the standard SV40 map. Integration of SV40 DNA in transformed cells most likely occurs with a break in the late SV40 genes. In model A, most rounds of transcription recognize the early viral promoter (P_E) and terminator (T_E). Occasionally, transcription is initiated within the host genome and continues through the viral genes to the viral terminator T_E. This latter event is responsible for the low levels of transcription of anti-late RNA sequences and would provide some indication (based on which anti-late sequences are present) about the site of cleavage within the viral genome. Alternatively, extensive regions of one or both DNA strands may be transcribed, as is the case during the lytic cycle (model B). Subsequent degradation of all but the early viral sequences would lead to their abundance. Incomplete degradation of the anti-late RNA might result in its detection at a lower concentration, and the localization of these sequences to the 5′ RNA side of the early region may indicate that a putative processing enzyme proceeds in that direction. If model B is correct, the integration site in the viral DNA may be anywhere within the late gene region. Since the evaluations of transcription in transformed cells have thus far employed total cellular RNA, we do not yet know if the anti-late RNA sequences are present in the cytoplasm of transformed cells. If they are not, the analogy to transcription and processing in the lytic system might lead one to favor a model similar to B. ▭, Host cell DNA; ▬ viral DNA; P_E and T_E, putative promoter and terminator for transcription of the early SV40 genes; — abundant viral RNA sequences; ---- scarce RNA sequences; A–K, *Hind* fragments of SV40 DNA; (d) a specific cleavage site or (×) a random cleavage site within the late SV40 genes for integration into transformed host cell DNA (From KHOURY et al., 1975)

which consists of large molecules (50–70S). Viral RNA sequences in the cytoplasm are considerably smaller (<26S). Although it has not been rigorously proven, it seems likely that these large nuclear SV40 specific RNA molecules are precursors to the cytoplasmic RNA. Furthermore, DARNELL and his colleagues have presented evidence that some of these high molecular weight virus specific molecules contain covalently linked SV40 and host RNA sequences (LINDBERG and DARNELL, 1970; WALL and DARNELL, 1972). If these studies are correct, they provide a strong argument for the co-transcription of covalently linked viral and host cell DNA sequences.

As will be described, there is abundant evidence for the recombination of viral and host cell DNAs during the lytic infection, particularly after infections

at high input multiplicities (LAVI and WINOCOUR, 1972; TAI et al., 1972; MARTIN et al., 1973). Whether such recombination is related to integration and whether it is in fact a necessary step during productive infection remains to be determined. Nevertheless, some of the best data for co-transcription of host and viral RNA sequences comes from studies of the SV40 lytic cycle. High molecular weight (HMW) RNA selected late in the lytic cycle was found to anneal specifically to SV40 DNA immobilized on filters. JAENISCH (1972) suggested that the RNAse-sensitive tails on these HMW molecules represented the presence of covalently linked host cellular and viral RNA sequences. A subsequent analysis by ROZENBLATT and WINOCOUR (1972) extended these findings by demonstrating that a population of HMW RNA molecules, isolated from a productive SV40 infection, could be annealed to SV40 DNA-containing filters, eluted, and reannealed to filters containing monkey kidney cell DNA.

Studies of the RNA sequences transcribed from the nondefective adenovirus-SV40 hybrid viruses provide further evidence for the co-transcription of host and integrated viral genomes. The findings of covalently linked Ad2 and SV40 RNA sequences (OXMAN et al., 1974) and the analysis of the SV40 portion of this message (KHOURY et al., 1973c), suggests that a specific strand orientation of integration exists within the hybrid virus.

Thus, the minus strand of SV40 is linked to the strand of Ad-2 DNA which is transcribed in the region of the integration site. This orientation allows for the transcription of the early SV40 mRNA rather than the "antisense" sequences.

B. Adenoviruses

The first detection of adenovirus specific-RNA in transformed cells was reported by FUGINAGA and GREEN (1966) using the DNA-RNA filter-hybridization technique. The adenovirus RNA present in transformed cells appeared to be a subset of the early virus-specific mRNA; the size distribution of nuclear adenovirus RNA was larger than that of the cytoplasmic virus-specific RNA fraction (WALL et al., 1973). The subsequent development of liquid-phase hybridization techniques using separated strands of restriction enzyme fragments of the adenovirus genome, has given a clearer picture of adenovirus DNA transcription. Using these methods, FLINT et al. (1975) analyzed the RNA sequences in five Ad-2 transformed cell lines and found that they represented limited and discrete segments of the genome. An Ad-2-specific RNA species common to all of the transformed cell lines examined was complementary to about 10 percent of the *l* strand sequences located at the left end of the physical map of the Ad-2 genome. In addition, RNA sequences, complentary to approximately 7 percent of the *h* strand at the right end of Ad-2-DNA, were detected in two transformed cell lines. These two segments of the Ad-2 genome represent portions of the early region of viral DNA. Unlike the expression of virus-specific RNA in SV40-transformed cells, however, only a portion of the early region of Ad-2-DNA is transcribed into stable mRNA in transformed cells. It should be noted that any virus-specified proteins in these

Ad-2 transformed cell lines must be encoded by one of the subsets of the early region of the genome.

C. Herpesviruses

Little data is available regarding the transcription of herpesvirus DNA (HSV-1 or HSV-2) in transformed cells. This has been due, in part, to (1) the unavailability, until recently, of virus-free transformed cells lines, and (2) the large size of the HSV genome. If HSV-transformed cells contain only a segment of the viral DNA, comparable in size to that found in adenovirus or SV40 transformed cells, or if only a limited region of the DNA is transcribed, an analysis of viral specific RNA sequences becomes a difficult undertaking. These obstacles notwithstanding, FRENKEL and her colleagues (personal communication) have detected limited amounts of HSV-specific RNA in certain hamster cell lines transformed by UV-irradiated HSV-2 (DUFF and RAPP, 1971).

An RNA transcript, corresponding to approximately 5 percent of the HSV-2 genome has been reported to be present in cells derived from a human cervical carcinoma (FRENKEL et al., 1972).

In recent studies KIEFF and his associates (personal communication) have employed highly radiolabeled EBV DNA probes to analyze transcription in certain EBV "transformed" lymphocyte cultures. The extent of transcription seems to be dependent on the state of the viral genome in the transformed cells:

1. In "leaky" virus-producing transformed cultures, approximately 50 percent of the DNA or one full-strand equivalent is transcribed.

2. In transformed, non-virus-yielding, but IUdR inducible lines, the equivalent of 60 percent of one strand is transcribed into stable mRNA.

3. Only 10 percent of a DNA-strand equivalent of RNA is synthesized in nonleaky, noninducible transformed cells.

VI. Orientation of the Integrated Viral Genome

With respect to the orientation of the integrated viral genome itself, there are two major questions to be asked: (1) is the viral genome inserted intact or does it exist as a series of fragmented DNA segments, and (2) are the cleavage site(s) within the viral genome random or specific? Unfortunately, there is little available data to answer either of these questions and therefore our considerations will be largely speculative.

As mentioned above, no Ad-2 transformed cell line contains a complete copy of the viral genome. Since some of these lines contain only noncontiguous portions of the viral DNA, it could be concluded that fragmented segments are integrated. The differential representation of portions of the SV40 genome in one transformed cell line (SVT2) suggests that the more frequently represented segment of the genome may be integrated as fragments (BOTCHAN et al., 1974). On the other hand, there appears to be at least one full copy of the viral DNA present in all SV40 transformed cells and this particular set

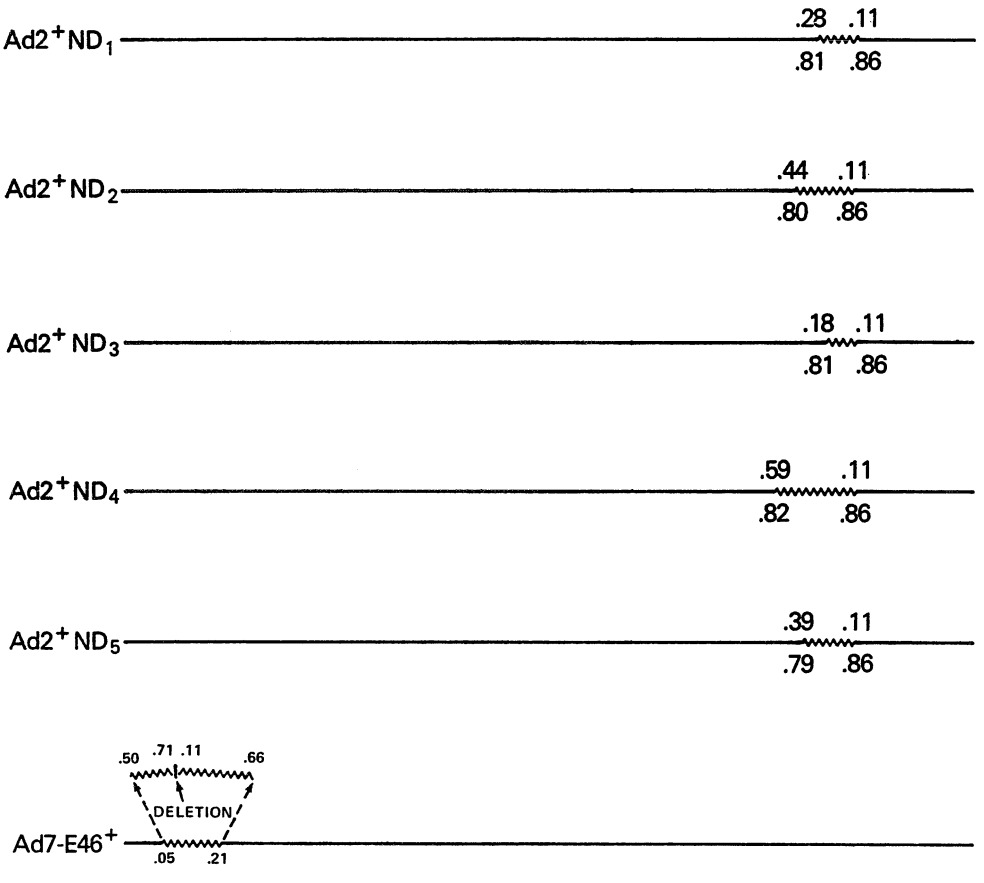

Fig. 4. Diagrammatic maps of the covalent adenovirus-SV40 hybrid viruses. The sites of integration of SV40 segments (〰) in Ad-2 DNA (—) for the nondefective hybrids Ad-2+ ND 1–5, and in Ad-7 DNA (—) for the defective hybrid E46+ as indicated. SV40 map units are noted above the lines and adenovirus map units below. The SV40 segments in Ad-2+ ND 1–5 are located at the right hand ends of the standard Ad-2 map. The SV40 segments in E46+, exhibiting both a tandem repeat and a deletion, have been arbitrarily located at the left hand end of the molecule (KELLY, 1975). See text and references (KELLY and ROSE, 1972; KELLY and LEWIS, 1973; MORROW et al., 1973; and KELLY, 1975)

of sequences may be intact. Such a suggestion is appealing since it is possible to "induce" intact replicating SV40 genomes from most SV40 transformed cell lines (see above). It should be noted, however, that the distribution of segments of the viral genome in transformed cells, as examined in these experiments, represents the result of selection through numerous cell generations and may bear little similarity to the state of the genome during the initial transformation event.

With regard to the presence of specific or random breaks in integrated viral genomes, we derive some information from the segments of viral DNA in adenovirus-SV40 hybrid molecules (Fig. 4). The cleavage sites within the SV40 segments present in these hybrid molecules suggest that a site at approx-

imately 0.11 map units may be preferentially cleaved during the integration process. This site appears as an SV40 terminus in both the nondefective hybrid viruses and in E46+. While a locus at 0.14 adenovirus map units appears to be a common integration site for all the nondefective hybrids, there is the possibility that these viruses evolved from one another by successive SV40 deletions. Thus one must reserve judgement about "hot spots" in the adenovirus molecules.

VII. Specificity of the Host Cell Integration Site

While the integration site of SV40 DNA in adeno-SV40 hybrid virus genomes has been fairly precisely localized (see above), much less is known about the location of viral DNA in cellular DNA. The main stumbling blocks have been the enormous size disparity (10^4–10^6) between the molecular weights of most oncogenic viral DNAs and the cell genome, and our lack of knowledge regarding the physical and biochemical organization of cellular DNA. GELB and MARTIN (1973) mechanically fragmented the DNAs of three SV40 transformed mouse cell lines and fractionated each preparation on a basis of reiteration frequency. The reassociation of radiolabeled SV40 DNA was then examined in the presence of reiterated or unique DNA from each cell line. In all cases, the labeled DNA probe reannealed more rapidly in the presence of nonreiterated DNA. Some variability, however, was observed among the three cellular DNA preparations. In one, the reiterated fraction seemingly had no influence on the reassociation of the radiolabeled viral DNA; in another, the reiterated fraction effected a significant acceleration although it was not as efficient as the unique fraction. GELB and MARTIN (1973) concluded that the cellular DNA sequences adjacent to the integrated viral genome were enriched for nonreiterated sequences. Their data also suggested that the integration site in each cell line was different.

BOTCHAN et al. (1973) approached this problem by digesting the DNA from an SV40-transformed mouse cell line with a bacterial restriction endonuclease and then determining the size and distribution of viral DNA. Using a cell line with 1–2 viral DNA equivalents/cell (SVT2), and R·*Eco* RI, which cleaves SV40 DNA at one unique site, BOTCHAN et al. (1973) predicted that two viral DNA fragments would be detected if the entire SV40 genome was integrated into host cell DNA by a single crossover. Each would contain viral and cellular DNA sequences. When the digestion products were analyzed by agarose gel electrophoresis and DNA-cRNA filter hybridization, two SV40 DNA-containing fragments, with molecular weights of 3.1×10^6 daltons and 1.8×10^6 daltons, respectively, were observed. The two DNA species contained 70 and 30 percent, respectively, of the SV40 DNA sequences present in SVT2 cells. These results imply that viral DNA is integrated in a limited number of cellular sites since SV40 DNA was present in two cleavage fragments of rather discrete size. Superficially, these data suggest that the SV40 genome was cleaved

0.3 map units from the R·*Eco* RI site during its integration into cellular DNA. The report by BOTCHAN et al. (1974) describing the differential representation of various portions of SV40 DNA in SVT2 transformed cells, however, makes this interpretation open to question.

Several groups have attacked the question of the specificity of the host cell integration site by examining the chromosomes prepared from virus transformed cells. Taking advantage of the unilateral loss of human chromosomes from mouse-human somatic cell hybrids, WEISS (1968) and WEISS et al. (1970) fused SV40 transformed human cells with mouse cells and followed the disappearance of SV40 T-antigen. The results of these studies indicated that T-antigen could still be detected in cell hybrids containing only a few human chromosomes; the T^- subclones of one hybrid retained 1–2 human chromosomes. These experiments were interpreted as indicating multiple integration sites in different chromosomes or the selective integration of SV40 DNA into specific human chromosomes which were preferentially retained in mouse-human cell hybrids. CROCE et al. (1973), using chromosome-banding techniques to identify the retained human chromosomes, reported the concordant segregation of SV40 T-antigen and the human chromosome C-7 in hybrid cells derived from the fusion between mouse and SV40 transformed human cells. All of the 71 cell hybrids examined, which synthesized SV40 T-antigen, contained the C-7 chromosome. Conversely, three of 15 T-antigen negative hybrid clones contained this chromosome. The T-antigen positive clones could also be induced to synthesize viral capsid antigen and SV40 virions following fusion with permissive green monkey cells (CROCE et al., 1974a). It should be noted that the virus rescued from T-antigen positive mouse-human cell hybrids is a "defective" form of SV40 which produced "microplaques" in monkey cells. Although this system is not entirely analogous to SV40 transformed 3T3 cells from which nondefective, fully infectious forms of virus can be readily recovered, the data suggest that SV40 genetic information is associated with a specific human chromosome. A similar segregation of T-antigen expression and a specific green monkey chromosome has recently been reported (CROCE et al., 1974b).

VIII. Biochemical Basis of the Integration Event

It has been presumed that animal viral genomes, like their prokaryote counterparts, become integrated into cellular DNA by a recombinational event. While little hard data are available on this point, ample evidence has been accumulated, particularly with the papovaviruses, to suggest that the covalent linkage of viral and host DNA sequences is not an uncommon event. During productive infection of green monkey cells with high input multiplicities of SV40, variable amounts of defective, supercoiled progeny viral genomes appear (YOSHIIKE, 1968; ALONI et al., 1969; LAVI and WINOCOUR, 1972). It has been shown that the molecular rearrangements that occur involve deletions, inversions, and duplications of viral DNA as well as the insertion of host cell

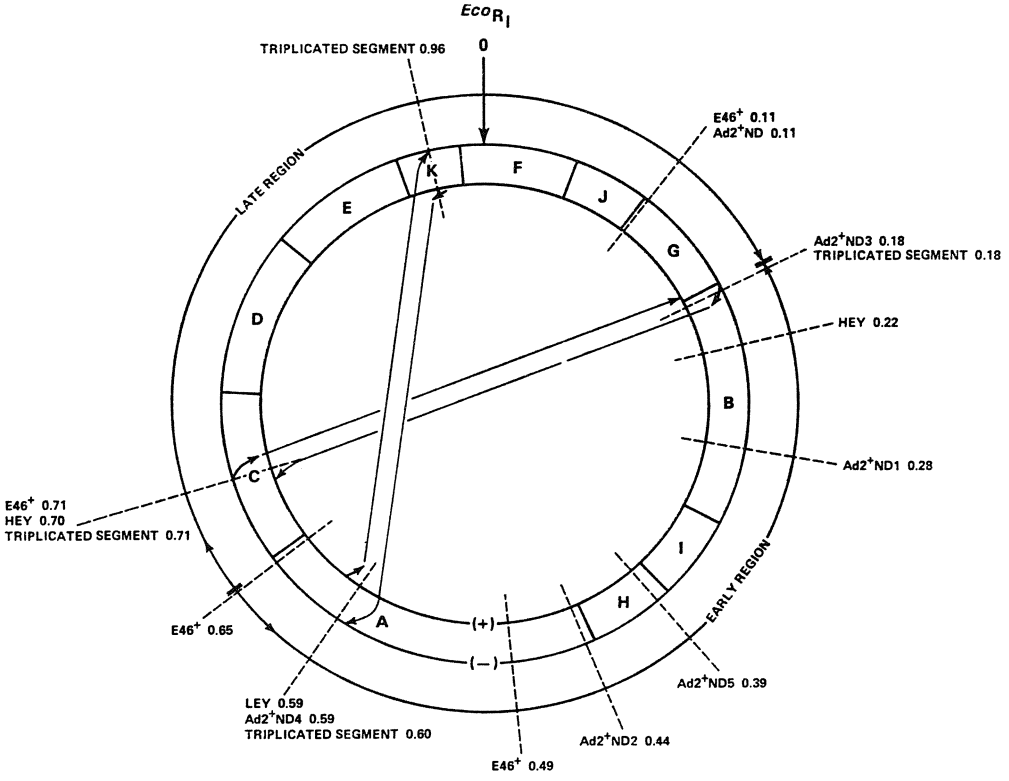

Fig. 5. The physical map of SV40 DNA containing putative recombination sites. The map positions of the termini of the SV40 segments contained in the Ad-2+ ND (KELLY and LEWIS, 1973; MORROW et al., 1973). HEY and LEY (KELLY et al., 1974) and, E46+ (KELLY, 1975), hybrid viral genomes as well as the triplicated DNA segment (KHOURY et al., 1974b) were located on the physical map of SV40 DNA

DNA (TAI et al., 1972; LAVI et al., 1973; ROZENBLATT et al., 1973; BROCKMAN and NATHANS, 1973; MARTIN et al., 1973). Unless great care is taken during their propagation, the genomes of papovaviruses, at least, readily undergo significant alteration, presumably through recombination.

One interesting example of the type of rearrangement that can occur was described by FAREED et al. (1974) and involved a triplication of approximately one-third of an SV40-like genome. Analysis of this altered viral DNA was facilitated by the absence of any cellular DNA and the triplication of the R·*Eco* RI site. It was possible, by nucleic acid hybridization studies as well as heteroduplex mapping experiments to show (1) that SV40 DNA sequences, topographically located in two separate and identifiable regions on the physical map of the viral genome, became covalently linked, and (2) that the two segments were fused by joining sequences present on the plus strand of the parental DNA molecule to sequences that were originally located on the minus DNA strand (KHOURY et al., 1974b). A recombinational event, involving sequences located at 0.18 and 0.71 SV40 DNA map units, would lead to such an inversion;

a subsequent deletion could result in the generation of a one-third sized DNA fragment (KHOURY et al., 1974b).

The analyses of such molecular rearrangements take on added significance if one assumes that altered viral genomes arise by recombinational mechanisms similar to those occurring during the integration process. We have endeavored to compare the four sites in SV40 DNA, involved in the generation of the one-third genome-sized DNA segment, with the map positions of other putative recombination loci. Eleven such sites are depicted on the physical map of SV40 shown in Fig. 5. They represent, for the most part, the termini of SV40 DNA segments present in adeno-SV40 hybrid viral genomes. Four of the 11 sites are involved in more than a single recombinational event. It is noteworthy that three of these were also associated with the appearance of the one-third-sized SV40 DNA segments (map positions 0.18, 0.60, and 0.71). Thus the molecular rearrangements accompanying the tandem triplication of SV40 DNA sequences may involve some of the same viral DNA sites that play a role in integration. Since it seems quite likely that the early region of the SV40 genome is not interrupted when viral DNA is integrated into the transformed cell genome (see above), one may conclude that a break must be introduced somewhere in the late region (0.66 to 0.17 map units) of the viral genome (Fig. 3). It is tempting to assign such a role to the site located at approximately 0.71 map units which includes the termini for the SV40 DNA segment in HEY (KELLY et al., 1974) and E46+ (KELLY, 1975) adeno-SV40 hybrid viral genomes (Fig. 4), and coincides with a locus involved in the generation of one-third-sized SV40 DNA molecules.

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The Replication of Papovavirus and Adenovirus DNA

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With 6 Figures

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I. Introduction

The animal viruses with DNA genomes have been classified into five groups—parvoviruses, papovaviruses, adenoviruses, herpesviruses, and poxviruses—in order of increasing complexity (MELNICK, 1974). Each of these virus groups have unique features which provide favorable experimental material for the investigator. The papovaviruses and adenoviruses have been especially useful in the study of DNA structure and replication. The availability of large quantities of viral DNA and the comparatively small genome size of these viruses have permitted experimental approaches leading to a detailed understanding of the primary (DAHR et al., 1974a, b; FIERS et al., 1974; ROBERTS et al., 1974), secondary, and tertiary (VINOGRAD and LEBOWITZ, 1966) structure of these DNA molecules. Restriction enzymes have been employed to generate defined fragments of these genomes which in turn have been used to study DNA replication and transcription and to localize viral mutants on the physical map of these genomes (Tables 2 and 3). With both the adenoviruses and papovaviruses the outlines of a DNA replication scheme are now available, the replicative intermediates have been visualized and several viral and cellular functions involved in DNA replication have been characterized (LEVINE, 1974).

This review will attempt to summarize the recent developments in the study of SV40, polyoma, and adenovirus DNA replication. These viruses are considered in the same review to permit the reader to glean whatever general principles may emerge from these studies. SV40, polyoma, and adenoviruses replicate in the nucleus of monkey, mouse, or human cells. The small size of their viral genomes makes it likely that a number of cellular functions are called upon during the replication of viral DNA. In this regard these studies should lead to a better understanding of the eukaryotic DNA replication process. Both papovaviruses and the adenoviruses can cause tumors in animals and transform cells in culture (TOOZE, 1973). The availability of temperature-sensitive mutants, in both of these virus groups, has made it possible to identify viral gene products that are required for viral DNA replication (TEGTMEYER, 1972; WILLIAMS et al., 1974; GINSBERG et al., 1974). Interestingly, some of these same functions are also required for viral transformation of cells in culture (BUTEL et al., 1974; WILLIAMS et al., 1974; GINSBERG et al., 1974;

Table 1. The physical properties of SV40, polyoma and adenoviruses

Size of virion[1]	SV40-polyoma	Human adenoviruses
	45 mμ	80 mμ
Symmetry[1]	Icosahedron T = 7 laevo (polyoma) T = 7 dextro (SV40)	Icosahedron T = 25
MW of virion	25×10^6	175×10^6
MW of DNA[2]	3×10^6	$20–25 \times 10^6$
Conformation of DNA[2]	Form I is double stranded, closed circular and supercoiled	Double stranded and linear (when extracted with SDS, pronase and phenol)
S_w^{20}[3]	Form I 21S Form II 16S	31S
Base composition[4]	SV40: 41% G + C polyoma: 48% G + C	Group A: 47–49% G + C group B: 50–53% G + C group C: 56–60% G + C
Unusual base sequence features[5]	—	Inverted terminal repeat of about 100–150 nucleotides
Denaturation maps	SV40[6] polyoma[7]	Adenoviruses types 2, 5, 12, Celo[8, 9, 10, 11]
Nearest neighbor analysis	SV40[12] polyoma[13]	Adenoviruses type 5[12]

[1] MATTERN et al., 1967; ANDERER et al., 1967; HORNE et al., 1959; VALENTINE and PEREIRA, 1965.
[2] CRAWFORD, 1965; KLEINSCHMIDT et al., 1965; GREEN et al., 1967; VAN DER EB et al., 1969; VINOGRAD et al., 1965.
[3] WATSON and LITTLEFIELD, 1960; CRAWFORD, 1965.
[4] CRAWFORD, 1963; CRAWFORD and BLACK, 1964; PINA and GREEN, 1965.
[5] GARON et al., 1972; WOLFSON and DRESSLER, 1972).
[6] MULDER and DELIUS, 1972; YOSHIIKE et al., 1972.
[7] FOLLETT and CRAWFORD, 1968.
[8] DOERFLER and KLEINSCHMIDT, 1970.
[9] ELLENS et al., 1974.
[10] DOERFLER et al., 1973.
[11] YOUNGHUSBAND and BELLETT, 1972.
[12] SUBAK-SHARPE et al., 1966.
[13] MORRISON et al., 1967.

KIMURA and ITAGKI, 1975; MARTIN and CHOU, 1975; TEGTMEYER, 1975; BRUGGE and BUTEL, 1975; OSBORN and WEBER, 1975). Thus the study of DNA replication is not only useful because it is an integral part of the lytic infection, but DNA replication is clearly relevant to our understanding of viral transformation and the regulation of viral and cellular gene expression.

Table 1 reviews the physical properties of SV40, polyoma, and the human adenoviruses. The large amount of information already available with these viruses should permit a very sophisticated analysis of DNA replication, genetic recombination, viral genetic structure, and gene regulation in eukaryotic cells.

II. Polyoma-SV40 — Introduction

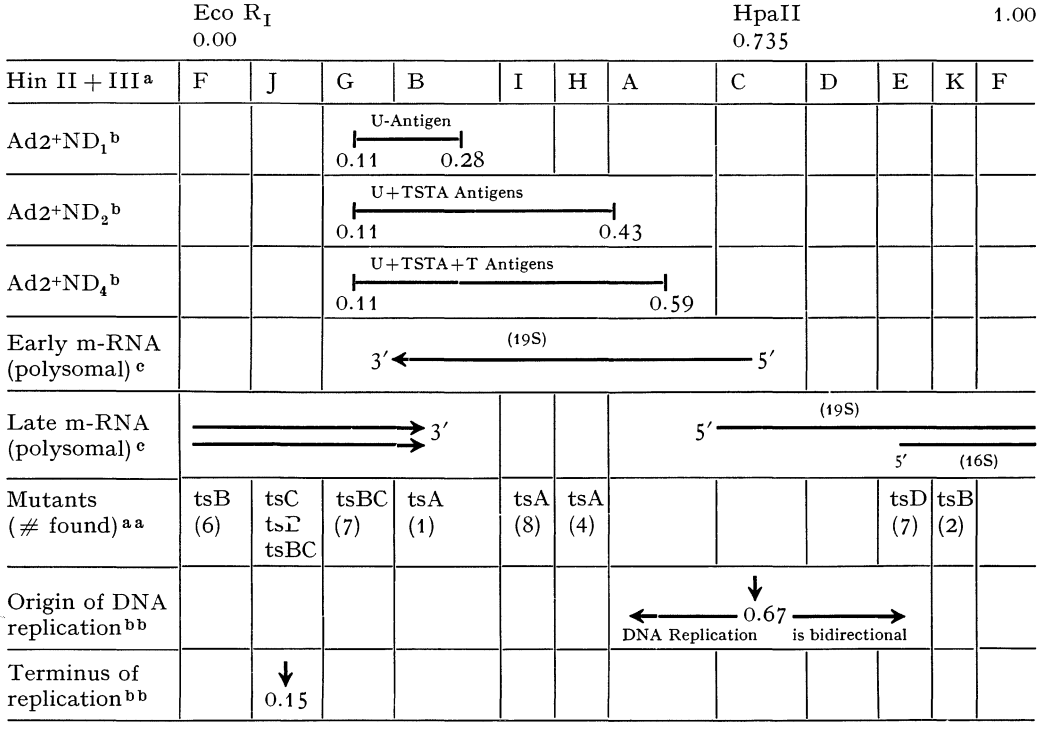
The papovaviruses (MELNICK, 1962) have been divided into two groups: (a) papillomaviruses and (b) the polyoma-SV40 group. Virtually all our information about DNA replication has come from the study of SV40 and polyoma, which are the only two viruses to be considered here. These viruses have an icosahedral structure (T =7) and the particle weight of the virion is about 25×10^6 (ANDERER et al., 1967). The virion is composed of two or three viral structural proteins (MW, 45000, 35000, 25000) (ESTES et al., 1971) surrounding a core consisting of DNA and three or four proteins that are similar if not identical to cellular histones (FREARSON and CRAWFORD, 1972; PETT et al., 1975).

Polyoma replicates in mouse cells and transforms a number of rodent cells in culture. SV40 replicates in African green monkey cells and can transform human and monkey cells as well as rodent cells in culture. All of the biochemical and genetic studies with these two viruses indicate that they are essentially similar in their viral functions, the replication of viral DNA and their effects upon the host cell (TOOZE, 1973). For this reason information obtained with either one of these viruses alone, will be combined in this review to give a more complete picture of viral DNA replication.

A. Lytic Infection

Shortly after infection of permissive cells a virus specific transcript can be detected on the polysomes (WEINBERG et al., 1972). This polysomal RNA migrates in acrylamide gels at 19S and has an estimated molecular weight of $900–1000 \times 10^3$ (WEINBERG et al., 1974). This species of m-RNA will hybridize with about 30–40 percent of a restricted portion (Hin, A, H, I, B) of the E or minus strand of the SV40 genome (Table 2) (KHOURY et al., 1973; SAMBROOK et al., 1973; KHOURY et al., 1975). The size of an SV40 transcript from 40 percent of the genome is expected to be about 600000 MW and so this transcript of 19S, (900000–1000000 MW) is apparently too large to be only E strand specific viral RNA (WEINBERG et al., 1974). It remains possible then that cellular RNA sequences could increase the size of this transcript. On the other hand this molecular weight estimate from acrylamide gels may not be very accurate. The early region of SV40 polysomal RNA (Table 2) can theoretically code for one protein fo 70–100 thousand MW or several proteins of this total molecular weight range. Three distinct immunologic activities have been detected at early times after SV40 infection of permissive cells. The tumor antigen (T-antigen) (BLACK et al., 1963) and U-antigen (LEWIS and ROWE, 1971) are clearly early viral induced antigens with a nuclear and perinuclear location, respectively. A tumor specific transplantation antigen (TSTA) has also been described (HABEL, 1961; SJOGREN et al., 1961) and may appear on the surface of infected cells at early times after infection (GIRARDI and DEFENDI, 1970). Temperature-sensitive mutants have been isolated with SV40 (TEGTMEYER and OZER, 1971; CHOU and MARTIN, 1974; KIMURA and DULBECCO, 1972; KIT et al., 1970)

Table 2. Location of SV40 transcripts, antigens, mutants, and replication sites on the hind II + III cleavage map



[a] Danna and Nathans, 1971; Morrow and Berg, 1972; Mulder and Delius, 1972.
[b] Kelly and Lewis, 1973.
[c] Khoury et al., 1973; Sambrook et al., 1973; Weinberg et al., 1974; Khoury et al., 1975.
[aa] Lai and Nathans, 1974a, b.
[bb] Nathans and Danna, 1972; Fareed et al., 1972.

and polyoma (Fried, 1965a; Eckhart, 1969; DiMayorca et al., 1969) and generally fall into three complementation groups (tsA, B, and C), and two restricted or noncomplementing groups (tsD and tsBC which does not complement B or C mutants) (Chou and Martin, 1974; see also Robb et al., 1974, for a different analysis of complementation testing). Only one group of these mutants, the tsA class, codes for an early function (Fried, 1965a; Tegtmeyer and Ozer, 1971). The tsA gene product is required for the initiation of each round of SV40 DNA replication (Tegtmeyer, 1972). The initiation of late transcription requires viral DNA replication (and therefore requires tsA function), but, once late viral RNA synthesis begins, viral DNA replication is no longer necessary for continuous transcription (Cowan et al., 1973). The late transcripts found on the polysomes are of two size classes, 16S (600000 MW) and 19S (900000 MW) and hybridize to the L or plus strand of a restricted portion of the SV40 genome (Hin, C, D, E, K, F, J, G) (Khoury et al., 1973; Sambrook et al., 1973; Weinberg et al., 1974; Khoury et al., 1975) (Table 2).

The 16S and 19S RNA's are derived from overlapping regions of the SV40 genome (Weinberg et al., 1974). Early transcripts (19S) continue to be synthesized late in infection (Aloni et al., 1968; Oda and Dulbecco, 1968a, b). The late transcripts apparently code for virion proteins VP-1 (45000 MW), VP-2 (35000 MW), and VP-3 (25000 MW) (Walter et al., 1972; Tegtmeyer, 1974). Cellular DNA synthesis (Hartwell et al., 1965; Hatanaka and Dulbecco, 1966), cellular histone synthesis (Winocour and Robbins, 1970), and some cellular enzyme activities, involved in the biosynthesis of deoxypyrimidines and DNA (see Tooze, 1973 for a compilation of these data), are all markedly stimulated after SV40 and polyoma infection. The cellular histones in association with viral DNA (Green et al., 1971; White and Eason, 1971) and capsid protein (McMillen and Consigli, 1974; Sen et al., 1974) are then assembled into mature virions. TsB, C, and BC mutants are required for late viral functions (Robb et al., 1974; Chou and Martin, 1974). TsD mutants do not synthesize any known viral products (at either early or late times after infection) at nonpermissive temperatures. Since these tsD mutants map in the late region of the SV40 genome (Table 2) it is likely that they code for a late function virion protein (Robb et al., 1974) and the defect at nonpermissive temperatures is probably a block in the uncoating step of the SV40 virion.

B. The Structure of SV40 and Polyoma DNA

1. Secondary and Tertiary Structure

The DNA extracted from SV40 and polyoma virions contains two or sometimes three discrete species of DNA that have been separated and identified by band sedimentation in sucrose or CsCl gradients (Watson and Littlefield, 1960; Weil and Vinograd, 1963; Crawford, 1963). These three forms of DNA have been named components I (21S), II (16S), and III (14–12S). Component I is composed of two continuously closed circular polynucleotide strands, base-paired in the normal Watson-Crick helix. This double-stranded helix is further twisted about itself to form superhelical turns (tertiary structure) (Vinograd et al., 1965). Component II DNA is a double-stranded circular DNA molecule containing one or more chain interruptions in the phosphodiester backbone which then allow free rotation of the DNA strand opposite the polynucleotide chain break (Vinograd and Lebowitz, 1966). Component III DNA is composed almost entirely of linear, double-stranded, cellular DNA packaged into pseudovirions during lytic infection (Michel et al., 1967; Winocour, 1969; Levine and Teresky, 1970; Trilling and Axelrod, 1970).

As a consequence of the closed circular and superhelical properties of component I DNA it has a more compact shape (Vinograd et al., 1965), a lower intrinsic viscosity (Opschoor et al., 1968), and is more resistant to shear forces (Weil and Vinograd, 1963) than is component II DNA. The two continuously closed polynucleotide strands of component I DNA are topologically interwound and cannot be separated by pH's or temperatures that denature component II DNA (Vinograd et al., 1965, 1968; Crawford and Black, 1964).

In 0.1N NaOH, component II DNA denatures and the two polynucleotide strands separate into a linear (16S) and a circular component (18S). Under the same conditions component I DNA undergoes a helix-coil transition into a very compact cyclic coil that sediments at 53S in alkaline CsCl gradients (VINOGRAD et al., 1965). Component I DNA molecules intercalate lower amounts of planar dyes like ethidium bromide (EtBr) than do relaxed circular molecules (CRAWFORD and WARING, 1967; BAUER and VINOGRAD, 1968) and this observation has been employed to separate these two forms of DNA in EtBr-CsCl density gradients where form I DNA has a higher buoyant density (RADLOFF et al., 1967).

Is not yet clear why all naturally occurring closed circular DNA molecules contain superhelical turns. Two different mechanisms have been postulated: (a) the superhelical turns result from a difference in the pitch of the Watson-Crick helix in vivo and in vitro (VINOGRAD and LEBOWITZ, 1966; WANG et al., 1967; WANG, 1969) or (b) the tertiary structure of DNA in vivo, which is altered during isolation, results in superhelical turns in vitro (WORCEL and BURGI, 1972). While either of these mechanisms appears feasible no experimental test of these alternatives has been formulated.

2. The Primary Structure of SV40 DNA

The sequence of polynucleotides in SV40 DNA is under intensive investigation (ZAIN et al., 1973; DAHR et al., 1974a, b; FIERS et al., 1974) and almost 50 percent of this sequence is presently known. A particularly interesting observation, relevant to DNA replication, concerns the region of the genome where SV40 polysomal transcripts begin (5′ end of these molecules). The 5′ ends of the early and late cytoplasmic transcripts (Table 2) overlap each other (are complementary to each other) (DAHR et al., 1974a and personal communication). This overlap region appears to encompass the probable origin of DNA replication. It is of some interest then that this region of the genome (around 0.67 units, see Table 2) contains symmetric repeated sequences of polynucleotides that are rich in runs of adenylic and thymidylic acid residues (DAHR et al., 1974a and personal communication).

3. Restriction Enzyme Cleavage Maps

Physical maps of the SV40 genome have been obtained with restriction enzymes Hin d II + III (DANNA and NATHANS, 1972), Hpa I (SACK and NATHANS, 1973), Eco RII (SUBRAMANIAN et al., 1974), and Hae (HUANG et al., 1973; LOBOWITZ et al., 1974; SUBRAMANIAN et al., 1974). The enzymes Eco RI (MULDER and DELIUS, 1972; MORROW and BERG, 1972) and Hpa II (SHARP et al., 1973) each cleave SV40 at a single unique site (Table 2). These fragments have been employed in nucleotide sequence analysis (SUBRAMANIAN et al., 1974), mapping of polysomal transcripts (KHOURY et al., 1973; SAMBROOK et al., 1973; WEINBERG et al., 1974; KHOURY et al., 1975), determining the

origin and direction of DNA replication (Nathans and Danna, 1972; Thoren et al., 1972; Fareed et al., 1972; Laipis et al., 1975), and mapping temperature sensitive (Lai and Nathans, 1974b) and other kinds of mutants (Mertz et al., 1974; Lai and Nathans, 1974a) (Table 2). Polyoma DNA is cut into eight fragments by Hpa II (Griffin et al., 1974) and only one cleavage site for Eco RI is present in this genome.

C. Genetics of SV40 and Polyoma

Over 300 independently isolated temperature-sensitive mutants of polyoma and SV40 have now been characterized (Eckhart, 1969; DiMayorca et al., 1969; Tegtmeyer and Ozer, 1971; Kimura and Dulbecco, 1972, 1973; Chou and Martin, 1974). Based upon the type of complementation test employed, these mutants fall into three to five groups (A, B, C, BC, D) (Tegtmeyer and Ozer, 1971; Chou and Martin, 1974; also see Robb et al., 1974).

1. TsA mutants. Of the 300 temperature-sensitive mutants so far isolated, the A group has been the only early gene function detected in SV40 or polyoma. This could be due to one of three reasons (1) SV40 contains only one early gene function, (2) SV40 contains other early gene functions but temperature-sensitive mutants cannot be obtained with these other functions, (3) other early functions do exist but represent a very small portion of the total early genome size. There appear to be at least two early SV40 antigens (T and U) (Black et al., 1963; Lewis and Rowe, 1971) but it is not clear whether these are coded for by the viral genome. It remains possible that T and U antigens share amino acid sequence homologies (e.g., U is a proteolytic breakdown product of T) or are related in some other manner.

The A gene product is synthesized early and late times after lytic infection and is required for viral DNA replication (Fried, 1970; Tegtmeyer and Ozer, 1971). This protein functions at the initiation step of each round of viral DNA replication (Tegtmeyer, 1972; Francke and Eckhart, 1973).

Several polyoma tsA mutants fail to synthesize T-antigen at the non-permissive temperatures (Oxman et al., 1972; Paulin and Cuzin, 1975). Cells infected with SV40 tsA mutants at nonpermissive temperatures produce a 100000 MW protein that is rapidly turned over (proteolysis). This protein can be efficiently precipitated out of solution by antiserum from animals bearing SV40 tumors (Tegtmeyer, 1974). While these results are consistent with the possibility that T-antigen is the A gene product, other explanations remain possible and indeed feasible.

The tsA gene product is also involved in viral transformation. It appears to be required for the initiation (Fried, 1965b) and maintenance of the transformed phenotype (colony morphology, topoinhibition, growth in agar) (Butel et al., 1974; Kimura and Itagki, 1972; Martin and Chou, 1975; Tegtmeyer, 1975; Brugge and Butel, 1975; Osborn and Weber, 1975).

2. TsB, C, and BC mutants. TsB and C mutants function late after lytic infection and represent likely one or more viral coat protein genes (Robb et al.,

1974; TEGTMEYER et al., 1974). The tsBC class of mutants are unusual in that they complement tsA class mutants but not tsB nor tsC class mutants (CHOU and MARTIN, 1974). With more stringent complementation conditions all of TEGTMEYER's late mutants fall into this category (one late mutant class called tsB mutants) for complementation. However, TEGTMEYER's tsB mutants have been further divided into two subgroups based upon their phenotypes (OZER and TEGTMEYER, 1972; ROBB et al., 1974). It is of some interest to recall that late cytoplasmic viral transcripts are of two sizes (16S and 19S) and contain overlapping sequences in the region where tsB, tsBC, and tsC mutants have been mapped (Table 2). This situation could give rise to a class of BC mutants. This type of hypothesis predicts amino acid sequence homology in two or more of the viral coat proteins (VP1, 2, or 3).

3. TsD mutants. This class of mutants is noncomplementing when tested against tsA, B, or C groups ((DULBECCO and ECKHART, 1970; ROBB and MARTIN, 1972; ROBB et al., 1974; CHOU and MARTIN, 1974). These mutants map in late region of the SV40 genome (LAI and NATHANS, 1974a) (Table 2) and all the available evidence indicates that these mutants alter a virion protein that prevents viral DNA uncoating at the nonpermissive temperatures (ROBB et al., 1974; ROBB and MARTIN, 1972).

It is somewhat surprising then that there have been a number of reports that tsD mutants are involved in the maintenance of the transformed phenotype. Polyoma ts3 (a class D mutant)-transformed BHK cells show a temperature-dependent effect upon the cell surface change, detected by wheat germ agglutinin (ECKHART et al., 1971) and the ability to initiate cellular DNA synthesis in a confluent monolayer (topoinhibition) (DULBECCO and ECKHART, 1970). SV40 ts101 (a class D mutant)-transformed 3T3 mouse cells have a temperature-dependent regulation of SV40 T-antigen production (ROBB, 1973). These data do not agree with the observation that all tsD mutants, so far examined, map in the late region of the genome (LAI and NATHANS, 1974a, b; Table 2). This region was not even transcribed in nine different SV40-transformed cell lines that were tested by KHOURY et al. (1973).

A genetic map employing recombination frequencies has not yet been constructed with these mutants. However, NATHANS and his collaborators have devised a test to rescue temperature-sensitive mutants by restriction enzyme DNA fragments from the wild type virus (LAI and NATHANS, 1974a, b). This approach has placed the tsA, B, C, BC, and D class mutants onto Hin d II + III fragments of the physical map of SV40 (Table 2). While the early region of the SV40 genome covers Hin A, H, I, and B fragments, 12 independent tsA mutants map in Hin H and I, and only one (tsA-7) maps in Hin B. All the tsD mutants examined so far map in Hin E while tsB, tsC, and tsBC mutants have been located in Hin K, F, J, and G fragments. Curiously, no mutants presently map in Hin A, C, or D which represents some 43 percent of the SV40 genome. There certainly may be relatively restricted sites in a protein where a mutation results in a temperature-sensitive phenotype and these results may reflect this possibility.

BERG and his collaborators (SHENK et al., 1974) have employed the single strand specific nuclease S-1 to map the location of deletions, insertions, and temperature-sensitive mutants of SV40 in heteroduplex molecules composed of mutant and wild type DNAs. Thus a second procedure is now available for the location of SV40 mutants on the physical map of the genome.

D. Properties of Replicating SV40 and Polyoma DNA

The replicative intermediates of SV40 or polyoma DNA have been isolated by a variety of different techniques. Each of these procedures takes advantage of the unique properties of replicating DNA. In all cases viral DNA was labeled with either a radioisotope (^{3}H-thymidine) or a heavy density probe (bromodeoxyuridine) for a short period of time. Viral DNA was then selectively fractionated from cellular DNA by the procedure of HIRT (1967) or a variation of this devised by BOURGAUX et al. (1969, 1971).

The replicative intermediates of SV40 or polyoma DNA sediment in neutral pH sucrose gradients at 25S, ahead of form I viral DNA (LEVINE et al., 1970). This unusually rapid sedimentation rate was not due to association of proteins or RNA with the replicating DNA, since pronase and RNase treatment did not alter these sedimentation properties (LEVINE et al., 1970). Rather, the fast sedimentation behavior of these SV40 DNA intermediates appears to be due to increased mass and the compact structure of these DNA molecules (JAENISCH et al., 1971; SEBRING et al., 1971). The unusual compact nature of the SV40 replicative intermediates was demonstrated by centrifugation of replicating DNA in ethidium bromide (EtBr)-CsCl density gradients. The replicative intermediates in this dye-density gradient distributed themselves in a broad band between component I DNA (1.592 gms/cm^3) and component II DNA (1.552 gms/cm^3) (JAENISCH et al., 1971; SEBRING et al., 1971). Electronmicrographs of these replicating molecules (Fig. 1) showed two untwisted loops of equal size (presumed replicated region) attached to a superhelical tail (presumed unreplicated region) (JAENISCH et al., 1971; SEBRING et al., 1971). Those replicating molecules obtained from the lower density portions of the EtBr-CsCl gradient (near or at the position of component II DNA) had almost completed the replication process and did not contain a superhelical tail (Fig. 1), while molecules from the higher density regions of this gradient were in earlier stages of the duplication process (JAENISCH et al., 1971; SEBRING et al., 1971). The presence of a superhelical tail in the replicative intermediates and the density position of these molecules in EtBr-CsCl gradients (between form I and II viral DNA) suggested that the unreplicated region of these molecules bound less EtBr dye and behaved as a closed circular molecule. On the other hand, the presumed replicated regions of these molecules bound more dye and behaved like relaxed circular SV40 DNA. This explains why those replicative intermediates just beginning the duplication process have a heavier density than those molecules that have almost completed their duplication. If this explanation is correct, then the template polynucleotide strands

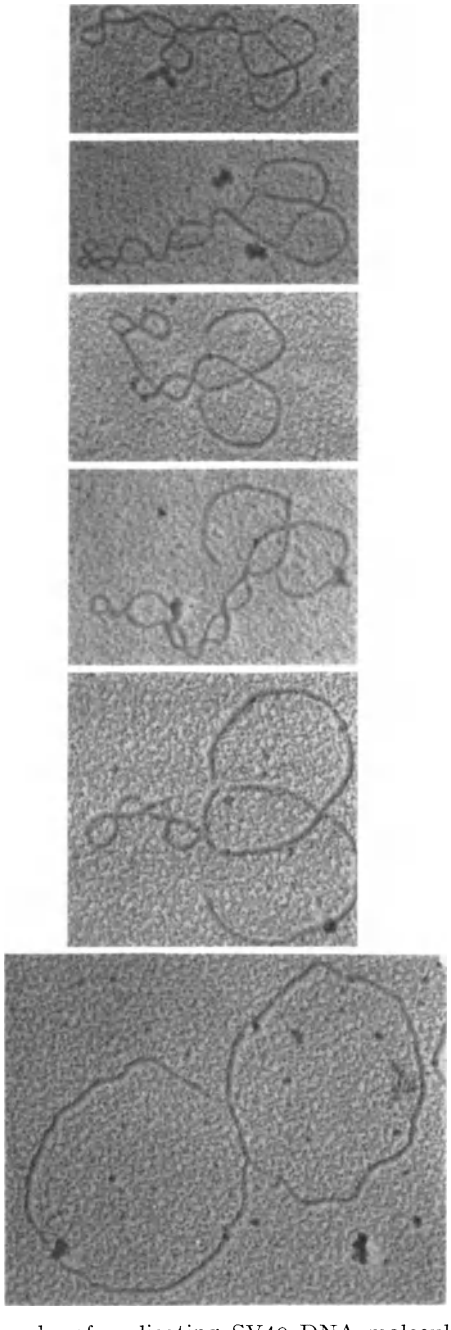


Fig. 1. Electron-micrographs of replicating SV40 DNA molecules. Viral DNA was obtained and prepared for microscopy as described by JAENISCH et al. (1971)

of the replicative intermediate should be continuously closed circles, while the newly replicated portions of these molecules (relaxed loops of equal sizes) should contain linear, progeny polynucleotide strands, hydrogen bonded to the separated template strands (SEBRING et al., 1971; MAYER and LEVINE, 1972).

This model was tested (JAENISCH et al., 1971) by treating these replicative intermediates with pancreatic DNase to convert the closed circular replicative forms (like component I DNA) to relaxed replicating circles (like component II DNA). This DNase treatment shifted the SV40 replicative intermediates to the lighter density position in an EtBr-CsCl density gradient, just as it shifts the density of component I DNA (heavy density) to the lighter density component II DNA. Thus, one break in the phosphodiester backbone of component I DNA or the replicative intermediate relaxed these molecules and permitted more EtBr to bind to these DNA's (lighter density position). Employing a different approach, SEBRING et al. (1971) labeled the template polynucleotide strands of SV40 replicative intermediates with ^{14}C-thymidine, by labeling for 2 hours followed by a 1.25 hr chase period. The replicative intermediates were then isolated by an EtBr-CsCl density gradient and sedimented in alkaline pH sucrose gradients. Under these conditions closed circular DNA (form I DNA) undergoes a helix-coil transition and sediments as a compact cyclic coil at 53S. The template polynucleotide strands of the replicating DNA, isolated from different density positions in the EtBr-CsCl gradient, sedimented between 36–47S consistent with the closed circular nature of these partially replicated molecules.

Closed circular and superhelical SV40 component I DNA sediments at 21S in a neutral pH sucrose gradient. The addition of increasing concentrations of EtBr first unwinds the negative superhelical turns, resulting in a slower sedimentation rate, and then winds in new superhelical turns of the opposite sense (positive turns) resulting in a more rapid sedimentation rate (BAUER and VINOGRAD, 1968). The closed circular nature of the template strands of SV40 replicative intermediates was confirmed by demonstrating this same effect of different EtBr concentrations upon the sedimentation rate of replicating SV40 DNA (MAYER and LEVINE, 1972; SEBRING et al., 1974). In addition, these experiments demonstrated that the sense of the superhelix in SV40 replicating DNA was the same (negative sense) as is found in component I mature viral DNA. In fact the replicative forms of SV40 have a slightly higher superhelical density than mature component I viral DNA (SEBRING et al., 1974) which is consistent with the possibility that there are denatured regions of DNA ahead of the replicating forks (SEBRING et al., 1974).

The presence of single-stranded regions of SV40 DNA at the position of the replication forks has been observed in electron micrographs of replicating molecules (JAENISCH et al., 1971). The single-stranded specific nuclease from *Neurospora crassa* (RABIN et al., 1972) has also been employed to demonstrate such single-stranded regions in replicating polyoma DNA (BOURGAUX and BOURGAUX-RAMOISY, 1971). Benzoylated-naphthoylated DEAE-cellulose chromatography has been used to separate the replicative forms of SV40 (LEVINE et al., 1970) and polyoma (BOURGAUX et al., 1971) DNA from mature form I DNA. The replicative intermediates bind more strongly to this resin than component I DNA. Presumably the benzoyl and naphthoyl groups in this column base stack with the nonhydrogen bonded bases (single stranded regions) of the replicative intermediates.

E. The Mechanism of Replication of SV40 and Polyoma DNA

The replication of SV40 and polyoma DNA can be divided into four distinct stages (LEVINE, 1974; Fig. 2): (1) initiation, (2) polynucleotide chain propagation or elongation, (3) segregation of the two progeny molecules, and (4) maturation of these newly made daughter molecules.

1. Initiation of DNA Replication

Initiation of each round of polyoma or SV40 DNA replication requires protein synthesis (GERSHON and SACHS, 1964; KIT and NAKAJIMA, 1971; KANG et al., 1971). At least one of the proteins required for initiation is the SV40 tsA gene product (FRANCKE and ECKHART, 1973; TEGTMEYER, 1972). Cells infected with tsA mutants at the nonpermissive temperature synthesize a 100000 MW protein that is turned over rapidly and is precipitated by antibody to T-antigen (TEGTMEYER, 1974). In addition, many SV40 tsA mutants produce a reduced level of T-antigen at the nonpermissive temperature (ROBB et al., 1974), and some polyoma tsA mutants fail to produce T-antigen under nonpermissive conditions (OXMAN et al., 1972; PAULIN and CUZIN, 1975). The appearance of T-antigen during lytic infection is controlled by an early viral function (GILDEN et al., 1965; RAPP et al., 1965; Table 2). These observations are consistent with the possibility that T-antigen is coded by, or is regulated by, the SV40 A gene product. CARROLL et al. (1974) have shown tat T-antigen can exist in several aggregated forms, the smallest of which sediments at 5S in high ionic strength buffers. All of these aggregated forms of T-antigen bind to double stranded calf thymus DNA cellulose columns in low ionic strength buffers at pH 6.2. Some heterogeneity exists in the properties of T-antigen eluted from DNA cellulose. One fraction of this antigen eluted at low ionic strength, pH 8.0 buffers, while a second fraction elutes at pH 8.0 at high salt concentrations. T-antigen seems to have only a poor affinity for single-stranded calf thymus DNA under these conditions (CARROLL et al., 1974). T-antigen also binds to SV40 DNA (OSBORN, WEBER, and LEVINE, unpublished observations; CARROLL, personal communication), and the question of specificity of T-antigen binding to DNAs remains to be investigated.

The origin of SV40 DNA replication is at an unique site in the chromosome (0.67 map units, Table 2) (NATHANS and DANNA, 1972; FAREED et al., 1972). A similar observation has now been made for polyoma DNA where the origin is located at 0.29 percent from the Eco RI site (CRAWFORD et al., 1974). When SV40 is passaged at high multiplicities of infection, defective viral chromosomes evolve containing deletions, duplications, and substitutions with cellular DNA (UCHIDA et al., 1968; YOSHIIKE and FURUNO, 1969; LAVI and WINOCOUR, 1972; TAI et al., 1972; BROCKMAN et al., 1973; ROSENBLATT et al., 1973).

One of the striking features of these variant chromosomes is the consistent retention and frequent repetition of the region of the SV40 genome containing the origin of DNA replication (BROCKMAN et al., 1974; FAREED et al., 1974; FOLK and WANG, 1974; KHOURY et al., 1974b; ROBBERSON and FRIED, 1974).

These observations strongly suggest that the origin of DNA replication is an essential, cis-acting site, required for the DNA replication process.

GRIFFITH et al. (1975) have detected (by electron-microscopy) one or more globular protein "knobs" associated with SV40 DNA in a high salt (1M NaCl or 5M CsCl) stable complex. The high salt resistant DNA-protein complex has been obtained from mature SV40 virions or the nucleoprotein complex (GREEN et al., 1971) extracted from SV40 infected cells. These globular proteins are associated with SV40 DNA at a restricted site in the genome and have been mapped at about 0.7 units in the physical restriction enzyme map (GRIFFITH et al., 1975). This site is close to the origin of DNA replication (0.67 units) and this fact has given rise to the speculation that these globular proteins could be involved in initiation of DNA replication. The properties of this salt-resistant tightly bound protein(s) isolated from mature virions are similar to those expected from the phenotype of tsD mutants. These mutants apparently code for a virion protein and tsD mutants fail to express any viral functions at the nonpermissive temperature (CHOU and MARTIN, 1974). The salt-stable protein-DNA complex might then be involved in regulatory processes during transcription and/or DNA replication (GRIFFITH et al., 1975).

The unique nature of the origin of SV40 DNA replication suggests that the proteins involved in the start of DNA replication must recognize a unique base sequence or structure (DNA-protein complex) in the SV40 chromosome. It is not presently clear how this is accomplished but the unusual base sequence repeats (see section B.2) (DAHR et al., 1974a) near the origin of replication, and the presence of the globular salt-resistant proteins (GRIFFITH et al., 1975) fit these criteria.

2. Polynucleotide Chain Propagation

Once DNA synthesis has begun, replication proceeds bidirectionally from the unique origin (DANNA and NATHANS, 1972; FAREED et al., 1972; JAENISCH et al., 1971; BOURGAUX and BOURGAUX-RAMOISY, 1971). Replicative intermediates labeled for very short time intervals (15–45 seconds at 37° C) with ^{3}H-thymidine contain small nascent DNA chains that sediment at 4S (about 150 nucleotides) in alkaline pH sucrose gradients. These fragments can be chased into longer growing progeny strands of SV40 DNA and therefore likely represent true intermediates of the new daughter strands during elongation (FAREED and SALZMAN, 1972; MAGNUSSON et al., 1973). These fragments are similar (but smaller) to the Okazaki pieces found in replicating *Escherichia coli* chromosomes (OKAZAKI et al., 1968). Oligoribonucleotides have been detected in polyoma DNA at the 5′ ends of many of the 4S fragments (MAGNUSSON et al., 1973; PIGIET et al., 1974; REICHARD et al., 1974; HUNTER and FRANCKE, 1974b). This RNA species is homogeneous in size (about a decanucleotide long), and is clearly not composed of an unique sequence of oligoribonucleotides (REICHARD et al., 1974). The 5′ end of this RNA preferentially begins with either AMP or GMP while the 3′ end (closest to the 4S DNA fragment) can be

composed of any of the four nucleotides (AMP, GMP, UMP, CMP) (REICHARD et al., 1974; PIGIET et al., 1974). The presence of an "initiator RNA" molecule satisfies the primer requirements for DNA polymerase. Each 4S fragment then is initiated by an RNA molecule (of a relatively constant size) during discontinuous growth of the polynucleotide chain.

This discontinuous mode of synthesis can be exaggerated by the addition of inhibitors of DNA synthesis such as hydroxyurea (HU) (MAGNUSSON et al., 1973; MAGNUSSON, 1973; LAIPIS and LEVINE, 1973) or fluorodeoxyuridine (Fudr) (SALZMAN and THOREN, 1973). These inhibitors apparently limit the levels of one or more deoxyribonucleoside triphosphates and thus slow the rate of polynucleotide chain elongation (SKOOG and NORDENSKJOLD, 1971; LAIPIS and LEVINE, 1973). Under these conditions, the 4S viral DNA fragments are synthesized at a slow rate. The low levels of the deoxyribonucleoside triphosphates appear to drastically reduce the ability of these 4S fragments to be joined to the growing polynucleotide strand (Fig. 2). These 4S fragments are not adjacent to the longer growing strands of viral DNA but are separated by gaps (single-stranded regions) of one or more nucleotides (LAIPIS and LEVINE, 1973; OTTO and REICHARD, 1975). The existence of such gaps was demonstrated by treating (in vivo) the SV40 replicative intermediates with T-4 DNA polymerase and *E. coli* DNA-ligase in a repair or gap-filling reaction. Neither of these enzymes alone was sufficient to join the 4S fragments to the longer progeny strand of DNA. If the 4S fragment was adjacent to the growing progeny strand of DNA, and contained the proper 5′-PO_4 and 3′-OH groups, ligase alone could seal these 4S fragments to the growing DNA strand. When both DNA polymerase and *E. coli* ligase were employed in vitro, up to 80 percent of the 4S fragments were joined to the longer growing polynucleotide chain of SV40 DNA (LAIPIS and LEVINE, 1973) demonstrating the need for both DNA synthesis and ligation to fill the gaps between the 4S fragment and the progeny strand of DNA. It is not unreasonable to suspect that such gaps are generated by the removal of the oligoribonucleotide initiator RNA's at the 5′ end of the 4S fragments. RNase H would be a good candidate for the excision of these ribonucleotide sequences.

HU and Fudr lower the levels of dXTP's in the cell and this appears to severely inhibit the gap-filling process while 4S fragments continue to be synthesized, albeit at a slower rate (MAGNUSSON et al., 1973; SALZMAN and THOREN, 1973; LAIPIS and LEVINE, 1973). This differential inhibition of these two processes (synthesis of 4S fragments and gap-filling) has been explained by postulating that different DNA polymerases, with different Kms for the dXTPs are involved in these two distinct steps of polynucleotide chain elongation (MAGNUSSON et al., 1973; SALZMAN and THOREN, 1973; LAIPIS and LEVINE, 1973). A similar conclusion has been deduced from studies with in vitro nuclei isolated from polyoma-infected cells (FRANCKE and HUNTER, 1975; OTTO and REICHARD, 1975). When relatively crude nuclei preparations (containing contaminating cytoplasmic fractions) are employed to study polyoma DNA replication in vitro, most of the viral DNA replicative intermediates continue

their synthesis of viral DNA and some of these molecules complete replication to produce a component I DNA (Winnacker et al., 1972; Magnusson et al., 1972; Hunter and Francke, 1974a). Highly purified nuclei from polyoma infected cells have a greatly reduced capacity to continue viral DNA synthesis. Predominantly 4S fragments are synthesized in these "purified" nuclei and the gap-filling steps are severely inhibited. The addition of cytoplasmic factors to these nuclei restores their ability to fill the gaps between 4S fragments and continue elongation (Francke and Hunter, 1975). The cytoplasmic alpha or maxi DNA polymerase (Chang and Bollum, 1971; Weissbach et al., 1971; Sedwick et al., 1972) may be one of these factors (the beta or mini polymerase has a nuclear location) but is clearly not the only cytoplasmic component required to complete this system (Franke and Hunter, 1975). The addition of *E. coli* DNA polymerase I and calf thymus DNA-ligase to purified polyoma-infected cell nuclei restored the gap-filling ability of these nuclei (Otto and Reichard, 1975).

The picture of polynucleotide chain elongation that emerges from these studies can now be summarized (Fig. 2). The DNA helical strands ahead of the replication fork are either denatured with the aid of cellular DNA unwinding protein (Herrick, 1973), or separated by the synthesis of short oligoribonucleotide primers which are hydrogen bonded to one or both strands of DNA. These oligoribonucleotides are then employed as primers for the synthesis of short 4S oligodeoxyribonucleotides. RNase H may then remove the initiator RNA leaving a gap between the 4S fragment and the growing longer polynucleotide strand. DNA polymerase (possibly different from the one that synthesized the 4S fragments) then is employed to fill in these gaps and DNA-ligase is required to seal the 4S fragment to the growing chain of viral DNA. All known DNA polymerases require a primer that contains a free 3′OH group for the addition of 5′-triphosphate deoxyribonucleosides. Both sides of the replication fork (Fig. 2) contain the long linear growing strands of DNA, one side terminates in a $5'PO_4$ group and the other a 3′OH group, as is required by the antiparallel nature of these DNA molecules. The presence of an initiator RNA molecule and the discontinuous mode of replication with the synthesis of 4S fragments explains how one side of the replication fork is synthesized in the 5′ to 3′ direction (as is required by the properties of DNA polymerase) even though the longer progeny DNA chain terminates on a 5′-phosphate group (Fig. 2). On the other side of this fork however the progeny DNA terminates in a 3′OH group and synthesis thus could be either continuous or discontinuous. Several groups (Fareed et al., 1973; Magnusson et al., 1973; Francke and Hunter, 1974; Laipis and Levine, 1973) have studied this question and there is some disagreement in their conclusions. If DNA replication was discontinuous on both sides of the replication fork, then 4S fragments would be synthesized from DNA strands with complementary polynucleotide sequences. If, on the other hand, DNA was synthesized discontinuously on one strand (with a 5′-phosphate terminus) and continuously on the complementary strand (3′-hydroxyl terminus) then the collection of 4S fragments should not hybridize

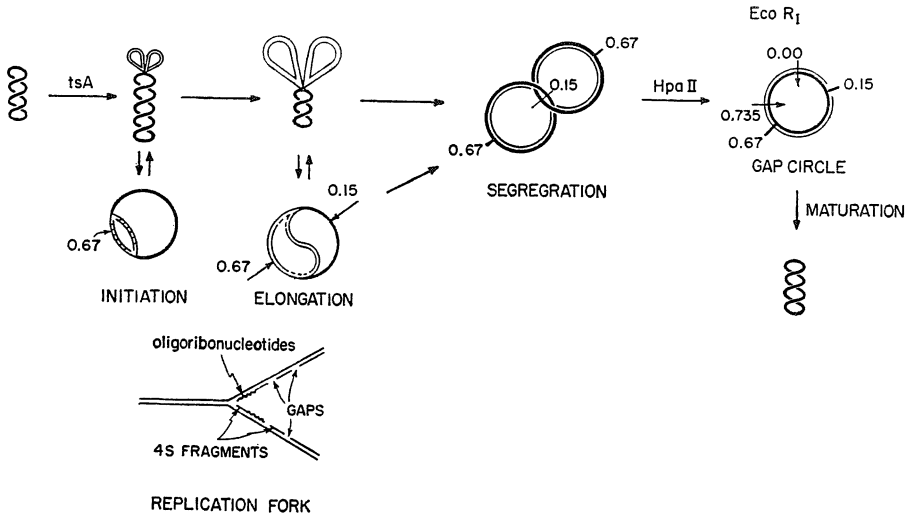


Fig. 2. A model depicting the stages in SV40 DNA replication. Initiation of DNA synthesis occurs at a unique site (0.67) in the genome and requires the A gene product. Polynucleotide chain elongation proceeds from this origin site bidirectionally by addition of 4S fragments of DNA at replication fork. The closed circular and superhelical replicative intermediates are in equilibrium with the active relaxed replicative form. When replication proceeds about 180° around the circle (0.15 units) segregation of the two daughter molecules takes place resulting in a 16S circular molecule containing a single-stranded region or gap at terminus (0.15) of replication. The restriction enzymes Eco R_I (cuts at 0.00) and Hpa II (cuts at 0.735) have been employed to localize position of gap in this post-segregational intermediate. Maturation of this DNA then requires a DNA polymerase and polynucleotide ligase activity to produce a closed circular mature viral DNA molecule

with themselves. In a number of experiments to test whether the 4S fragments would self-anneal or not, FAREED et al. (1973) and LAIPIS and LEVINE (1973) found relatively high levels of self-annealing for SV40. A similar result was obtained for polyoma by PIGIET et al. (1973) while FRANCKE and HUNTER (1974) have detected low levels of self-complementary 4S fragments. The latter group have also presented kinetic arguments to support their contention that replication is continuous on one of the DNA strands of polyoma (FRANCKE and HUNTER, 1974). This point remains in some dispute.

3. Segregation of the Two Daughter Molecules

The steps in polynucleotide chain elongation continue until the two replication forks approach each other at the terminus region, which is 180 degrees from the origin (0.15 units on the restriction enzyme map) (DANNA and NATHANS, 1972). The two interlocked replicating circles then separate forming a 16S circular intermediate (FAREED et al., 1973; LAIPIS and LEVINE, 1973; LAIPIS et al., 1975). This intermediate contains a closed circular template strand and a linear progeny strand that is shorter than a unit length SV40 linear molecule (LAIPIS and LEVINE, 1973; LAIPIS et al., 1975). This 16S intermediate

thus contains a single-stranded region of DNA at or near the terminus of replication (Laipis et al., 1975) and segregation occurs prior to the completion of the entire length of SV40 DNA. It is not yet clear, however, whether all of the segregated intermediates contain this characteristic gap (single-stranded region) at the terminus of replication. It remains possible that one progeny DNA molecule has completed the duplication process and the complementary daughter molecule is segregated containing a short single-stranded region. Nor is it clear which enzyme activities are involved in segregation or even how the two interlocked daughter molecules separate (presumably through a linear intermediate) and reform circular DNAs.

Failure to segregate the two replicating circles apparently generates a catenated (interlocked) dimer (Jaenisch and Levine, 1973). These catenated dimers are themselves unstable and decay, via a recombination event, into either two monomers or a concatenated (circular) dimer (Jaenisch and Levine, 1973). Protein synthesis may well be required for efficient segregation because cycloheximide treatment of SV40-infected cells increases the frequency (2–4 times) of catenated dimers (Jaenisch and Levine, 1972, 1973).

4. Maturation of Progeny Molecules

The postsegregational intermediates of SV40 DNA are composed of a circular template molecule and a linear strand of newly synthesized progeny DNA (Fareed et al., 1973). As discussed above, this intermediate contains a gap (single-stranded region) at the terminus region of replication (Laipis et al., 1975). While *E. coli* DNA-ligase alone failed to convert these molecules to closed circles, T-4 polymerase plus *E. coli* ligase repaired this DNA (at 30 to 50 percent efficiency) and permitted ring closure to occur (Laipis and Levine, 1973; Laipis et al., 1975). Thus in SV40-infected cells, both DNA polymerase and polynucleotide ligase are required to finish replication after segregation, and generate mature closed circular DNA molecules.

All of these stages of DNA replication are summarized in Fig. 2.

F. The Unwinding Problem during DNA Replication of Closed Circular Molecules

During semiconservative replication, the two template strands must separate to permit continued synthesis. Because of the topological constraints of closed circular molecules (Vinograd and Lebowitz, 1966) for every turn of the Watson-Crick helix (secondary structure) removed, either a negative superhelical turn must be removed or a positive superhelical turn is put into the tertiary structure. The replicative intermediates as isolated from infected cells contain closed circular template strands of DNA (Jaenisch et al., 1971; Sebring et al., 1971) and the unreplicated portion of these molecules contains superhelical turns in the same sense (negative) as mature component I SV40 DNA (Mayer and Levine, 1972; Sebring et al., 1974). Because of this, it

appears unlikely that extensive template strand separation and therefore polynucleotide chain elongation is occurring on the closed circular replicative intermediates. It becomes necessary then to introduce an interruption or break in the phosphodiester backbone of the template DNA to permit DNA synthesis (SEBRING et al., 1971; MAYER and LEVINE, 1972). This may be accomplished by a nuclease and DNA ligase acting in concert or by a superhelical untwisting activity that appears to combine these two activities (CHAMPOUX and DULBECCO, 1972; WANG, 1971).

As DNA replication proceeds the progeny strands become a larger percentage of the replicative intermediate DNA and the target size of the template DNA per molecule therefore decreases. If the activity responsible for relaxing these DNA molecules acts randomly throughout the genome, then this predicts that newly initiated DNA molecules would be relaxed more frequently (have a larger target for the relaxation enzymes), and replicate at a faster rate than almost completed replicative intermediates. Several experimental observations support this hypothesis. Labeling of the replicative intermediates with short pulses of ^{3}H-thymidine preferentially labels the newly initiated replicative intermediates (LEVINE et al., 1970; MAYER and LEVINE, 1972). In addition, the pool of replicative molecules contains a larger proportion of almost completed DNA intermediates than those molecules just beginning replication (LEVINE et al., 1970; MAYER and LEVINE, 1972; HUNTER and FRANCKE, 1974). These results are most simply interpreted as a decreasing rate of replication with an increasing degree of duplication. They also indicate that the closed circular replicative intermediates isolated from SV40-infected cells (JAENISCH et al., 1971; SEBRING et al., 1971) are "waiting forms" not actively involved in polynucleotide chain elongation. This is consistent with the observation that one round of SV40 DNA replication appears to take between 5–15 min to complete in vivo (LEVINE et al., 1970; BOURGAUX et al., 1971; DANNA and NATHANS, 1972). The rate of polynucleotide chain elongation of mammalian cell DNA is about 2 μ per min and it should then take about 25 sec (SV40 DNA is 1.66 μ and replication is bidirectional) to complete one round of SV40 DNA replication in the absence of any rate limiting step. The relaxation of the late replicative intermediates of polyoma or SV40 DNA may then give rise to the slow, rate limiting step in DNA replication (LEVINE et al., 1970; MAYER and LEVINE, 1972).

G. Polyoma and SV40 Nucleoprotein Complexes as the Natural Template for DNA Replication

In all the experiments discussed previously, the replicative form of SV40 and polyoma DNA have been prepared for analysis after cell lysis in SDS at high salt concentrations. This removes all of the protein normally required for the replication process. Employing a different cell lysis procedure (Triton X100 and 0.25 M NaCl), GREEN et al. (1971) have recovered polyoma DNA in close association with cellular and/or viral proteins. A similar nucleoprotein complex

has been recovered from SV40-infected cells (WHITE and EASON, 1971). These DNA protein complexes sediment at about 50S in neutral sucrose gradients. Replicating SV40 DNA can be found in this nucleoprotein complex form and sediments at the leading edge of the 50S peak (WHITE and EASON, 1971; LEVINE et al., 1974). The protein to DNA ratio of this complex is about 0.82–0.95 and the major proteins associated with viral DNA appear to be cellular histones and some viral coat proteins (SEN et al., 1974; MCMILLEN and CONSIGLI, 1974). Indeed this complex appears to be a precursor of mature virus (MCMILLEN and CONSIGLI, 1974).

Like mammalian DNA, SV40 DNA is apparently duplicated in close association with cellular histones. Indeed, the SV40 nuclear protein complex is associated with the superhelical untwisting activity described by CHAMPOUX and DULBECCO (1972) that may be involved in relaxation of the SV40 replicative intermediates (SEN and LEVINE, 1974). It should be informative to employ this replicating DNA-nucleoprotein complex as a template in future studies.

III. Adenoviruses — Introduction

The adenovirus group comprises about eighty different viruses with similar structure that cause respiratory diseases in their natural hosts and can induce tumors when injected into newborn rodents (TRENTIN et al., 1962). In addition to the thirty-three serologically distinct types of human origin, adenoviruses have been found in a variety of animal species such as monkeys, dogs, birds, and mice.

Like the papovaviruses, the adenoviruses have an icosahedral structure (T-25) but are much larger and more complex. The virion particle weighs about 175×10^6 daltons and contains 12–14 percent DNA. The virion of human serotype 2 (Ad 2), which has been studied in detail, is composed of three major capsid proteins (hexon, penton base, and fiber) and a core consisting of two different core proteins which are strongly bound to the DNA. Using polyacrylamide gel elctrophoresis in the presence of denaturing agents like sodium dodecyl sulphate (SDS) at least nine different polypeptides can be detected in the purified virion. Five of these can be ascribed to the capsid and core proteins while the exact location of the other four polypeptides in the virion is not well established (PHILIPSON and LINDBERG, 1974). In contrast to the papovaviruses, no evidence exists for the presence of cellular histones in the adenovirus particle.

A. Lytic Infection

The lytic cycle has been most intensively studied of types 2 and 5 adenoviruses with human cells (HeLa or KB) as the permissive host. The virus adsorbs, presumably to a specific receptor site at the plasma membrane, penetrates into the cell and is uncoated within 15 min after attachment to the cell surface (SUSSENBACH, 1967; LONBERG-HOLM and PHILIPSON, 1969). The DNA enters the nucleus and within 1–2 h after infection the first viral transcripts

can be detected in the cytoplasm. These early viral transcripts represent about 22 percent of the genome coding capacity. They hybridize with both strands (poly UG-light and heavy density) of the DNA and come from at least four distinct regions of the adenovirus genome (SHARP et al., 1974; PHILIPSON et al. 1974; CRAIG et al., 1974) (Table 3). At least three early infected cell specific proteins have been detected by SDS-polyacrylamide gel electrophoresis (RUSSELL and SKEHEL, 1972; ANDERSON et al, 1973; WALTER and MAIZEL, 1974). One of these proteins has been isolated in a native form, based upon its ability to bind to single stranded DNA (VAN DER VLIET and LEVINE, 1973). This protein has a molecular weight of 72000 and is coded for by one of the early transcripts from the Ad2 Eco R_I B fragment (Table 3) (ANDERSON, LEWIS and GESTELAND, personal communication). The mutant H5ts125 (for nomenclature see GINSBERG et al., 1973) appears to be a temperature-sensitive mutant in an early viral gene that codes for this DNA-binding protein (VAN DER VLIET et al., 1975). H5ts125 and H5ts36 (WILLIAMS et al., 1974; GINSBERG et al., 1974) are temperature-sensitive mutants in two different (complementing) adenovirus genes, both of which are required for viral DNA replication, at early times after infection. Viral DNA replication begins at 6–10 hr after infection and coincides with a gradual decreases in cellular DNA synthesis. Viral DNA synthesis is required to initiate transcription of some late viral genes (GINSBERG, personal communication). The polysomal late transcripts come from 90–100 percent of the adenovirus genome but one class of early m-RNA may not be synthesized at late times (LUCAS and GINSBERG, 1971). Early and late species of polysomal viral RNA have been mapped on the Eco R_I cleavage map and these data are presented in Table 3. By 18 hours after infection 60 percent of the m-RNA synthesized is of viral origin (RASKAS and OKUBO, 1971) and 80 percent of the polypeptides made are viral structural proteins (WHITE et al., 1969). There are at least 15 distinct genetic complementation groups that function at late times (WILLIAMS et al., 1974; GINSBERG et al., 1974) as determined with the H5ts mutants. Late proteins have been detected by SDS-polyacrylamide gel electrophoresis and several of the virion proteins appear to be made as precursor polypeptide chains with a larger molecular weight which are then cleaved prior to or during viral assembly (ANDERSON et al., 1973). The virions are assembled in the nucleus where large amounts of capsomers and viral DNA accumulate, only about 10 percent of which are finally assembled into progeny virus.

Details of the mechanism of adenovirus DNA replication have only been studied with a limited number of adenoviruses. In this review we will deal exclusively with the results obtained from four human types (Ad2, Ad5, Ad12, and Ad31) and from an avian adenovirus, chick embryo lethal orphan virus (CELO). This virus is morphologically similar to the human adenoviruses but lacks the common group specific antigen. Its DNA is about 20 percent larger and the polypeptides derived from the virion, in particular the penton, differ from the human types (LAVER et al., 1971). Other recent review articles dealing in a more general way with the adenoviruses can be found elsewhere (SCHLESINGER, 1969; GREEN, 1970; PHILIPSON and LINDBERG, 1974).

B. The Structure and Properties of Adenovirus DNA

The DNA in adenovirus particles is tightly associated with the core proteins and can be extracted by treatment with a proteolytic enzyme like papain or pronase followed by phenol and/or sodium dodecyl sulphate (GREEN and PINA, 1964; VAN DER EB et al., 1969). Electron microscopy and sedimentation have been used to show that adenovirus DNA extracted in this manner has a linear duplex structure and that both polynucleotide strands of the molecule are continuous throughout their length (GREEN et al., 1967; VAN DER EB et al., 1969; YOUNGHUSBAND and BELLETT, 1971). The human adenovirions contain DNA molecules with molecular weigths in the range of $20–25 \times 10^6$ daltons, which may code for 25–50 polypeptides of average size (VAN DER EB and VAN KESTEREN, 1966; GREEN et al., 1967). Since the virion polypeptides represent about 35 percent of the coding capacity of the viral genome, adenovirus DNA must code for a considerable number of still unknown nonstructural proteins.

Based on their oncogeneicity the human adenoviruses have been divided into three groups A, B, and C. DNA from the highly oncogenic group A viruses (Ad12, Ad18, and Ad31) has a low guanine and cytosine (G+C) content (47–49 percent) while Ad3, Ad7, Ad8, Ad11, Ad14, and Ad16 from the weakly oncogenic group B have DNA's with 50–53 percent G+C. Ad1, Ad2, Ad5, and Ad6 of the nononcogenic group C viruses have the highest G+C content, 56–60 percent (PINA and GREEN, 1965). A similar relation between oncogenicity and G+C content of the DNA has not been found for the simian adenoviruses (GOODHEART, 1971).

The homology between human serotypes in the same group and between serotypes from different groups has been studied by DNA-DNA hybridization and heteroduplex mapping. DNA-DNA hybridization showed that DNA molecules from serotypes of group A share 80–85 percent of the base sequences, from group B 70–100 percent from group C 85–95 percent (GREEN, 1970). On the other hand, it was shown that serotypes from different groups have only 10–25 percent of their polynucleotide sequences in common.

The relationship between different serotypes was elegantly studied by heteroduplex mapping (GARON et al., 1973). As expected, the most extensive heterologies are observed in heteroduplexes prepared with DNA molecules from serotypes belonging to different groups (A,B,C). Within groups, heteroduplex molecules contained two specific regions in which the heterologies are mostly marked (about 0.5–0.6 and 0.8–0.9 map units, Table 3). These regions map at similar positions among heteroduplexes within each group. Using the single-strand specific endonuclease from *Neurospora crassa*, BARTOK et al. (1974) were able to digest specifically the heterologous regions from heteroduplexes of Ad2 and Ad5 DNA after which three specific double-stranded fragments were obtained.

1. Structural Properties of Adenovirus DNA

Several studies deal with the question whether adenovirus DNA has the ability to circularize. Short cohesive single-stranded molecular ends have never

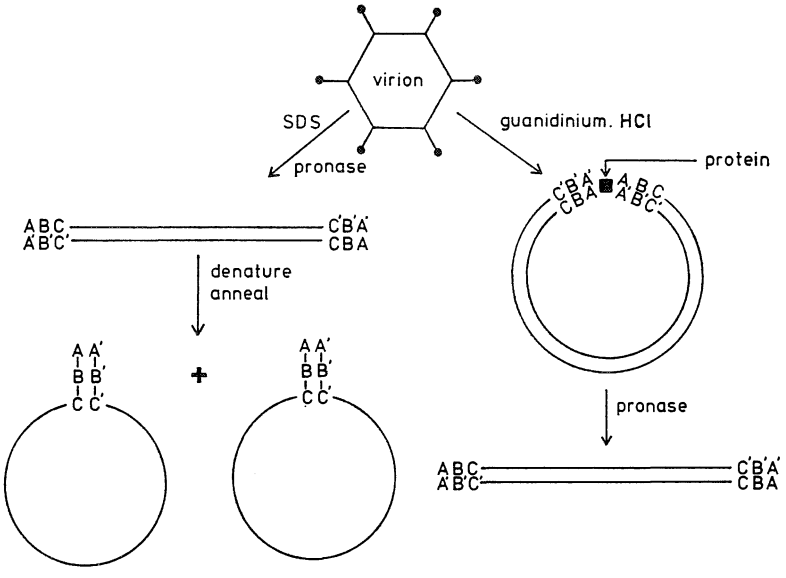


Fig. 3. Schematic diagram of different structural forms of adenovirus DNA. Treatment of isolated virions with pronase and sodium dodecyl sulphate yields linear double-stranded DNA molecules. After denaturation and annealing at low DNA concentrations single-stranded circles appear due to the presence of an inverted terminal repetition in adenovirus DNA. Extraction of virions with guanidinium HCl releases a circular DNA-protein complex which is linearized by a subsequent pronase digestion

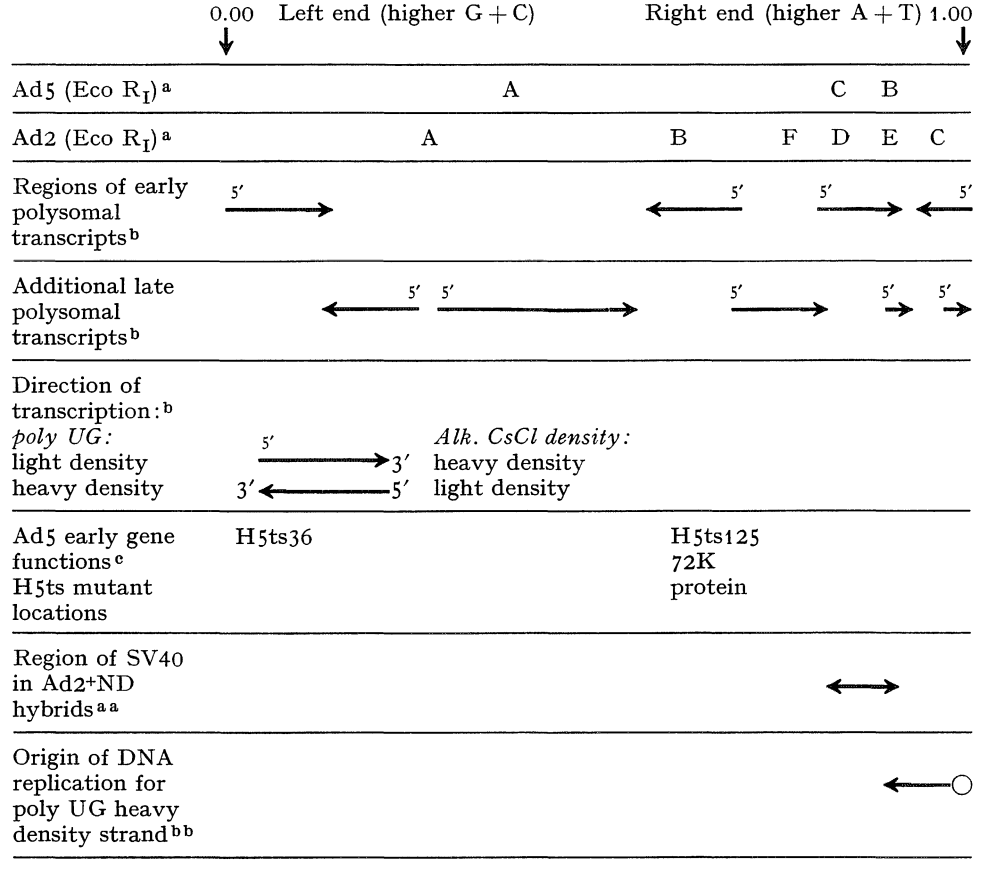
been detected, neither was circularization possible after digestion of native adenovirus DNA with *E. coli* exonuclease III, indicating that adenovirus DNA is not terminally redundant (GREEN et al., 1967; YOUNGHUSBAND and BELLETT, 1971). On the other hand, both strands of human and avian adenovirus DNA are able to form single-stranded circles after denaturation of native DNA followed by renaturation at low DNA concentrations (GARON et al., 1972; WOLFSON, and DRESSLER, 1972; ROBINSON and BELLETT, 1975) (Fig. 3).

Single-stranded circles cannot be generated if a limited number of nucleotides (350–1400 for the human serotypes and 110 for CELO virus) have been removed from the 3′-ends of the native DNA by digestion with *E. coli* exonuclease III before denaturation and annealing (GARON et al., 1972; ROBINSON and BELLETT, 1975). More direct measurements with the help of restriction enzymes which cleave native adenovirus DNA in or close to the terminal repetition show that the inverted terminal repetition of Ad2 DNA is 100–140 basepairs long (ROBERTS et al., 1974). Determination of short nucleotide sequences at the termini of Ad2 and Ad5 DNA has led to the following structure for both types of DNA (STEENBERGH et al., 1975).

$$\begin{array}{ll} {}^{3'}\text{OH-GpTpApGp-Cp Cp}\text{———}\text{GpGp-CpTpApGp}^{5'} \\ {}^{5'}\quad \text{pCpApTp Cp-GpGp}\text{———}\text{Cp Cp-GpApTpG-OH}_{3'} \end{array}$$

These sequences are consistent with an inverted terminal repetition and indicate that the repetition extends to the very ends of the molecule. Inverted

Table 3. Location of adenovirus type 2 transcripts, H5ts mutants, SV40 insertion and Ad5 replication site on the eco R_I cleavage map



[a] MULDER et al., 1974.
[b] SHARP et al., 1974; PHILIPSON et al., 1974; CRAIG et al., 1974.
[c] GRODZICKER et al., 1974a; GESTELAND and ANDERSON, personal communication.
[aa] KELLY and LEWIS, 1973; MORROW et al., 1973.
[bb] ELLENS et al., 1974; SUSSENBACH et al., 1974.

terminal repetitions have also been detected in linear DNA molecules from other sources, e.g., Tetrahymena mitochondrial DNA and adenovirus-associated virus DNA (ARNBERG et al., 1975; KOCZOT et al., 1973). It has been suggested that these inverted terminal repetitions may be involved in the replication of linear molecules, but their actual function is still obscure.

Another interesting property of adenovirus DNA has been discovered by BELLETT and coworkers. They isolated DNA from CELO and Ad2 virions in the absence of a proteolytic enzyme employing an extraction with guanidinium hydrochloride. The isolated DNA had a circular duplex structure while treatment of the double-stranded circles with pronase converted the circles into linear molecules (Fig. 3) (ROBINSON et al., 1973). Similar circular structures

have also been observed for Ad5 DNA (VAN WIELINK, personal communication). They consist of linear duplex molecules which are kept in a circular form by a protein linker. The proteins cannot be removed from the DNA by boiling of the DNA-protein complex in sodium dodecyl sulphate, suggesting that they are covalenty linked to the DNA (ROBINSON and BELLETT, 1974). The function of these DNA-protein complexes is still unknown.

The distribution of adenine-thymine and guanine-cytosine basepairs in Ad2, Ad5, Ad12, and CELO DNA has been studied by partial denaturation mapping (DOERFLER and KLEINSCHMIDT, 1970; ELLENS et al., 1974; DOERFLER et al., 1972; YOUNGHUSBAND and BELLETT, 1972). In all types of DNA a unique denaturation pattern was observed indicating that adenovirus DNA is not circularly permuted, but that all molecules have a specific nucleotide sequence. The high percentage of homology between Ad2 and Ad5 is reflected in the extensive similarity of their partial denaturation patterns. Both DNA molecules show regions rich in adenine-thymine at the left side of the molecule between 0.0 and 0.1 on the scale of fraction length (Table 3), while two other early melting regions are found at the right side of the molecule between 0.5 and 0.6 and between 0.8 and 1,0, respectively (DOERFLER and KLEINSCHMIDT, 1970; ELLENS et al., 1974). Since the DNA of Ad2 and Ad5 have asymmetric distribution of guanine-cytosine basepairs along the molecule, the two halves of the genome obtained by controlled shear can be separated by equilibrium density centrifugation of half molecules in Hg(II)-Cs_2SO_4 density gradients (KIMES and GREEN, 1970; DOERFLER and KLEINSCHMIDT, 1970; GRAHAM et al. 1947a). However, other methods must be employed if a more specific fragmentation of adenovirus DNA is required.

2. Restriction Enzyme Maps

The restriction enzymes Eco R_I (PETTERSSON et al., 1973; MULDER et al., 1974) and HpaI (SHARP et al., 1974; PHILIPSON et al., 1974; MULDER et al., 1974) have been employed to generate unique fragments of Ad2 and Ad5 DNA that have been ordered into a physical map of the genome (Table 3). These fragments have been especially useful in the study of viral transcription and determining which portions of the adenovirus genome can be found in transformed cells (GALLIMORE et al., 1974).

A comparison of the Eco R_I cleavage sites of two closely related adenovirus serotypes 2 and 5 indicates several interesting similarities and differences. Ad2 DNA is cleaved into six fragments as shown in Table 3. Ad5 DNA is cleaved into three fragments with molecular weigths of A, 17.4×10^6; B, 3.7×10^6; C, 1.7×10^6. The order of these fragments is A:C:B with the largest fragment A at the left end of the molecule (MULDER et al., 1974). The cleavage sites between fragments A:B, B:F, and E:C in Ad2 DNA are absent in Ad5 DNA while the remaining two sites (F:D and D:E) are identical in Ad2 and 5 DNA (Table 3).

3. Separation of Complementary DNA Strands

Separation of the intact complementary strands of Ad2, Ad5, Ad7, and Ad12 DNA has been performed using poly UG or poly IG (LANDGRAF-LEURS and GREEN, 1971; PATCH et al., 1972; TIBBETTS et al., 1974). The two complementary strands bind these copolymers to a different extent which leads to different buoyant densities in CsCl. Complementary strands of Ad2 and Ad5 DNA have also been separated by alkaline CsCl equilibrium density centrifugation (SUSSENBACH et al., 1973; SHARP et al., 1974). The buoyant densities of the two strands in alkaline CsCl differ 2–4 mg/ml which is sufficient for separation. The heavy strands of Ad2 and Ad5 DNA obtained by the poly UG procedure have the lower buoyant density in alkaline CsCl (TIBBETTS et al., 1974; VLAK, personal communication) (Table 3). The complementary strands of fragments of Ad2 DNA produced with restriction enzymes have been isolated either by annealing of the denaturend fragments in the presence of an excess of one of the intact complementary strands followed by separation of the partial duplex and the remaining single strand DNA, or by gel electrophoresis of the denatured fragments (TIBBETTS and PETTERSSON, 1974; SHARP et al., 1974). The separated strands of Ad2 DNA fragments have successfully been used in the analysis of transcription of Ad2 DNA (Table 3).

4. Biological Activity of Purified Adenovirus DNA

Although it was known that simian adenovirus DNA itself was infectious (BURNETT and HARRINGTON, 1968), human adenovirus DNA has been considered to be noninfectious. NICOLSON and MCALLISTER (1972) using DEAE dextran first found infectivity with a very low efficiency for purified Ad1 DNA while GRAHAM and VAN DER EB (1973a) using DNA-calcium phosphate coprecipitates obtained much higher efficiencies of infectivity with Ad5 DNA. Employing the latter technique, the transforming activity of adenovirus DNA could also be demonstrated (GRAHAM and VAN DER EB, 1973b). Fragmentation of the DNA destroys the infectivity but hardly affects the transforming activity (GRAHAM et al., 1974a). Transformation studies with specific fragments of Ad5 and Ad2 DNA, produced with the aid of restriction enzymes, led to the conclusion that the genes responsible for the transforming activity are located in the utmost left 7 percent of the viral genome (Table 3) (GRAHAM et al., 1947a, b). The same part of the Ad2 genome is present in all Ad2-transformed cell lines examined and is likely to be necessary for the maintenance of the transformed state of these cells (GALLIMORE et al., 1974).

C. Genetics of Adenoviruses

The class of mutations which has been most frequently used in genetic studies of adenovirus is the temperature-sensitive conditional lethal type. These mutants are unable to multiply at high temperatures, usually 38–40° C. They have been isolated after treatment with a variety of mutagens including

Table 4. Classification of temperature-sensitive mutants into complementation groups

Serotype[b]	Number of mutants tested	Number of different complementation groups found	Number of early classes (no viral DNA synthesis at 39.5°)	Genetic map obtained by recombination analysis	References
H2	20	13	0	Linear	BEGIN and WEBER, 1975
H5	51	16 } 17[a]	1 } 2[a]	Linear	WILLIAMS et al., 1974
	15	6	2	—	GINSBERG et al., 1973
	10	—	—	—	TAKAHASHI, 1972
H12	10	—	—	—	LUNDHOLM and DOERFLER, 1971
	34	13	3	—	SHIRPKI et al., 1972
	10	6	2		LEDINKO, 1974
	3	3	1	—	RUBENSTEIN and GINSBERG, 1975
H31	12	8	1	—	SUZUKI et al., 1972
CELO	49	5[c]	—	—	ISHIBASHI, 1971

[a] A total of 17 different complementation groups, 2 of which are early classes, were found after comparison of the mutants.
[b] The nomenclature proposed by GINSBERG et al. (1973) was used.
[c] The CELO mutants could be classified into 5 functional groups on the basis of the different morphological changes they induced in infected chicken kidney cells. No complementation analysis was performed in this case.

hydroxylamine, nitrous acid, nitrosoguanidine, and 5-bromodeoxyuridine. At present, temperature-sensitive mutants have been described for the human types Ad2, Ad5, Ad12, and Ad31 and for the avian adenovirus CELO. Using complementation analysis these mutants could be classified into a number of functional groups, with a maximum of 17 complementation groups for Ad5. This is $^1/_3$–$^1/_2$ of the total number of genes theoretically possible based on the genome size.

A summary of the results obtained by complementation analysis is given in Table 4. Unfortunately the various Ad12 mutants have not yet been compared and therefore the total number of functional Ad12 groups is unknown. Intertypic complementation has been observed between Ad5 and Ad12 for a number of ts mutants (WILLIAMS et al., 1974; GINSBERG et al., 1974). This indicates the presence of a significant number of common functions in these two serotypes, in spite of the large differences in nucleotide sequences revealed by partial denaturation of heteroduplexes (see Section III B).

1. Location of Ad5 Mutations on the DNA Molecule

Preliminary genetic maps have been constructed for Ad2 and Ad5 based on recombination frequencies in two factor crosses (BEGIN and WEBER, 1975; WILLIAMS et al., 1974). Both maps show a linear order for the ts mutants

tested. This is in agreement with the linearity of the DNA molecule and suggests that during recombination the DNA may not be in a circular form.

In order to understand the relation between the order of mutations on the genetic map and the actual physical location of the mutations on the DNA molecule, GRODZICKER et al. (1974a) used an ingenious approach. They isolated wild type recombinants from an intertypic cross between Ad5 ts mutants and ts mutants from the nondefective Ad2-SV40 hybrid Ad2+ND_1 (see Section IV B). The latter ts mutants are all located on the Ad2-part of the hybrid genome (GRODZICKER et al., 1974a). Intertypic recombination can occur because the genomes of Ad5 and Ad2 are closely related. Such a cross is expected to yield a wild type recombinant with a hybrid genome, containing Ad5 DNA sequences in the region where the Ad2+ND1 mutation was located, and Ad2+ND1 sequences at the location of the original Ad5 mutation. Subsequent analysis of the recombinant DNA using the restriction enzymes Eco RI and Hpa I revealed which region of the recombinant genome contained Ad5 sequences and which Ad2+ND1 sequences. This analysis was possible because the restriction enzyme cleavage maps of Ad2 and Ad5 are different (see Table 3). In this way the crossover points in those molecules yielding wild type recombinants could be established and a physical map of the mutant order was obtained. This physical map correlates well with the genetic map based upon recombination frequencies and shows that one of the early mutants, H5ts36, maps at the molecular left side of the molecule. While the other, H5ts125, maps at the position of the Ad2 Eco RI B fragment (see Table 3). Mutants defective in hexon transport map at 65–80 percent from the left molecular end while another late mutant, H5ts22, defective in fiber synthesis, maps about 10 percent from the right end of the DNA molecule.

2. Properties of early Temperature-Sensitive Mutants Defective in Viral DNA Synthesis

Most of the ts mutants characterized so far are defective in a late function and only a limited number of DNA negative mutants have been isolated. These early mutants do not synthesize viral DNA at the nonpermissive temperature. Two early classes were found in Ad5, three in Ad12, and one in Ad31.

The three classes of Ad12 mutants, H12tsA, B, and C, were investigated in some detail. All three classes are T-antigen positive and all these mutants inhibit cellular DNA synthesis at the same rate as wild type virus, at the nonpermissive temperature (SHIROKI and SHIMOJO, 1974). Several experiments were performed to establish which step in viral DNA synthesis was blocked with these various mutants. When mutant-infected cells were grown at the permissive temperature and subsequently shifted to the nonpermissive temperature, Ad12 DNA synthesis was inhibited in all three classes of mutants, albeit at different rates. Class tsA mutants stop faster (within 60 min) than tsB or tsC mutants. Density shift experiments performed at nonpermissive temperatures showed the absence of a second round of viral DNA replication at

the nonpermissive temperature with all three mutant classes. On the basis of these data and pulse-chase experiments, SHIROKI and SHIMOJO (1974) concluded that all three functions were required for the initiation of Ad12 DNA replication. However, this preliminary conclusion has not yet been verified by analysis of the various forms of replicating DNA present after a shift-up in temperature, a method employed to establish the initiator function of the tsA gene product of polyoma and SV40 (TEGTMEYER, 1972; FRANCKE and ECKHART, 1973).

A number of experiments have been performed with the two classes of Ad5 DNA negative mutants. These mutants fall into two early complementation groups: (1) H5ts125 and (2) H5ts36, H5ts149.

a) H5ts125 can serve as a helper virus for multiplication of type 2 adeno-associated virus at 39.5°C, as can H5ts149 (GINSBERG et al., 1974). The biochemical mechanism of this helper function is unfortunately unknown.

b) Genetic dominance studies have shown that the H5ts125 gene product is required in stoichiometric amounts for virus production. Similar results were found for several late mutants defective in capsid protein synthesis. The H5ts36 gene product, however, is required in catalytic amounts (AUSTIN, YOUNG, and WILLIAMS, unpublished results).

c) When infection is carried out at 32°C and the mutant-infected cells are shifted to 39.5°C, viral DNA synthesis is reduced significantly (80–95 percent) in H5ts125 infected cells within 30 min. H5ts36 infected cells continue to synthesize Ad5 DNA for at least 6 hr after the temperature shift, followed by a decline in the rate of viral DNA synthesis (LEVINE et al., 1974; VAN DER VLIET and SUSSENBACH, 1975). Cells infected with H5ts149 exhibit an intermediate behavior, with 50 percent reduction 1 hr after temperature increase (GINSBERG et al., 1974). These results indicate strongly that both classes of mutant gene products (H5ts125 and H5ts36-H5ts149) are continually required for an optimal rate of viral DNA replication.

d) Analysis of pulse-labeled viral DNA synthesized in H5ts125-infected cells after a shift up in temperature did not show any accumulation of replicating intermediates, suggesting that chain propagation could occur normally under nonpermissive conditions (VAN DER VLIET and SUSSENBACH, 1975). This notion was confirmed by a study of viral DNA synthesis at 39°C in isolated nuclei obtained from mutant-infected cells. In isolated muclei, replicating molecules can be completed, but initiation of new replication rounds does not occur even with wild type virus (VAN DER VLIET and SUSSENBACH, 1972). No difference in rate of DNA synthesis between wild type and H5ts125 was observed in this nuclear system (VAN DER VLIET, unpublished results). Therefore the H5ts125 gene product does not seem to be required for chain propagation or termination of replicating molecules, but may be needed for initiation. Density shift experiments performed with H5ts125 infected cells shifted to 39.5°C show the absence of molecules replicating more than once, in agreement with an initiation function for the H5ts125 gene product (VAN DER VLIET and SUSSENBACH, 1975).

In summary, these results show that at least 2 viral gene functions are required for replication of Ad5 DNA. One of these, the H5ts125 gene product has been isolated and characterized as a DNA binding protein present in large amounts in the infected cells (VAN DER VLIET et al., 1975) and a number of its properties will be described in section F. 2. The second gene product (H5ts36 and H5ts149) has not been characterized yet. It should have a catalytic activity serving in some step during initiation of adenovirus DNA replication. Experimental evidence obtained by WILLIAMS et al. (1974) suggests that the H5ts36 gene product is also required for the initiation, but not for the maintenance of the transformed state in rat embryo cells.

D. The Isolation and Properties of Replicating DNA

The replication of adenovirus DNA starts between 6–10 hr postinfection in the nucleus of infected cells. At 13 hr postinfection the synthesis of adenovirus DNA has reached a maximal rate, while the synthesis of host cell DNA is almost completely inhibited (GINSBERG et al., 1967; MANTYJARVI and RUSSELL, 1968). The inhibition of host DNA synthesis facilitates considerably the study of viral DNA replication, since radioactive DNA precursors are thus exclusively incorporated into viral DNA.

The question at which site viral DNA replication takes place in the cell nucleus has been tackled by several investigators using electron-microscopic autoradiography. Replicating Ad12 DNA, pulse-labeled for 3 min with ^{3}H-thymidine, is found over the entire nucleus and not preferentially at the nuclear membrane. After a one hour chase period the distribution of radioactivity over the nucleus has not changed (SHIROKI et al., 1974). Similar autoradiographic results were also obtained by SIMMONS et al. (1974) for Ad2 and by VLAK et al. (1975b) for Ad5. These studies clearly show that replication of adenovirus DNA takes place throughout the cell nucleus and no preferential site has yet been implicated.

Replicating adenovirus DNA has been obtained from isolated nuclei of infected cells (SUSSENBACH and VAN DER VLIET, 1972a; VAN DER VLIET and SUSSENBACH, 1972) as well as from intact cells (BELLETT and YOUNGHUSBAND, 1972; VAN DER EB, 1973; PETTERSSON, 1973; BOURGAUX-RAMOISY et al., 1974; ELLENS et al., 1974). The newly synthesized DNA has been labeled by addition of radioactive thymidine to intact infected cells or by incubation of isolated nuclei in the presence of radioactive deoxyribonucleoside triphosphates. The isolated nuclei continue to support viral DNA synthesis and permit rapid pulse-labeling procedures and density shift experiments. Although viral DNA synthesis in the isolated nuclei proceeds at a slow rate and initiation of new rounds of replication have not been observed (VAN DER VLIET and SUSSENBACH, 1972), no substantial differences in the properties of replicating DNA from isolated nuclei and intact cells have yet been detected.

Replicating viral DNA can be separated from host DNA by the HIRT (1967) extraction procedure. Lysis of cells or nuclei by sodium dodecyl sulphate and

pronase, followed by addition of NaCl, generates a precipitate of high molecular weight host DNA, which can be removed by centrifugation (HIRT, 1967) Further separation of viral DNA from proteins and low molecular weight host DNA can be obtained by isopycnic centrifugation in CsCl. An improved separation is possible if the viral DNA is density-labeled with bromodeoxyuridine.

Replicating viral DNA, labeled for short periods of time, sediments in neutral sucrose gradients more rapidly than mature adenovirus DNA (SUSSENBACH and VAN DER VLIET, 1972a; VAN DER VLIET and SUSSENBACH, 1972; BELLETT and YOUNGHUSBAND, 1972; VAN DER EB, 1973; BOURGAUX-RAMOISY et al., 1974). During a subsequent chase period, the labeled replicative intermediate shifts to the position of mature adenovirus DNA in these neutral pH sucrose gradients, indicating the transient role of the rapidly sedimenting DNA in replication.

Sedimentation of pulse-labeled replicating DNA in alkaline pH sucrose gradients has shown that the new DNA strands have lengths equal to or shorter than mature DNA strands (HORWITZ, 1971; SUSSENBACH and VAN DER VLIET, 1972a; BELLETT and YOUNGHUSBAND, 1972; VAN DER EB, 1973; ROBIN et al., 1973). Single-stranded newly synthesized DNA longer than genome length has never been observed excluding a rolling circle type of replication. Replicating DNA has a higher buoyant density in CsCl than mature DNA (PEARSON and HANAWALT, 1971; SUSSENBACH and VAN DER VLIET, 1972a; BELLETT and YOUNGHUSBAND, 1972; VAN DER EB, 1973; PETTERSSON, 1973). The increased buoyant density is caused by the presence of extensive regions of single-stranded DNA in replicating molecules as shown by its strong binding to benzoylated-naphthoylated DEAE (BND) cellulose and by electron microscopy (SUSSENBACH et al., 1972; BELLETT and YOUNGHUSBAND, 1972; VAN DER EB, 1973; PETTERSSON, 1973; ELLENS et al., 1974). Removal of the single-stranded DNA by the single-strand specific endonuclease from *Neurospora crassa* reduces this high buoyant density of replicating Ad2 DNA. About 30 percent of the replicating DNA molecule appears to contain single-stranded regions (PETTERSSON, 1973), although ROBIN et al. (1973) have reported lower values. This discrepancy may be due to differences in the isolation procedures employed.

Electron microscopy of purified replicating Ad5 DNA has shown the presence of two classes of molecules: (1) linear duplex DNA molecules containing a branch (fork) with one arm composed of single-stranded DNA and the other arm double-stranded DNA and (2) unbranched molecules containing double-stranded and single-stranded regions (Figs. 4 and 5). Completely single stranded and totally double-stranded DNA molecules of genome size have also been observed (SUSSENBACH et al., 1972; VAN DER EB, 1973; ELLENS et al., 1974). In the branched intermediates, the lengths of the single-stranded arm and one of the double-stranded arms are always the same, while the combined lengths of one of these arms plus the remaining unreplicated duplex region are equal to the length of mature adenovirus DNA. Branched intermediates

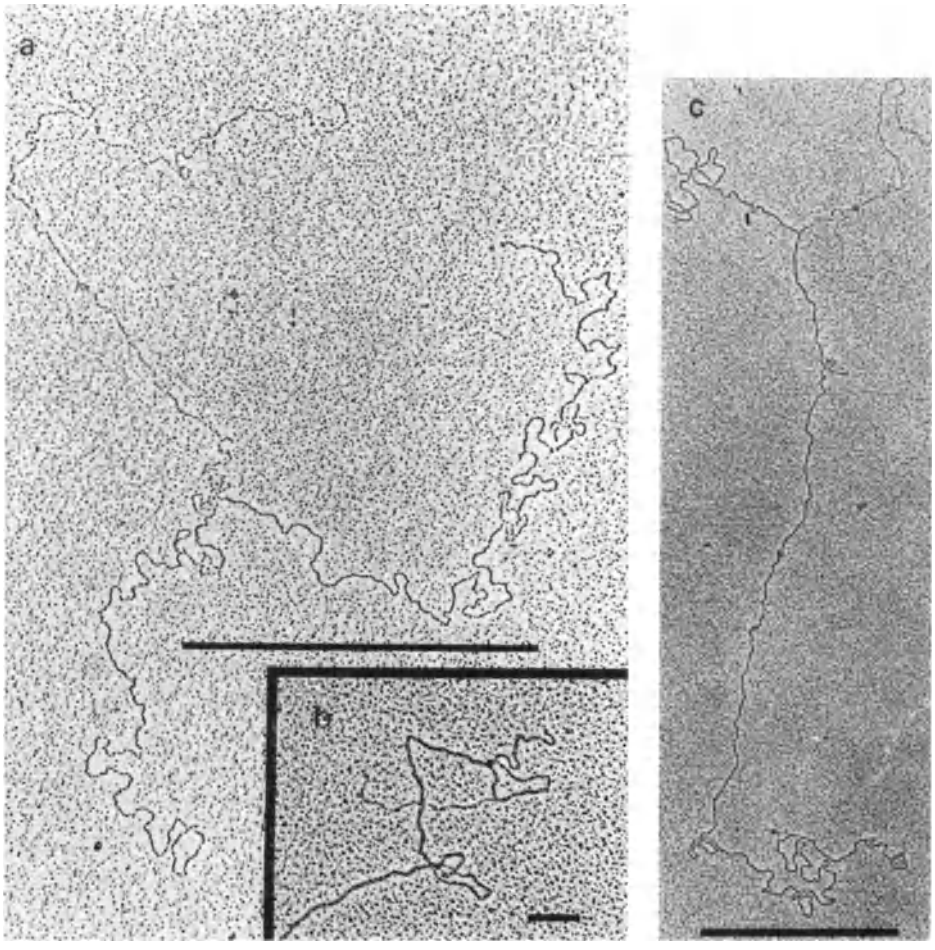


Fig. 4a—c. Branched replicative intermediates of Ad5 DNA isolated from purified infected nuclei. (a) Y-shaped molecule; displacement synthesis has progressed as far as 42% of the genome. Bar denotes 1 μm. (b) Detail of a Y-shaped molecule in which 3.5% of the genome has undergone displacement synthesis. Bar denotes 0.1 μm (c) Y-shaped molecules; the displaced single strand equals 23% of the genome. Bar denotes 1 μm. From Ellens et al. (1974)

with some double-stranded regions on the single-stranded arm have also been detected. Bourgaux-Ramoisy et al. (1974) have observed linear replicative intermediates of Ad2 DNA, which are almost free of single-stranded DNA. Circular replicating intermediates of adenovirus DNA have not been reported (Bellett and Younghusband, 1972; Doerfler et al., 1973).

Further analysis of replicating Ad2 and Ad5 DNA revealed that the newly replicated mature viral DNA had been synthesized in a semiconservative fashion (Pearson and Hanawalt, 1971; van der Vliet and Sussenbach, 1972). When single stranded full length DNA molecules were found they were of parental (template) origin only (Sussenbach et al., 1973). Hybridization of the single-stranded sheared regions (isolated by BND cellulose chromato-

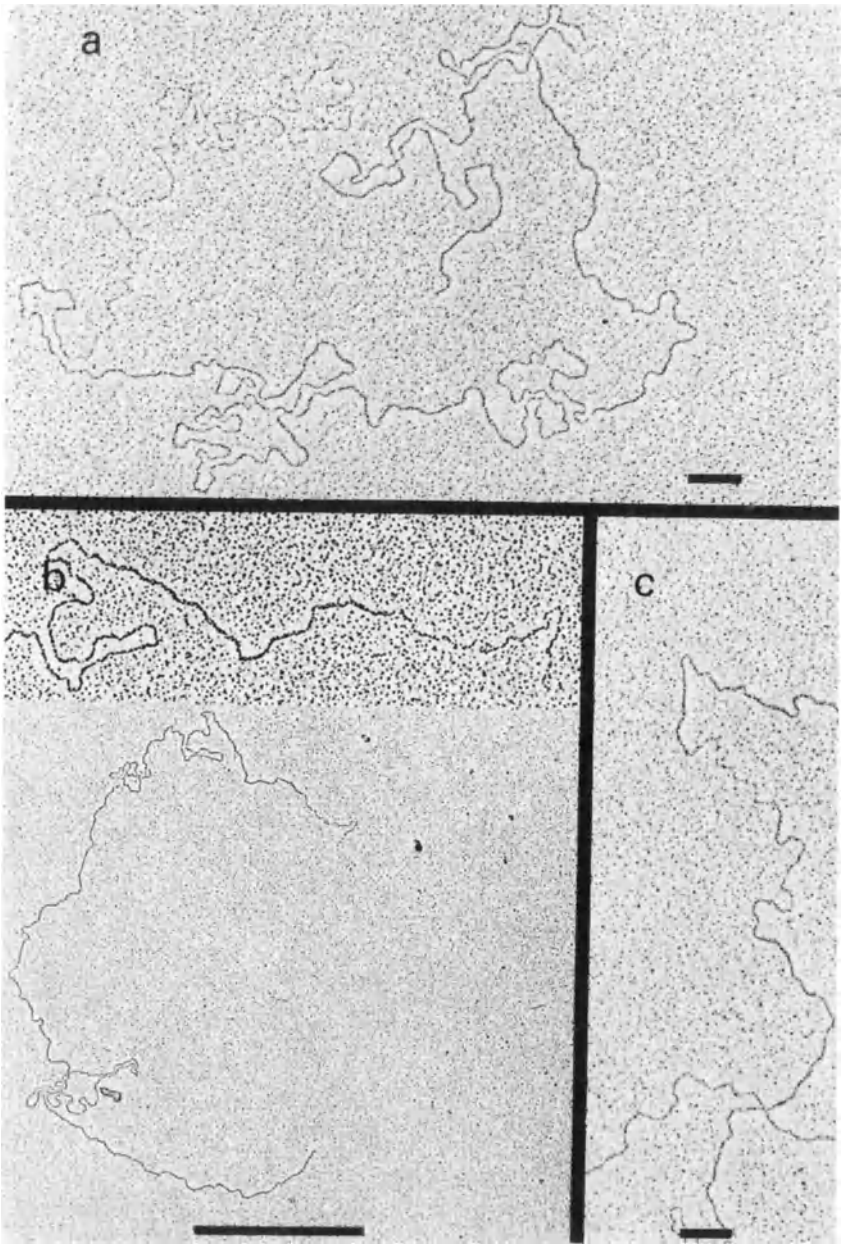


Fig. 5a—c. Unbranched replicative intermediates of Ad5 DNA. (a) Linear molecule with a single-stranded end representing 27% of the genome. Bar denotes 0.1 μm. (b) Linear molecule with a single-stranded end representing 7% of the genome. Bar denotes 1 μm. The inset shows an enlargement of the single-stranded end. (c) Detail of a linear molecule with a single-stranded gap of 3% of the molecular length. Bar denotes 0.1 μm. From ELLENS et al. (1974)

graphy) in replicating Ad5 DNA with separated complementary strands indicated that the single-stranded DNA represents predominantly one of the viral DNA strands (the strand with the higher buoyant density in alkaline

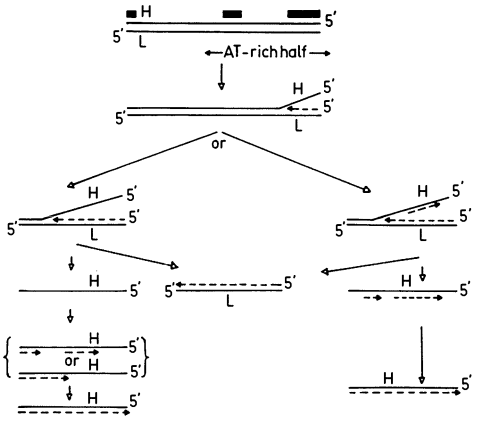


Fig. 6. Model for the replication of Ad5 DNA. Parental strands have been drawn as solid lines and new daughter DNA as dashed lines indicating the discontinuous character of their synthesis. Black bars in upper part indicate three prominent denaturation sites. The complementary strands with the lower and higher equilibrium density in alkaline CsCl have been indicated with L- and H-, respectively. Replication starts at right molecular end by displacement of parental H-strand. After completion of displacement synthesis, the displaced H-strand is converted into a duplex molecule by complementary strand synthesis. Occasionally complementary strand synthesis may start on the displaced strand before completion of synthesis. From SUSSENBACH et al. (1974)

CsCl which is the same strand with a light density position after poly UG hybridization) (SUSSENBACH et al., 1973). Partial denaturation mapping of branched intermediates of Ad5 DNA has shown that the double-stranded arm (newly replicated region), with the same length as the single-stranded arm, represents the adenine-thymine rich, molecular right end of the molecule (ELLENS et al., 1974) (Table 3). These replicative intermediates are visualized in Figs. 4 and 5 and a replication scheme derived from these data is presented in Fig. 6 (see Section E).

Apart from these replicating intermediates several other forms of intracellular viral DNA have been reported. In Ad2 as well as in Ad12 infected cells, double-stranded parental and newly synthesized viral DNA have been detected with lengths shorter than mature adenovirus DNA. These fragments fall into distinct size classes and it has been suggested that they originate by action of an adenovirus-associated endonuclease (BURLINGHAM and DOERFLER, 1971; SUSSENBACH, 1971; SUSSENBACH and VAN DER VLIET, 1972b). These DNA species are probably not involved in the replication of full length adenovirus DNA. On the other hand, it has been shown that viral DNA originating from both parental and progeny DNA may be integrated into host cell DNA (DOERFLER, 1970; BURLINGHAM and DOERFLER, 1971; BURGER and DOERFLER, 1974). The status of the integrated viral DNA and the physiologic function of this phenomenon are still unclear. WALLACE and KATES (1972) have isolated newly replicated Ad2 DNA in a rapid-sedimenting complex which also con-

tained protein and RNA. DOERFLER et al. (1971, 1973) has described a DNA-RNA-protein complex in Ad2-infected cells. The role of these complexes in DNA replication and/or transcription remains to be determined.

E. The Mechanism of Replication of Adenovirus DNA

The replication of adenovirus DNA is a complex process in which four distinct stages can be recognized: (1) initiation, (2) elongation, (3) termination, and (4) maturation of the segregated progeny molecules.

1. Initiation of DNA Synthesis

The study of initiation of adenovirus DNA replication has mainly taken the form of structure characterization of early replicating DNA and localization of the origin of DNA replication. Early replicating intermediates of Ad5 DNA were obtained by synchronizing the initiation of replication using hydroxyurea (HU) (SUSSENBACH and VAN DER VLIET, 1973). This drug inhibits DNA synthesis presumably by acting on ribonucleoside diphosphate reductase, leading to diminished deoxyribonucleoside triphosphate pools (SKOOG and NORDENSKJOLD, 1971). Addition of HU (10^{-2} M) to Ad5-infected cells at the beginning of the infection completely blocks viral DNA synthesis. After removal of the drug viral DNA synthesis starts almost immediately, although it takes about 20 min before a normal rate of viral DNA synthesis is resumed (SUSSENBACH and VAN DER VLIET, 1973). Replicating Ad5 DNA, isolated 5–10 min after release of this HU block, consists mainly of linear branched (with a fork) intermediates with one single-stranded arm. Completely or partially single-stranded DNA (with no fork region) does not appear until a later stage (Fig. 6) (ELLENS et al., 1974). This indicates that the branched intermediates represent early stages in replication. Their structure suggests that replication starts at the end of the molecule (right end) and proceeds by displacement of one of the parental strands. The position of the origin of replication of Ad5 DNA has been localized by digestion of pulse-labeled early replicating intermediates with the restriction enzyme Eco RI (ELLENS et al., 1974; SUSSENBACH et al., 1974). The radioactivity is specifically found in the terminal fragment (B fragment in Ad5 DNA, see Table 3) representing the molecular A–T rich right end of the genome. These data indicate that replication always starts from the same molecular end. These observations are in agreement with the results obtained with partial denaturation mapping of the branched replicative intermediates (ELLENS et al., 1974).

It appears then, that replication of Ad5 DNA starts at the molecular right end of the molecule and proceeds by displacement of one of the parental strands (the strand with the higher buoyant density in alkaline CsCl). The exact position of the origin of replication of Ad5 DNA is still unknown, but it must be located within 700 base pairs from the molecular right end as concluded from the shortest branched intermediates observed in the electron

microscope (ELLENS et al., 1974). Taking into consideration that DNA replication always proceeds in the 5′ → 3′ direction, the polarity of both complementary strands can be derived from these results. The 5′ end of the alkaline CsCl density H strand (the poly UG, L-strand) must then be located at the molecular right end of the molecule while the 5′ end of the alkaline CsCl density L-strand is positioned at the left end (SUSSENBACH et al., 1973; ELLENS et al., 1974). This assignment for the polarity of Ad5 DNA is in agreement with the results from transcription studies with Ad2 DNA (SHARP et al., 1974).

HORWITZ (1974), WINNACKER, PEARSON, and PETTERSSON (personal communications) and SUSSENBACH (unpublished results) have isolated mature Ad2 and Ad5 DNA, preferentially labeled in the terminus region. This was done by labeling the replicative intermediates with ^{3}H-thymidine and isolating the first molecules that appear as mature (31S) viral DNA. Digestion of this DNA with restriction enzymes (DANNA and NATHANS, 1972) has led to the conclusion that the termini of replication are present at both molecular ends of adenovirus DNA. Assuming a unidirectional mode of synthesis, this means that origins of replication must be present at both ends of the DNA molecule. These observations are not in conflict with the location of the Ad5 origin of displacement synthesis at the molecular right end as discussed above. A second origin, at the left molecular end, is probably involved in the conversion of the displaced parental single-strand into a duplex molecule some time after replication initiates at the molecular right end of the DNA molecule.

2. Polynucleotide Chain Elongation

The synthesis of the new adenovirus DNA strands proceeds in a semiconservative fashion (VAN DER VLIET and SUSSENBACH, 1972; BELLETT and YOUNGHUSBAND, 1972). The rate of synthesis has been estimated between 0.5 and 2.0 μm/min (PEARSON and HANAWALT, 1971; VAN DER EB, 1973; BELLETT and YOUNGHUSBAND, 1972). The new strands may be elongated continuously or discontinuously by addition of short stretches of nucleotides to the growing chain. In several other systems, chain propagation proceeds in a discontinuous fashion via so-called OKAZAKI pieces (OKAZAKI et al., 1968; MAGNUSSON et al., 1973; PIGIET et al., 1973; FAREED and SALZMAN, 1972) (Section II E. 2). The size of these OKAZAKI pieces ranges from 4S for SV40 and polyoma virus DNA up to 10S for *E. coli* DNA. However, the analysis of pulse-labeled human adenovirus DNA from infected cells did not show the involvement of OKAZAKI pieces in adenovirus DNA replication (HORWITZ, 1971; VAN DER EB, 1973). On the other hand, BELLETT and YOUNGHUSBAND (1972) observed that CELO DNA replication proceeds via 9–12S DNA pieces. Recent studies have shown that the replication of human adenovirus DNA can also occur discontinuously. Short 12S pieces of Ad2 and Ad5 DNA accumulate in the presence of HU (VLAK et al., 1975a; WINNACKER, 1975) or during synthesis in isolated nuclei of Ad2-infected cells (WINNACKER, 1975), and the DNA fragments can be chased into longer DNA strands of genome

size. These DNA fragments self-anneal to a considerable extent and hybridize with separated complementary strands of adenovirus DNA, indicating that they originate from both polynucleotide strands (VLAK et al., 1975a). The inability to detect the OKAZAKI pieces in adenovirus-infected cells with very short pulse-labeling times, in the absence of HU, could be due to a rapid gap-filling and/or sealing reaction of these DNA products.

The presence of large single-stranded regions of viral DNA in the replicative intermediate suggests that the synthesis of the two complementary DNA strands proceeds in a highly asynchronous fashion. From the structure of these branched intermediates (Figs. 4–6) it can be deduced that first one DNA strand is synthesized by displacement synthesis, while at a later time the second template strand is duplicated. The asynchrony in the synthesis of both complementary strands of Ad5 DNA can be exaggerated during viral DNA synthesis after release of a HU block. Under these conditions only displacement synthesis takes place, leading to an accumulation of single-stranded DNA (SUSSENBACH et al., 1973). Based upon these considerations it seems likely that the displacement synthesis and the complementary strand synthesis respond in a different manner to limiting levels of dXTPs. This may be due to different requirements for initiator proteins or DNA polymerases. The balance between the two synthetic processes may depend on the cell and virus type involved, the multiplicity of infection, and the physiologic state of the cell. However, much more information is required to clarify this problem.

3. Termination and Maturation of Postsegregational Intermediates

The steps involved in termination and segregation of adenovirus DNA remain ill-defined. HORWITZ (1974), WINNACKER and PETTERSSON (personal communication) have found that termination of Ad2 DNA replication takes place at both molecular ends, while PEARSON (personal communication) also detected termination internally in the molecule. Termination of replication at both molecular ends as well as at two internally localized sites has also been found for Ad5 DNA (SUSSENBACH, unpublished results). Electron microscopy has shown that several unbranched intermediates contain small single-stranded gaps (ELLENS et al., 1974), suggesting that filling of these gaps may be a post-segregational step. VAN DER EB (1973) observed that in a pulse-chase experiment, a fraction of apparently mature molecules of Ad5 DNA behaved for a relatively long time as replicating molecules on BND-cellulose columns. It is possible that these molecules may contain a number of small single-stranded gaps, which await conversion into duplex DNA. In short, these data indicate that the displacement synthesis of Ad2 and Ad5 DNA may terminate at the molecular left end, while complementary strand synthesis probably terminates at the molecular right end. The molecules that have completely segregated (DNA strand separation) may well contain internal regions of single-strand DNA, that are filled in by a postsegregational step comprised of gap-filling reactions (see Section II, E. 4).

4. The Mechanism of Replication

The adenovirus replicative intermediates, described above, best fit a model for replication in which linear intermediates are involved (SUSSENBACH et al., 1972; ELLENS et al., 1974; SUSSENBACH et al., 1974) (Fig. 6). In this model, replication of Ad5 DNA starts at the A–T rich end of the molecule displacing one of the viral DNA strands. After completion of the displacement synthesis, the displaced single-strand template is converted into a duplex molecule by so-called complementary strand synthesis which may start at different sites along the molecule. The complementary strand synthesis sometimes starts before completion of the displacement synthesis. This model explains all of the types of replicative intermediates observed. The synthesis of the two new strands could proceed discontinuously via 12S OKAZAKI pieces.

The major difficulty with a mechanism of replication involving linear intermediates is the conservation of the genetic information at the ends of the molecule. Since all known DNA polymerases require a 3′-OH group as a primer, which may be provided by a short stretch of RNA, replication of a linear DNA results in molecules containing either RNA at the 5′ end, or if the RNA is removed, a single-stranded 3′-end. Circularization or concatenization of linear genomes has already been proposed as a general possibility to overcome this problem (WATSON, 1972). However, this solution needs the presence of a terminal redundancy, which is absent in adenovirus DNA.

ROBINSON et al. (1973; 1974) have suggested that the circular DNA protein complex plays a role in replication and therefore conservation of the ends of the molecule. This may act directly by linking both ends of the molecule or, replication may proceed via covalently closed circular intermediates as shown for the replication of mitochondrial DNA (ROBBERSON et al., 1972).

It is possible that the ends of linear molecules have a specific structure permitting synthesis of the molecular ends without circularization. CAVALIER-SMITH (1974) has suggested that linear molecules may replicate their 5′-ends without circularization, if palindromic sequences are present at the ends. Our knowledge of the polynucleotide sequences at the ends of adenovirus DNA is not sufficient to evaluate this possibility.

F. Proteins Involved in Adenovirus DNA Replication

In spite of our increasing knowledge of the mechanism of viral DNA replication, relatively little is known about the proteins involved in this process. From the genetic analysis (Section III C), it is expected that at least two, may be three viral gene functions are required for the replication of adenovirus DNA. This number is relatively low, however, compared to the larger herpes viruses where at least eight complementation groups have been allocated to gene functions related to viral DNA synthesis (BROWN et al., 1973). This suggests that adenoviruses probably rely upon the DNA replication machinery of the host cell.

In contrast to cellular DNA synthesis and to the replication of papovavirus DNA (Section II, E. 1) adenovirus DNA replication is not, under all conditions, inhibited when protein synthesis is blocked in infected cells. When cycloheximide is added late in infection, that is 3–4 hr after the appearance of newly synthesized Ad2 DNA, replication (initiation, elongation, and termination) proceeds normally for at least 6 hr (HORWITZ et al., 1973). Addition of cycloheximide, prior to the synthesis of viral DNA, blocks DNA synthesis completely. A similar resistance to cycloheximide late in infection has been reported by YAMASHITA and GREEN (1974).

The observation that adenovirus DNA replication is not dependent upon continued protein synthesis might be explained by an excess of proteins required for replication late in infection. This unusual property may well facilitate the isolation and detection of such proteins.

1. Induction of Host Enzymes

Several enzymes exhibit an increased activity in response to adenovirus infection (GREEN, 1970; TOOZE, 1973). The degree of enhancement of enzymatic activity depends upon the host cell employed and is maximal in primary cells (e.g., human embryonic kidney cells), probably due to lower resting cell levels. The most intensively studied enzyme is thymidine kinase. KIT et al. (1970) observed a 2–4 fold enhancement in thymidine kinase activity in human (KB) as well as simian (CV-1) cells after infection with Ad2. This stimulation did not occur, however, in thymidine kinase deficient mutant cells, indicating that a cellular enzyme was stimulated after infection Moreover, the enzyme isolated from infected cells has similar molecular and enzymatic properties (e.g., electrophoretic mobility, sedimentation coefficient, and phosphate donor specificity) as the enzyme from uninfected cells (KIT et al., 1974; POSTEL and LEVINE, 1975)

In view of the single-stranded character of replicating adenovirus DNA, it would be of interest to know whether a viral-coded or virus-modified DNA polymerase exists in the infected cells. Human embryonic kidney cells infected with Ad2, Ad12 (LEDINKO, 1968), or Ad31 (SUZUKI and SHIMOJO, 1971) show a 3–5 fold enhancement in DNA polymerase activity. This increase in activity has not been observed in infected human KB cells. The origin of DNA polymerase(s) involved in replication of adenovirus DNA is still unknown.

2. Viral Coded Proteins

DNA cellulose chromatography of protein extracts obtained from infected cells has been employed to isolate and characterize one of the proteins involved in viral DNA replication (VAN DER VLIET and LEVINE, 1973). This procedure, originally developed by ALBERTS and co-workers (ALBERTS and HERRICK, 1971), selects for those proteins that are capable of binding to DNA. Using Ad5 infected monkey cells to eliminate the large excess of coat proteins syn-

thesized in human cells, VAN DER VLIET and LEVINE (1973) detected two infected cell specific proteins that bind tightly to single-strand DNA cellulose columns. The molecular weights of these proteins (in SDS-polyacrylamide gels) were 72000 and 48000. The same proteins can be detected in Ad5 infected human and hamster cells, and similar proteins have now been detected in Ad2 and Ad12 infected monkey or human cells (LEVINE et al., 1974; ROSENWIRTH et al., 1975). The DNA binding proteins from Ad12 infected cells have molecular weights of 60000 and 48000 (ROSENWIRTH et al., 1975). In all cases these proteins are synthesized in large amounts (10^6–10^7 molecules per infected cell) starting early after infection, at about the same time as the onset of viral DNA synthesis. The production of these DNA binding proteins does not require either viral or cellular DNA synthesis and neither protein corresponds to the polypeptides found in purified virions (VAN DER VLIET and LEVINE, 1973).

For Ad2, 5, or 12, the smaller 48000 MW protein is really a collection of several polypeptide species of similar molecular weights (45000–50000), which have recently been resolving using autoradiographic analysis of the separating gels. Peptide maps of the 72000, 60000, and 48000 MW species have shown: (1) The 72000 MW proteins from Ad2 and Ad5 contain very similar tryptic peptides which are distinguishable from the tryptic peptides of the Ad12 60000 MW protein. (2) The 48000 MW species from Ad2 and Ad12 contain a subset or peptides all of which are found in the Ad2 and Ad12 72000 and 60000 MW proteins. These data strongly indicate that the DNA binding proteins with a low nuclear weight are proteolytic breakdown products of the larger species. (3) The 72000 MW proteins from Ad2 infected human and monkey cells have identical peptide maps. (4) Mock infected human and monkey cells contain two single-stranded DNA binding proteins of approximately 72000 and 48000 MW. The peptide maps of these proteins are totally different from the peptide maps of the Ad2, 5, or 12, 72000 and 60000 MW proteins (ROSENWIRTH, ANDERSON, LEVINE, 1975, in press).

Evidence that the DNA binding protein of 72000 MW is an early viral gene product required for adenovirus DNA replication, comes from a study of the production of DNA binding proteins in cells infected with Ad5 temperature-sensitive mutants required for viral DNA synthesis (H5ts36 and H5ts125). Normal levels of DNA binding proteins are produced in Ad5 wt-, H5ts36-, and H5ts125-infected cells at the permissive temperature and in Ad5 wt and H5ts36 at the nonpermissive temperature. H5ts125-infected cells at the nonpermissive temperature produce less than 5 percent of the wild type levels of the DNA binding proteins with 72000 MW and 48000 MW (VAN DER VLIET et al., 1975). A similar observation has now been made with the 60000 MW protein produced by the H12tsA275 mutant (ROSENWIRTH et al., 1975). The DNA binding proteins, isolated from H5ts125 infected cells grown at the permissive temperature, were more thermolabile for continued binding to single-stranded DNA than either the wild type protein or the H5ts36 proteins (VAN DER VLIET et al., 1975). These data strongly suggest that the DNA binding protein of

72000 MW is coded for by the H5ts125 adenovirus gene and that this protein is required for viral DNA replication. When early species of Ad2 specific polysomal m-RNA are translated in vitro, a 72000 MW species is synthesized. A small percentage of this protein chromatographs like authentic single-strand DNA binding protein on single-stranded DNA cellulose columns (LEVINE et al., 1975).

The adenovirus DNA binding protein resembles the T4 gene 32 protein (ALBERTS and FREY, 1970) in a number of its properties. Both of these proteins are produced in large quantities in infected cells, and conditional lethal mutants for these proteins (T-4 gene 32 and H5ts125) have been employed to demonstrate that these gene products act in a stoichiometric rather than a catalytic fashion. Both of these proteins are coded for by genes acting early after infection which are required for viral DNA replication. At least in the case of T-4, gene 32 protein is also required for genetic recombination (TOMIZAWA et al., 1966; BROKER and LEHMAN, 1971).

A more detailed understanding of the role of the DNA binding protein in the various steps of adenovirus DNA replication will depend upon further characterization of the physiology of H5ts125 and similar mutants. The available evidence (Section III, C. 2) indicates that the block in viral DNA synthesis in these mutants concerns some step during the initiation of new rounds of replication and not elongation or chain termination (VAN DER VLIET and SUSSENBACH, 1975; GINSBERG, personal communication).

An endonuclease activity specific for Ad2 and Ad12 infected cells has been studied by DOERFLER and co-workers. This protein is synthesized late in infection and can be isolated in close association with the penton base although it is not identical with this protein. The enzyme generates double-strand scissions in any kind of DNA, preferentially at GC rich regions. Its activity is inhibited to a greater extent by poly dG:dC than by poly dA:dT (BURLINGHAM et al., 1971). It has been suggested that this endonuclease has a role in the integration of viral DNA into host DNA during abortive as well as productive infection but definite proof of its origin (viral or cellular) and physiologic function is lacking.

IV. Adenovirus-SV40 Interactions

A. SV40 Complementation of an Adenovirus Late Event

The human adenoviruses undergo an abortive infection in African green monkey cells in culture (RABSON et al., 1964). In these cells, all of the adenovirus early functions are expressed and adenovirus DNA replicates normally (FELDMAN et al., 1966; FRIEDMAN et al., 1970). Late m-RNA species appear to be synthesized, processed, and transported to the cytoplasm as in permissive human cells (FOX and BAUM, 1972; BAUM et al., 1968). Late adenovirus m-RNA is even detected on the polysomes of monkey cells but late proteins are not translated or, if synthesized, are degraded rapidly (BAUM et al., 1972).

Coinfection of monkey cells with SV40 and adenovirus permits the production of late adenovirus proteins and infectious adenovirus is now produced (Rabson et al., 1964). The simplest interpretation of these data is that SV40 supplies or induces a function that permits translation of late adenovirus m-RNA. The available evidence indicates that an SV40 early function is required for this complementation (Friedman et al., 1970). The nature of this translational block to late adenovirus m-RNA is not at present clear.

B. Adenovirus-SV40 Hybrids

The successful replication of adenovirus in AGMK cells requires coinfection of these cells with SV40. On at least two distinct occasions (Rowe and Baum, 1964; Huebner et al., 1964; Lewis et al., 1969), the progeny of this coinfection have contained recombinant viruses or hybrid viruses, packaged in an adenovirus virion. This has occurred in spite of the fact that polynucleotide sequence homology has not been found between SV40 and any human adenovirus so far examined. Two classes of recombinants have been isolated: (1) defective for adenovirus replication and (2) nondefective for adenovirus replicative functions. (1) The defective hybrids represent adenovirus SV40 recombinants, where the SV40 insertion has deleted or destroyed some essential adenovirus genetic information. This defective recombinant (PARA or $E46^+$) requires a wild type adenovirus helper (Ad2 or 7 function equally well) to supply the deleted or altered adenovirus functions (see Tooze, 1973, for details). The population of these two viruses (defective recombinant and wild type adenovirus) can then replicate in monkey cells because the SV40 information supplies the late translational functions required for adenovirus growth. (2) The nondefective adenovirus-SV40 recombinants contain an insertion of SV40 DNA in the adenovirus genome. While a portion (5–7 percent) of the adenovirus genome is deleted, the recombinant is viable and able to plaque with single hit kinetics (Lewis et al., 1969) on monkey or human cells. Either nonessential adenovirus functions have been deleted or the SV40 insertion supplies these deleted functions.

The physical and biological properties of these defective and nondefective recombinants have been well characterized and are summarized in Table 5. These data permit several observations to be made: (1) The SV40 function required for complementation of a late adenovirus m-RNA translation in monkey cells maps in the early region of SV40 DNA (Kelly and Lewis, 1973; Morrow et al., 1973), and is most closely correlated with the presence of SV40 U antigen (see Table 2, and Section II, A). (2) The region of the adenovirus genome which is deleted in the nondefective recombinants (0.79–0.86) is also the region of DNA that tolerates drift of the polynucleotide sequences as determined when the different adenovirus serotypes are compared by heteroduplex mapping (see Section III, B. 1, Table 3). (3) One insertion point of the SV40 DNA in the adenovirus genome occurs at the identical place in the

Table 5. Physical, immunologic, and biological properties of adenovirus SV40 recombinants

Recombinant	SV40[a] insertion	Ad[a] deletion	SV40 Functions[b]			Growth in[c] Human	AGMK
			U	TSTA	T		
$Ad2^+ND_1$	17 % 0.11–0.28	5.4 % 0.80–0.86	+	−	−	+	+
$Ad2^+ND_2$	33 % 0.11–0.44	6.1 % 0.80–0.86	+	+	−	+	+
$Ad2^+ND_3$	7 % 0.11–0.18	5.3 % 0.81–0.86	−	−	−	+	−
$Ad2^+ND_4$	48 % 0.11–0.59	4.5 % 0.81–0.86	+	+	+	+	+
$Ad2^+ND_5$	28 % 0.11–0.39	7.1 % 0.79–0.86	−	−	−	+	−
Para-Ad7 $E46^+$ (defective)	75 % 0.11–0.86	10 %	+	+	+	+ with helper Ad	+

[a] KELLY and LEWIS, 1973; MORROW et al., 1973.
[b] LEWIS et al., 1974; LEWIS and ROWE, 1971.
[c] LEWIS et al., 1973.

SV40 genome in both the defective ($E46^+$) and nondefective ($Ad2^+ND$ series) hybrids which were isolated independently from each other (WEISSMAN, personal communication). The other SV40 insertion point is at a variable position in the SV40 chromosome. While this may reflect the selection pressure for including a specific region of the SV40 genome it is remarkable that two independent recombinants share a similar insertion point and this could indicate a special site for recombination in the SV40 genome.

GRODZICKER et al. (1974b) have isolated mutants of $Ad2^+ND_1$ that plaque on human cells but no longer yield plaques on AGMK cells. These mutants are then presumably defective in the SV40 gene function required for adenovirus late m-RNA translation. $Ad2^+ND_1$ infected cells produce a protein (32000 MW) not found in Ad2 infected cells which then may be coded for by the SV40 inserted genetic material. The $Ad2^+ND_1$ mutants no longer able to plaque on monkey cells do not contain this 32000 MW protein indicating it is either degraded or not synthesized properly in these mutants. Whether this protein is in fact U antigen remains to be determined.

From the properties of the $Ad2^+ND$ hybrids it has been possible to correlate early regions of the SV40 genome with the presence and absence of the three immunologic entities T, U, and TSTA produced by these recombinants in SV40 infected or transformed cells (see Tables 2 and 5). While these antigens are all located in the early region of the SV40 genome, it should be recalled that only one class of early temperature-sensitive mutants (tsA) has been isolated (see Table 2).

C. SV40 Complementation or Suppression of H5ts125 by SV40 for Adenovirus DNA Replication

The H5ts125 mutant identifies an adenovirus gene that produces a single-stranded specific DNA binding protein that is required for adenovirus DNA replication. Adenovirus DNA is not replicated in H5ts125 infected AGMK cells at the nonpermissive temperature, but adenovirus DNA is replicated at the permissive temperature in these cells (LEVINE et al., 1974). When AGMK cells are infected first with SV40 and then H5ts125 at the nonpermissive temperature, adenovirus DNA replicates normally and infectious adenovirus is produced (LEVINE et al., 1974, 1975). In this case SV40 can suppress or complement the defect in the adenovirus H5ts125 mutation. The adenovirus specific DNA binding protein cannot be detected in H5ts125 infected monkey cells or in SV40-H5ts125 coinfected monkey cells at the nonpermissive temperature, eliminating the possibility that SV40 produces a function that stabilizes the temperature-sensitive adenovirus DNA binding protein at 40°C (LEVINE et al., 1974). While SV40 tsB, C, and BC mutants will complement the H5ts125 mutant at nonpermissive temperatures, SV40tsA mutants fail to complement this adenovirus defect in viral DNA synthesis (LEVINE et al., 1974, 1975). These data indicate that one of three possible mechanisms could account for the suppression or complementation of H5ts125 by SV40: (1) SV40 tsA gene product can functionally substitute (initiate adenovirus DNA synthesis) for the adenovirus DNA binding protein; (2) SV40tsA gene product induces a viral or cellular function that suppresses or complements the H5ts125 function; or (3) SV40 DNA synthesis (dependent upon tsA function) is required for this apparent complementation of the H5ts125 gene. SV40 DNA replication may be required to increase the probability of recombination events between SV40 and adenovirus DNA or to increase the level of SV40 early and late functions which may be employed in this complementation. Both SV40 tsA and H5ts125 behave as initiation functions for viral DNA replication and it is possible that the tsA gene product can initiate DNA synthesis in type 5 adenovirus DNA, bypassing (suppression) the requirement for the H5ts125 protein. More experiments are required before a choice is made between these alternative mechanisms.

V. Adenovirus and SV40 DNA Replication — General Principles

A. Early Viral Functions

All of the temperature-sensitive early viral mutants isolated with SV40, polyoma, or the adenoviruses are required for viral DNA replication (see Section II, C; III, C). More precisely, these mutants (tsA, H5t125, H12tsA, B, C, and possibly H5ts36) are required for the initiation of viral DNA synthesis at a specific origin site in the viral genome. Because of the specificity of the origin site, recognition of polynucleotide sequence or structure is required, and it is not surprising that viral functions are involved here. It appears likely that, for the small DNA viruses, cellular functions are employed

for the elongation, termination, and postsegregational events (gap-filling events) observed with both of these viruses. Viral functions are required for the initiation of DNA replication.

Some of these same early viral gene functions employed in DNA replication are also required for the establishment and maintenance of the transformed cell phenotype (SV40tsA and H5ts36) (BUTEL et al., 1974; WILLIAMS et al., 1974; KIMURA and ITAGKI, 1974; MARTIN and CHOU, 1975; TEGTMEYER, 1975; BRUGGE and BUTEL, 1975; OSBORN and WEBER, 1975). Because of the limited coding capability of these small DNA viruses, viral gene products could well have multiple functions. Indeed the SV40tsA gene product appears to be involved in: (1) the initiation of viral DNA synthesis (TEGTMEYER, 1972), (2) the stimulation of cellular DNA synthesis in infected cells (CHOU and MARTIN, 1975), (3) the stimulation of cellular enzyme activities, like thymidine kinase, which is observed after lytic or abortive infection (POSTEL and LEVINE, unpublished observations), and (4) the establishment and maintenance of the transformed state (BUTEL et al., 1974; KIMURA and ITAGKI, 1975; TEGTMEYER, 1975; MARTIN and CHOU, 1975; BRUGGE and BUTEL, 1975; OSBORN and WEBER, 1975). It is possible that these seemingly unrelated phenotypes have a common underlying mechanism possibly grounded in the initiation of viral and cellular DNA synthesis (LEVINE, 1973).

The various types of interaction between SV40 and the human adenoviruses demonstrates the interchangeable nature and functional similarities of these early viral gene products. The complementation of H5ts125 DNA replication by SV40, but not SV40tsA, suggests that the SV40tsA gene product directly, or via the activation of cellular functions, can suppress or substitute the H5ts125 function. The ability of an SV40 early function to permit the translation of late adenovirus m-RNA in monkey cells again points to a unitary set of mechanisms acting at various stages during lytic infection. The mechanisms involved in the recombination between adenovirus and SV40 genomes, in the absence of apparent polynucleotide sequence homologies, is totally unexplored. If indeed these events are analogous to lambda integration into the *E. coli* chromosome (nonhomologous base sequence recombination) (see HERSHEY, 1971) a genetic and biochemical analysis of these events should lead to new insights concerning viral and cellular gene functions. The possibility that this might represent a good model for the integration of viral DNA into the cellular genome remains to be explored.

B. DNA Replication

A number of similarities as well as differences can be found in the mechanisms of SV40 and adenovirus DNA replication: (1) Both groups of viruses initiate replication at a unique, single, origin site. (2) SV40 DNA synthesis proceeds in a bidirectional symmetric fashion (both DNA strands replicated simultaneously) while adenovirus employs unidirectional synthesis in a displacement (one DNA strand lags in its replication) mode of synthesis. (3) OKE-

ZAKI like fragments appear to be an intermediate in the replication of both viral DNA's but the sizes of these fragments are different with SV40 (4S) and the adenoviruses (12S). (4) Similar types of evidence now exist for the use of two distinct DNA polymerases in the elongation of both of these viral DNA molecules. (5) At least some of the postsegregational intermediates of SV40 and adenoviruses appear to contain single-stranded gaps that are filled in with DNA polymerase and sealed by ligase after the segregation of the parental DNA strands. Such gapped molecules observed in other systems (BROKER and LEHMAN, 1971) are aggressive recombination intermediates. Given the fact that recombination imparts certain selective advantages, these postreplicative forms may be generated with good reason. The immediate product of replication could well be an excellent substrate for SV40-adenovirus recombination events and integration events into the cellular chromosome.

This review of the papovaviruses and adenoviruses makes it quite clear that specific nucleic acid-protein interactions play an integral role in lytic infection, DNA replication, and possibly transformation. The SV40 T-antigen binds to double-stranded viral and cellular DNA (see Section II, E. 1) and if this antigen is indeed the product of SV40 gene A, it is then clearly involved at the origin site of DNA replication as well as the maintenance of transformation. The SV40-polyoma nucleoprotein complex (see Section II, G) composed of cellular histones, capsid proteins, and containing a superhelical untwisting activity, provides an excellent model for cellular chromatin structure and replication. The adenovirus single-strand specific DNA binding protein is involved in the initiation of viral DNA replication at a unique site in the chromosome (like SV40tsA) and its function can be suppressed or complemented by SV40 but not SV40tsA mutants (at 40°C). These specific nucleic acid-protein interactions are clearly not limited to DNA protein complexes. The viral polysomal transcripts, complementary to specific DNA strands and derived from restricted portions of the viral genome (see Tables 2 and 3), point to a protein-RNA specificity at the transcription level or the posttranscriptional processing of these RNA's. The ability of an SV40 function or viral induced function, to relieve the translational block of late adenovirus m-RNA in monkey cells, further demonstrates that these viruses act upon the host cell at many levels of regulation.

Clearly there are a large number of unsolved problems that remain to be explored with these viruses. The isolation and characterization of early viral functions has just begun and should yield fruitful information pertaining to the mechanisms of DNA replication and transformation. The viral and/or cellular gene products involved in recombination, integration, and regulation of cellular and viral gene expression are all unknown. Those proteins that play a structural role (histones for SV40, H5ts125 protein for adenoviruses) and catalytic roles (H5ts36, perhaps SV40tsA) during replication need to be explored and defined. It is becoming clear that the questions being investigated in these viral systems may reveal the kind of general principle useful in many other eukaryotic systems as well.

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In vitro Translation of Adenovirus Messenger RNA

HEINER WESTPHAL[1, 2]

With 5 Figures

Table of Contents

I. Introduction

Among the tumor viruses, adenovirus is exceptionally well suited for studies of in vitro protein synthesis because the patterns of in vivo transcription have been carefully examined (for review, see SHARP and FLINT, 1976), and because a substantial amount of information has accumulated concerning the proteins specified in vivo in response to adenovirus infection.

Naturally, the rapid progress currently made in the cell-free translation of animal virus messenger RNA (reviewed by SHATKIN et al., in press) is not confined to the adenovirus system. Rather, the data to be discussed below may serve as an example how a functional assay for messenger RNA can supplement genetic and biochemical studies of tumor virus gene expression.

II. Adenovirus-Specific Proteins Synthesized In Vivo

Human adenovirus type 2 is most commonly used in studies concerning adenovirus molecular biology. In electron micrographs, the virus appears as an icosahedral particle with 20 equilateral triangular faces and 12 vertices. A dense core, containing the DNA, can be distinguished from the outer coat or capsid, which is composed of 240 hexon and 12 penton capsomers. The pentons,

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located at the vertices of the virion, consist of a penton base and a fiber projecting from it. The adenovirion proteins are synthesized on cytoplasmic polysomes shortly before virus maturation. As host cell protein synthesis gradually declines, they constitute a predominant proportion of newly made protein. A large body of information exists concerning their biochemical and immunologic properties (for review, see PHILIPSON and PETTERSSON, 1973).

Each of the major components of the virus capsid (hexon, penton base, and fiber) is composed of several identical or closely related polypeptides. Besides these the virus contains several other polypeptides which form distinct bands in SDS-polyacrylamide gel electropherograms (MAIZEL et al., 1968). Approximately 13 bands (*Roman numerals*, Fig. 1) can be distinguished. Five of these represent elements of the major capsid and core proteins (Table 1). Most of the minor virion polypeptides, e.g., IIIa, VI, VIII, and IX, appear to be closely associated with either the hexon or the penton capsomers (EVERITT et al., 1973).

A number of additional virus-specific polypeptides, not present in the virion particle, have been found in extracts of cells harvested at late stages of productive infection with adenovirus type 2 (ANDERSON et al., 1973; WALTER and MAIZEL, 1974). Arabic numerals (Fig. 1) indicate their molecular weights. Whereas some of these polypeptides may constitute components of nonstructural virus proteins, others have been shown to be precursors of virion polypeptides. Thus, a polypeptide of 20000 MW (termed *p-VII* in Fig. 1) is the precursor to core polypeptide VII, whereas the 27K and 26K polypeptides appear to be precursors of virion polypeptides VI and VIII, respectively (ANDERSON et al., 1973; WALTER and MAIZEL, 1974; ISHIBASHI and MAIZEL, 1974a; ÖBERG et al., 1975).

Early proteins synthesized as a result of adenovirus infection include DNA binding proteins (VAN DER VLIET and LEVINE, 1973; SHANMUGAM et al., 1975), some of which may be related to the tumor antigen (GILEAD et al., 1975), as well as a number of as yet undefined proteins whose polypeptide components have been detected in electropherograms of extracts of infected cells (RUSSELL and SKEHEL, 1972; ANDERSON et al., 1973; WALTER and MAIZEL, 1974; ISHIBASHI and MAIZEL, 1974b). Genetic evidence (VAN DER VLIET et al., 1975) suggests that at least one of the DNA binding proteins is a virus gene product.

III. Cell-Free Systems used in Adenovirus Messenger RNA Translations

The establishment of a cell-free system capable of promoting RNA translation in vitro is still considered an art by many. If so, it has at least become an increasingly popular art, for extracts from a wide variety of pro- and eukaryotic cells have been shown to serve this purpose well (ZUBAY, 1973; LAST and LASKIN, 1972; HASELKORN and ROTHMAN-DENES, 1973; LEDER, 1973). Two kinds of systems may be distinguished. The first group comprises crude cell (S30) extracts which contain all macromolecular components necessary to

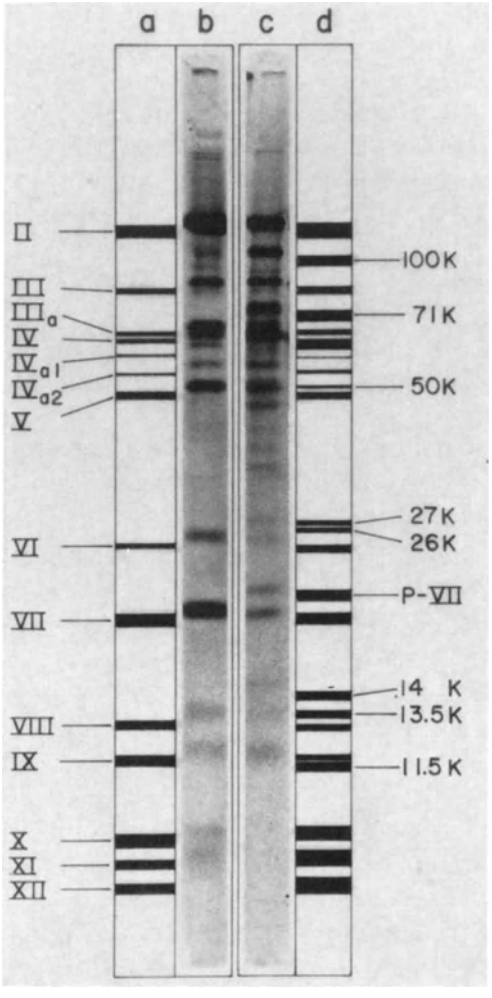

Fig. 1. SDS-polyacrylamide gel electrophoresis of adenovirion polypeptides (a, b), and of an extract from infected cells (c, d). The figure depicts actual autoradiograms of [^{35}S]-methionine-labeled polypeptides as well as line drawings of individual bands. Reproduced with permission from LEWIS et al. (1974)

promote protein synthesis in response to added messenger RNA. Systems belonging to the second group are composed of purified subfractions of the cell's translation apparatus, often derived from more than one cell source. Systems of the latter category are primarily designed to study the translational process itself. They have, however, also been used in cell-free synthesis of adenovirus polypeptides (ANDERSON et al., 1974).

With increasing progress in the field of in vitro translation, the quality standards for cell-free systems have become more stringent. Among the criteria generally applied are (1) efficiency of the system, conveniently measured in terms of incorporation of amino acids into acid-insoluble material; (2) reliable

translation of the information contained in added messenger RNA; (3) low background levels of protein synthesis in response to endogenous messenger RNA contaminating the system.

Many messenger RNAs are faithfully translated by cell-free systems derived from a wide spectrum of cell sources. For example, bacterial messenger RNAs have been translated in mammalian systems (AVIV et al., 1972), and vice versa (ATKINS et al., 1975). Therefore, the choice of system to use can often conveniently be adjusted to availability of biological material and ease of preparation. In this respect, the wheat germ system, originally introduced by MARCUS and FEELEY (1966), is very attractive because a local health food store plus the outfit of a standard biochemical laboratory will provide the material and equipment needed for its preparation (MARCUS, 1972; ROBERTS and PATERSON, 1973).

Aside from the macromolecules contained in cell extracts, an appropriate buffer system, containing all amino acids, K^+, Mg^{++}, ATP, GTP and an energy regenerating system is required for translation. Concentrations of K^+ and Mg^{++} are critical, and optima may vary for individual messenger RNA species. Systems optimized for the translation of a given messenger RNA may nevertheless vary in their capability of producing complete polypeptide chains instead of prematurely terminated molecules. Thus, the "art" of preparing a suitable translation system is to reconcile all these parameters in such a way that a maximal amount of reliable information is obtained.

IV. Viral Messenger RNA

The patterns of transcription at various times of the adenovirus life cycle will be reviewed by SHARP and FLINT in this series. Viral messenger RNA synthesis begins soon after infection, first in small quantities which rapidly rise during the course of infection. As synthesis of host cell ribosomal and messenger RNA is gradually shut down, viral RNA becomes the predominant form of RNA synthesized late in productive infection. The viral RNAs entering polysomal structures at the various stages of viral development may be divided into several classes according to (1) sedimentation velocity or electrophoretic mobility, (2) informational content, examined by in vitro translation, and (3) genetic origin determined by hybridization with various portions of the viral DNA.

RNA preparations used in cell-free synthesis of adenovirus proteins are commonly obtained by phenol extraction of infected cell cytoplasm or polysomes. A convenient method to remove contaminant ribosomal RNA is to select for poly(A) containing messenger RNA sequences, for instance by chromatography on oligo(dT) cellulose (AVIV and LEDER, 1972). If the genetic origin of messenger RNA is to be examined, the RNA has to be annealed to either total viral DNA or defined portions thereof prior to translation. Functional integrity and sufficient concentration of messenger RNA are obvious prerequisites for successful experimentation. Whereas damage inflicted upon

adenovirus messenger RNA by the various extraction and purification procedures appears to be minimal, logistical problems are often in the way of preparing sufficient quantities of certain species of messenger RNA, e.g., viral RNA synthesized in transformed cells, or the viral RNAs transcribed at initial stages of productive infection. Once prepared, the messenger RNA can be stored in liquid nitrogen without loss of biological activity.

V. Cell-Free Synthesis of Virion Polypeptides

OKUBO and RASKAS (1972) and WILHELM and GINSBERG (1972) reconstituted adenovirion protein synthesis in an incubation mixture containing ribosomes and supernatants of cells harvested late in infection, in addition to the standard ingredients of a cell-free system. They were able to demonstrate that polypeptides matching the size of known components of the adenovirus particle were released from polysomes. Moreover, radioactive proteins obtained in the in vitro reaction were specifically precipitated by serum directed against adenovirions or against individual capsid components (WILHELM and GINSBERG, 1972). Supernatants of infected cells containing macromolecular factors necessary to promote the in vitro reaction could, without adverse effect, be replaced by correspondent fractions of uninfected cells (OKUBO and RASKAS, 1972). Although not suited to demonstrate initiation of polypeptide synthesis in vitro or to study cell-free translation of individual messenger RNA fractions, these experiments gave a convincing account of the capability of polysomes to release adenovirion products which had, at least in part, been synthesized in vitro.

Encouraged by these results, a number of laboratories set out to test the capability of purified adenovirus messenger RNA to direct the synthesis of viral polypeptides in vitro. Structural elements of the adenovirus particle were the only proteins characterized well enough to serve as a reference in the analysis of polypeptides obtained by cell-free translation of messenger RNA. Hence, attention was primarily focused on those adenovirus messenger RNA species that are found in polysomes of cells harvested at late stages of productive infection when structural virus proteins are produced. Cell-free systems employed in these studies included S30 extracts derived from ascites tumor cells (AVIV et al., 1971) or from wheat germ (ROBERTS and PATERSON, 1973) or purified ribosomal subunits and pH 5 enzyme of ascites cells in connection with rabbit reticulocyte initiation factors (SCHREIER and STAEHELIN, 1973, modified by ANDERSON et al., 1974).

Adenovirus messenger RNA added to either one of these systems markedly stimulated in vitro protein synthesis, but so did the RNA from uninfected cells. The specificity of the translation became apparent when the products of both reactions were analyzed by SDS-polyacrylamide gel electrophoresis. Only the messenger RNA derived from infected cells, and not the one from uninfected cells, gave rise to polypeptides comigrating with adenovirion components (ERON et al., 1974a, b; ANDERSON et al., 1974; ÖBERG et al., 1975).

Matching pairs of in vitro and in vivo polypeptide bands were thus detected for all major virion components, with the notable exception of polypeptides VI, VII, and VIII which are cleaved in vivo from larger precursor molecules (ANDERSON et al., 1973; EVERITT and PHILIPSON, 1974; ISHIBASHI and MAIZEL, 1974a). When extracts of infected cells containing these precursors were included in the SDS-polyacrylamide gel analysis, corresponding in vitro polypeptides were readily detected (ANDERSON et al., 1974; ERON et al., 1974b; LEWIS et al., 1975). For two additional virus-specific polypeptides, 100 K and 11.5 K (see Fig. 1), in vitro equivalents were also found in the SDS-polyacrylamide gels (LEWIS et al., 1975). These two polypeptides have no known association with the virion particle.

A common difficulty encountered in the evaluation of electropherograms of cell-free translation products concerns the "background" of polypeptide bands that do not originate from viral RNA. All systems have a certain amount of endogenous protein synthesis presumably sustained by messenger RNA trapped in the cell extracts. Any exogenous RNA added to the system, be this messenger RNA from infected or mock-infected cells or even *Escherichia coli* ribosomal RNA, considerably enhances the level of endogenous polypeptide synthesis, possibly (ATKINS et al., 1975) because the added RNA protects endogenous messenger RNA from nuclease attack. Therefore, a sizable number of background polypeptide bands is always found among the translation products of viral messenger RNA. They can impede recognition of virus-specific products, especially in areas of the gel where many bands are clustered.

These difficulties notwithstanding, SDS-polyacrylamide gel electrophoresis has been the single most powerful technique in the examination of adenovirus-specific cell-free translation products. The information obtained by gel analysis is naturally limited to size and relative frequency of denatured polypeptides comigrating with the various markers. Additional criteria used to prove the identity between in vivo and in vitro products included (1) cochromatography on sodium dodecyl sulfate hydroxyapatite, (2) specific immunoprecipitation, and (3) matching tryptic peptide fingerprints.

MOSS and ROSENBLUM (1972) had shown that chromatography on hydroxyapatite in the presence of SDS separates polypeptides on the basis of criteria other than molecular weight. Accordingly, ERON et al. (1974a, b) fractionated in vitro translation products of late adenovirus messenger RNA and authentic in vivo viral products by this technique. For each of the polypeptides tested, the chromatographic behavior of in vivo and in vitro products was identical.

Using antisera directed against individual virus proteins, ERON et al. (1974a, b) and ÖBERG et al. (1975) were able to selectively precipitate the in vitro counterparts of several structural components of the virion (Fig. 2).

Finally, matching tryptic digest fingerprints corroborated the identity between several of the major virion components and the corresponding in vitro translation products (ERON et al., 1974a, b; ANDERSON et al., 1974) (Fig. 3).

Table 1 summarizes the data determined by the various laboratories on the cell-free translation products of messenger RNA extracted from cells at late

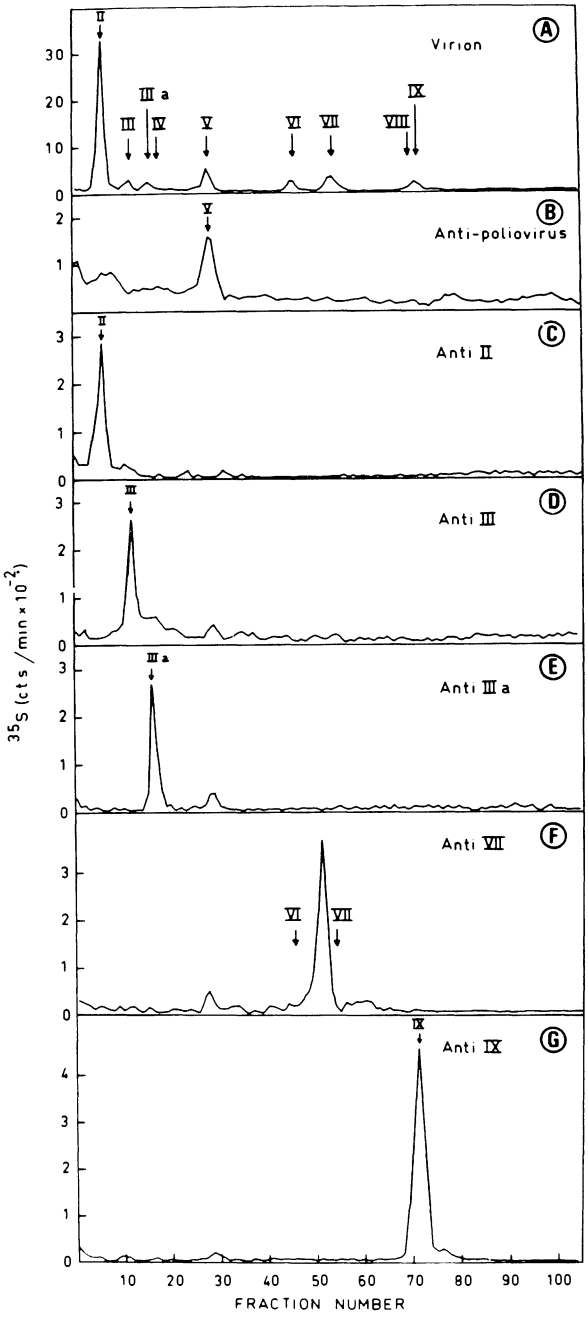

Fig. 2. SDS-polyacrylamide gel electrophoresis of cell-free translation products selected by immunoprecipitation with sera directed against individual adenovirus type 2 proteins. The [^{35}S]methionine-labeled in vitro polypeptides were translated from polysomal RNA of infected KB cells. Roman numerals refer to adenovirion proteins listed in Table 1. Details of the experiment may be found in the original article by Öberg et al. (1975) (reproduced with permission)

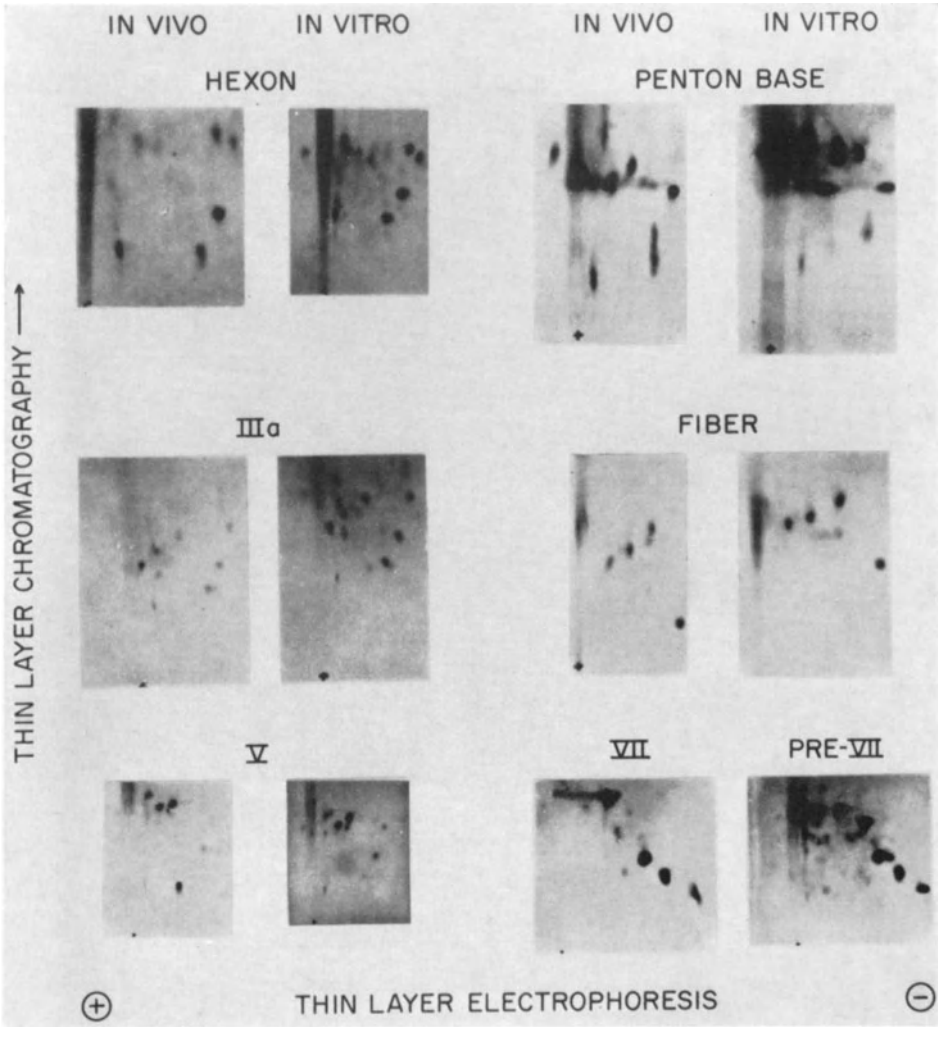

Fig. 3. Thin-layer chromatography of [^{35}S]methionine-labeled in vitro or in vivo adenovirus type 2 polypeptides. For details, see ERON et al. (1974a, b). Reproduced with permission from WESTPHAL et al. (1974)

stages of productive infection with adenovirus type 2. Most or all polypeptides found in the virion particle, or their respective precursors, are synthesized in vitro in response to this RNA. The native configuration of in vitro products, apart from that needed to react with antibody, has not yet been investigated. Therefore, we do not know at the present time whether proteins are assembled in vitro from the polypeptide elements in a manner analogous to the in vivo process.

In the initial experiments the relative amounts of in vitro translation products did not reflect those of the in vivo counterparts. Most notably, polypeptide II, the element of the hexon capsomer—in vivo the most abundant

Table 1. Adenovirion polypeptides synthesized in vitro

Polypeptide		MW	Method used for identification			
			SDS polyacrylamide gel electrophoresis	SDS hydroxyapatite chromatography	Immunoprecipitation	Tryptic peptide fingerprints
II	(hexon)	120000	+	+	+	+
III	(penton base)	85000	+	+	+	+
IIIa		66000	+	−	+	+
IV	(fiber)	62000	+	+	+	+
V	(core)	48000	+	+	+	+
pVI		27000	+	−	+	−
pVIII		26000	+	−	+	−
pVII	(core)	20000	+	+	+	+
IX		12000	+	−	+	−

The data have been extracted from the work of several laboratories as cited in the text. Only polypeptides identified by more than one technique are listed.

virus product—appeared as a comparatively faint band in most gel analyses. However, improvements of the cell-free systems, including careful control of Mg^{++} and K^{+} optima, and, most recently (ATKINS et al., 1975), the addition of polyamines to the reaction mixture, have now created conditions for in vitro translation which resemble more closely in vivo conditions.

VI. Translation of Messenger RNA Fractionated by Sucrose Gradient Centrifugation

Polysomes of cells harvested late in infection with adenovirus type 2 were found to contain distinct size classes of adenovirus RNA which appeared to originate from different portions of the viral genome (see SHARP and FLINT, this series). It seemed likely that these classes of adenovirus messenger RNA had distinct coding specificities. ANDERSON et al. (1974), WESTPHAL et al. (1974), and ÖBERG et al. (1975) therefore sedimented cytoplasmic or polysomal messenger RNA though sucrose gradients, translated individual fractions in the cell-free system, and analyzed the products by SDS-polyacrylamide gel electrophoresis. The result showed that RNAs coding for individual polypeptides had indeed been fractionated by this procedure. For instance, polypeptides III, II, pVII, IV, V, and IX were expressed in this order by RNAs with decreasing sedimentation velocities. As can be seen in Figure 4, only partial separation of individual messenger RNAs has been achieved so far, and it is therefore difficult to estimate the number of messenger RNA classes that can be separated by physical methods.

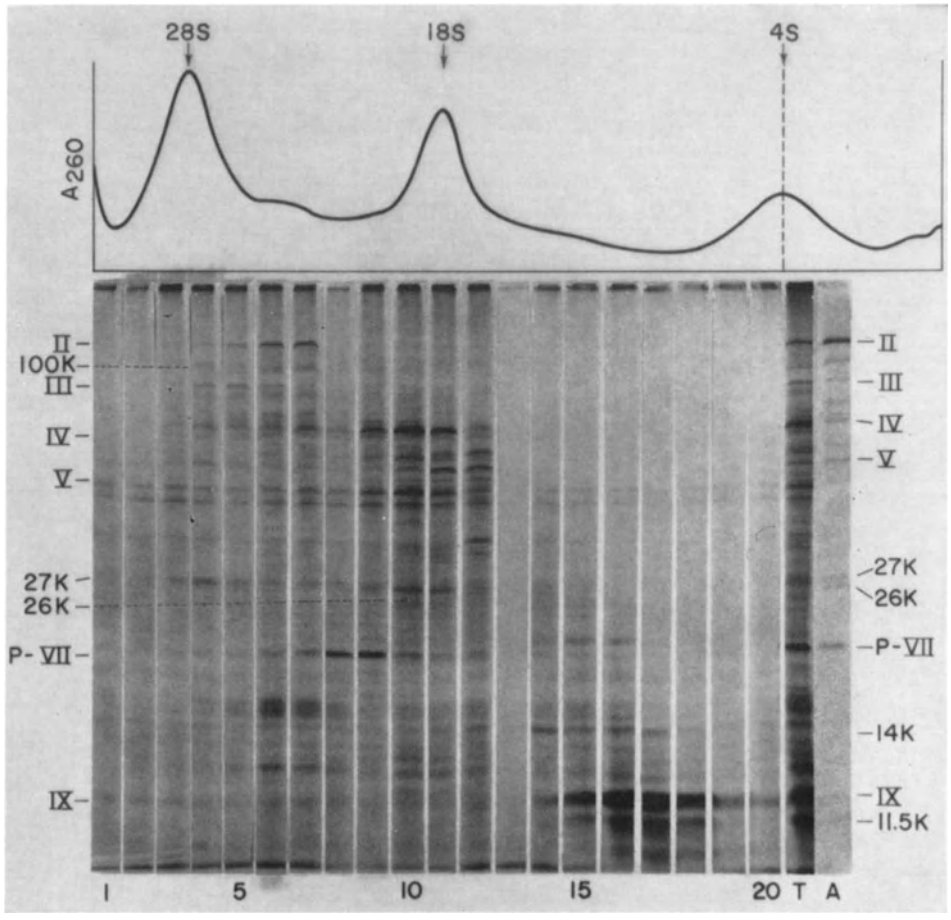

Fig. 4. SDS-polyacrylamide gel electrophoresis and autoradiography of the in vitro translation products of messenger RNA fractionated by sucrose gradient centrifugation. Cytoplasmic RNA was prepared from adenovirus type 2 infected KB cells and sedimented through a sucrose gradient. The upper diagram represents OD_{260} profile of ribosomal RNA contained in the preparation. RNA from individual fractions of the gradient was translated in vitro, and the reaction products were analyzed by SDS-polyacrylamide gel electrophoresis. The autoradiogram of the gel depicts the pattern of [^{35}S]methionine-labeled polypeptides obtained in the individual translations. The designations of polypeptide bands correspond to those of Figure 1. The cell-free translation products of unfractionated RNA (column *T*) and an in vivo labeled extract of infected cells (column *A*) are shown for comparison. Reproduced with permission from LEWIS et al. (1974)

There was a general correlation between the size of the polypeptide and the sedimentation rate of the correspondent messenger RNA, with at least one notable exception. The precursor of polypeptide VII was expressed by an RNA which, judged by its sedimentation velocity, appeared to be several times the size of a pVII codon. This finding raises interesting questions concerning the composition of pVII messenger RNA. Extra sequences surrounding the pVII message could either contain information for additional polypeptides or serve a

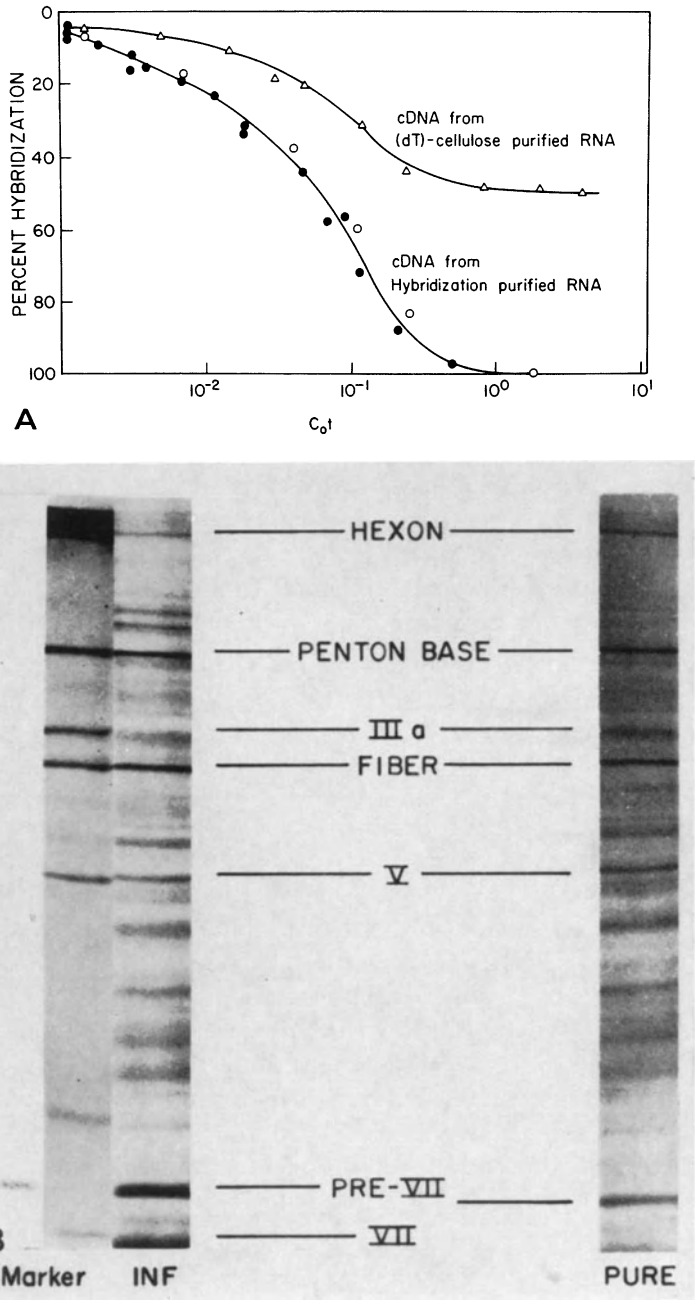

Fig. 5. (A) Reassociation kinetics of adenovirus type 2 DNA and synthetic DNA transcribed from adenovirus messenger RNA preparations. DNA complimentary either to crude polysomal RNA derived from infected cells (▲), or to RNA that had been further purified by preparative hybridization (○), was annealed with virion DNA. Reassociation of virion DNA alone was monitored in a reference assay (●). (B) SDS-polyacrylamide gel electrophoresis and autoradiography of [^{35}S] methionine-labeled polypeptides synthesized in vitro in response to crude (INF) or purified (PURE) adenovirus messenger RMA. For details, se WESTPHAL et al. (1974) (reproduced with permission)

hitherto unknown function. The fragmentation patterns of pVII messenger RNA obtained with RNase III, an enzyme able to recognize specific cleavage sites within an RNA chain, suggest that the translatability of this RNA is abolished even if cuts are introduced outside the pVII codon region (WESTPHAL and CROUCH, 1975). Therefore, whatever the function of these extra sequences may be, their integrity appears to be required for the proper expression of the pVII message.

VII. The Genetic Origin of Virus-Specific In Vitro Translation Products

The genetic capacity of the adenovirus genome is more than sufficient to code for all the virus-specific products that have so far been observed in cell-free translation assays. Concerning the major virion proteins, there was little doubt that they were expressed by the virus itself. However, among the less well characterized virus specific proteins, especially those not found in the virion particle, there could be one or the other component which was not virus-coded but, instead, a virus-induced host cell gene product. To distinguish between the two alternatives, ERON and WESTPHAL (1974) selected messenger RNA by hybridization to viral DNA prior to in vitro translation. The pattern of polypeptides obtained in response to the purified RNA was indistinguishable from that observed when a crude polysomal messenger RNA preparation was used (Fig. 5 B). This showed that all the previously identified in vitro polypeptides synthesized in response to late messenger RNA were indeed viral gene products. The conclusion was, of course, based on one assumption, namely, that hybridization to viral DNA had generated a preparation of pure viral messenger RNA. The experiment of Figure 5 A lends strong support this assumption. Using avian tumor virus RNA-dependent DNA polymerase, both the crude polysomal messenger RNA and the RNA selected by hybridization with viral DNA were transcribed in vitro into complementary DNA. The figure shows the reassociation kinetics of either of the two complementary DNAs with viral DNA, demonstrating that while crude polysomal messenger RNA preparations contained a considerable amount of nonviral sequences, these were completely removed by the subsequent purification step.

The successful in vitro translation of messenger RNA that had been hybridized with viral DNA immediately suggested a biochemical method for mapping of adenovirus gene function. The strategy was to anneal the RNA to defined portions of the DNA, prepared with the help of DNA restriction enzymes, and to then translate the individual RNA fractions in a cell-free system. Using this approach, LEWIS et al. (1975) have indeed obtained RNA fractions with distinct coding specificities, as judged by SDS-polyacrylamide gel electrophoresis of cell-free translation products. More efforts in this direction will hopefully soon establish a detailed physical map of all virus gene products known so far.

VIII. Translation Products of Early Messenger RNA

Several laboratories have recently begun to examine the cell-free translation products of messenger RNA extracted at early times of productive infection, i.e., before the onset of viral DNA replication. These studies are aimed at establishing the molecular correlates for virus gene functions mediating the initial events of viral development. So far, polypeptides of 11 000 MW (LEWIS et al., 1974), 55 000 MW and 73 000 MW (ERON and WESTPHAL, 1975) and of 71 000 MW (ATKINS et al., 1975) have been observed in the in vitro assays, employing crude preparations of early RNA. Naturally, the next task will be to translate pure early adenovirus messenger RNA in order to see which early virus-coded polypeptides can be discerned and in which way they can be related to the virus-specific proteins discovered in vivo.

IX. Outlook

Nonstructural adenovirus gene functions expressed in productively infected or in transformed cells will undoubtedly receive much attention in the course of future investigations. Using the techniques outlined in this review, it should be possible to establish molecular correlates for each of these gene functions, and to determine their position in the physical map of adenovirus DNA. In this way, cell-free translation is expected to complement both genetic and biochemical studies concerning the function of the individual virus gene products. Similar work in another tumor virus system is currently in progress (PRIVES et al., 1974; ROBERTS et al., 1975).

Another problem now being investigated concerns the restriction of human adenovirus growth in monkey cells, and its reversal by coinfection with SV40. The work of several laboratories (see ERON et al., 1975) points to a possible translational control of adenovirus growth in the heterologous host, which, if confirmed, would make this a fascinating research project.

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RNA Tumor Viruses and Human Cancer

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With 2 Figures

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I. Introduction

The considerable efforts in recent years to determine an etiology of human cancer with its importance for early diagnosis, prophylaxis, and therapy have associated, with increasing frequency, RNA tumor viruses with some human malignancies. Early electron microscopic and immunologic studies gained new significance with recent biochemical evidence: the discovery of an RNA-directed DNA synthesizing polymerase in RNA-tumor viruses and the elucidation of RNA tumor viral replication and transformation properties provided new techniques for the discovery of RNA tumor viruses and their association with human neoplasms.

It will be the purpose of this article to: (1) summarize our present knowledge of RNA tumor viruses as far as it is relevant to their detection in human tumors, (2) report on their associations with human cancer, and (3) discuss their possible role in human carcinogenesis. Reviews on related topics, but under different aspects, have been published by TEMIN and BALTIMORE, 1972; TOOZE, 1973; TEMIN, 1974; GREEN and GERARD, 1974; HIRSCH and BLACK, 1974; BAUER, 1974; BOLOGNESI, 1974; and GILLESPIE et al., 1975.

The literature up to December 1975 has been reviewed.

II. Molecular Biology of RNA Tumor Viruses

A. Detection and Identification

Members of the RNA tumor virus group (on morphologic-grounds termed A-, B-, and C-type particles, BERNHARD, 1960; see below) have long been known to cause malignancies in a variety of animals. In 1908, ELLERMAN and BANG were able to transmit leukemia of chicken by cell-free filtrates of leukemic cells. In 1911, ROUS induced sarcomas in chickens with filtrates from sarcomas. At that time, however, the significance of these findings was recognized only by few. With the availability of inbred mouse strains in the 1930s, new advances were made implicating tumor viruses in the etiology of mammalian cancer, and BITTNER (1936) demonstrated induction of mammary tumors in mice through a milk factor by showing that newborn mice from a strain with a high incidence of mammary tumors had a much lower incidence of tumors if they were removed from their mothers and fosternursed by low incidence C57 mice and vice versa.

The first conclusive evidence for an etiologic role of viruses in murine leukemia stems from GROSS (1951). He successfully transmitted leukemia in mice by inoculating very young C3H mice with AK-leukemic extracts. Another virus, causing myeloid leukemia in mice, was isolated by GRAFFI et al. (1955) from sarcoma filtrates and is referred to as the GRAFFI strain of murine leukemia virus (MuLV). In 1957, FRIEND obtained a filterable agent from the spleen of a leukemic Swiss mouse, which consistently produced, on serial transmission, a malignant disease of the hematopoietic system, and soon thereafter MOLONEY (1960) and RAUSCHER (1962) described viral agents that infected not only very young animals or certain genetic strains, but induced leukemia with an incidence of virtually 100 percent. Also the induction of murine sarcomas could be related to RNA tumor viruses, when HARVEY (1964) first described an "unidentified" virus from plasma of Moloney leukemia virus–infected rats, which caused the rapid production of anaplastic sarcomas, angiomas, and leukemias in mice, rats, and hamsters. MOLONEY (1966) recovered a rhabdomyosarcoma-inducing virus from sarcomas appearing in Balb/c mice after inoculation of high doses of mouse-derived Moloney leukemia virus, and FINKEL, ĖISKIS and JINKINS (1966) isolated a virus (FBJ) from a spontaneous murine osteosarcoma. KIRSTEN and MAYER (1967) described erythroblastosis and sarcomas in mice and rats after inoculation of spleen filtrates obtained from rats with erythroblastosis. SOEHNER and DMOCHOWSKI (1969) isolated from Moloney sarcoma virus stock a bone tumor virus which induces bone tumors in mice, rats, and hamsters (MSV-SD virus). OFFICER et al. (1973) encountered a neurotropic C-type virus which can induce paralysis of the lower limb in mice. Like several avian sarcoma viruses, the murine sarcoma viruses are defective and require for their replication and recovery superinfection with a leukemic "helper" virus (HARTLEY and ROWE, 1966; HUEBNER et al., 1966). Except for the FBJ virus none of these murine sarcoma viruses was isolated from naturally occurring sarcomas. BALL et al. (1973) and LO and BALL (1974) reported the isolation of a murine sarcoma virus under more controlled conditions from a mouse sarcoma induced with clone-purified murine leukemia virus.

Also, cat leukemias and sarcomas could be shown to be associated with (JARRET et al., 1964; KAWAKAMI et al., 1967) and transmitted by C-type viruses (JARRET et al., 1964; RICKARD et al., 1969; SNYDER and THEILEN, 1969; GARDNER et al., 1970), and more recently RNA tumor viruses have been isolated from primates (CHOPRA and MASON, 1970; KAWAKAMI et al., 1972; THEILEN et al., 1971) and from one species of snake (ZEIGEL and CLARK, 1969).

Mason-Pfizer monkey virus (MPMV) (CHOPRA and MASON, 1970) was isolated from a spontaneous mammary tumor of a rhesus monkey and, in one report, has been described to cause transformation in tissue culture (PIENTA et al., 1972). By serologic (NOWINSKY et al., 1971b) and ultrastructural (KRAMARSKY et al., 1971) comparison it differs from both B- and C-type particles, and probably forms a separate group. Similar particles have been described in cultures from the lactating mammary gland of a rhesus monkey,

designated X381 (AHMED et al., 1973; YANIV et al., 1974) and in HeLa cells (GELDERBLOM et al., 1974; BAUER et al., 1974; WATSON et al., 1974). Virions morphologically similar to the leukemia-sarcoma C-type viruses of chickens, mice, and cats were isolated from a fibrosarcoma of a woolly monkey (THEILEN et al., 1971) and from a lymphosarcoma, prolymphocytic form, of a gibbon ape (KAWAKAMI et al., 1972). The gibbon ape virus was also isolated from myelogenous leukemia of gibbons and reproduces the disease (KAWAKAMI, personal communication). The woolly monkey virus (simian sarcoma virus or SSV-1) induces fibromas and fibrosarcomas in marmoset monkeys (WOLFE et al., 1971) and can be rescued from woolly sarcoma virus-transformed cells by superinfection with a murine leukemia helper virus (SCOLNICK and PARKS, 1973). Woolly monkey and gibbon ape virus stocks contain nontransforming infectious leukemia viruses that can serve as helper for the rescue of murine-sarcoma virus (SCOLNICK et al., 1972b, c).

Considerable efforts have been made to isolate human candidate tumor viruses from human tumors or cell lines (PRIORI et al., 1971; MCALLISTER et al., 1972; STEWART et al., 1972a, b), but none of these isolates can be unambiguously called human. A C-type virus (ESP-1) produced by a culture from pleural effusion cells of a lymphoma patient (PRIORI et al., 1971; GALLO et al., 1971) contained murine leukemia virus group-specific antigen (GILDEN et al., 1971b) and is now believed to be a murine virus. RD-114, a C-type virus released from cultured rhabdomyosarcoma cells obtained from a cat brain tumor induced by the human RD-cell line and thought to be of human origin (MCALLISTER et al., 1972), was found by hybridization experiments (RUPRECHT et al., 1973b; GILLESPIE et al., 1973) to be rather an endogenous cat virus. It is unrelated to the feline leukemia-sarcoma viruses, but related to other endogenous cat viruses (LIVINGSTON and TODARO, 1973; FISCHINGER et al., 1973) and to an endogenous primate virus (M7) (SHERR et al., 1974b) isolated from a baboon placenta (KALTER et al., 1973b; BENVENISTE et al., 1974a) suggesting a common origin for these viruses (see below).

Most recently, RNA tumor virus-like particles have been observed in a variety of human malignancies, and the best evidence for their regular presence, thus far, has been assembled in the human leukemias. In this context the isolation of particles from human leukemic bone marrow cell lines by MAK et al., (1974a, b, 1975) and VOSIKA et al. (1975), and the continuous production of budding C-type viruses from cultured human acute myelogenous leukemia (AML) cells by GALLAGHER and GALLO (1975) deserve mention. The viruses show immunologic relatedness of their reverse transcriptase and of their group-specific antigen to the corresponding proteins of the simian sarcoma virus (SSV-1) and of the gibbon ape leukemia virus (GALV) (see below).

Endogenous viruses and the observation of virus-like particles in human malignancies and cell lines will be discussed further below. A classification of the RNA tumor viruses is presented in Table 1.

Table 1. RNA Tumor viruses[a] (as defined by morphology, biochemical, and biological properties)

1. Avian RNA tumor viruses
   (a) Avian leukosis-sarcoma (subgroups A, B, C, D, E, F, G)
   (b) Avian reticuloendotheliosis viruses
2. Mammalian C-type RNA tumor viruses
   (a) Murine leukemia-sarcoma viruses, strains Gross, Friend, Moloney, Rauscher, Kirsten, Harvey
   (b) Endogenous viruses of mice (AKR: N- and S-tropic; Balb/c: N- and S-tropic; xenotropic viruses of other mouse strains, etc.)
   (c) Feline leukemia-sarcoma viruses, strains Gardner, Rickard, Theilen
   (d) Endogenous viruses of cats (RD-114, CCC, etc.)
   (e) Hamster leukemia-sarcoma viruses
   (f) Rat leukemia viruses
   (g) Simian leukemia-sarcoma viruses, strains simian sarcoma (= woolly monkey), gibbon ape leukemia
   (h) Endogenous viruses of primates (M7, M28, other baboon viruses)
   (i) Virus like particles in human leukemias, lymphomas, and sarcomas; SSV-related C-type viruses
3. Reptilian RNA tumor viruses
   (a) Viper C-type virus
4. B-type viruses
   (a) Mouse mammary tumor virus
   (b) Viruslike particles in human milk
5. Mason-Pfizer monkey viruses (MPMV, X381, HeLa particles, AO-virus, etc.)
6. "Slow" (visna, maedi) viruses
7. Syncytium-forming "foamy" viruses

[a] Not all members of this class of viruses have been shown to cause tumors; therefore, the names "rnadna viruses" or "ribodeoxy viruses" have been suggested (compare TEMIN, 1974). For nomenclature and characterization see also DALTON et al., 1975.

RNA tumor viruses are identified and quantitated by numerous methods some of which will be summarized here. (1) Electron microscopic screening with thin sectioning and negative staining is used for the detection of the characteristic morphology of RNA tumor viruses (see below) in tissues and cell lines (BERNHARD, 1960; DMOCHOWSKI, 1960). (2) Radioactive labeling of virus-producing tissue cultures and subsequent density analysis will detect large quantities of virus. (3) A sensitive and specific test is the reverse transcriptase assay which, for better specificity, can be combined with inhibition of the reverse transcriptase by specific antisera (SCHLOM et al., 1971a; SCOLNICK et al., 1972b, c). (4) The detection of viral nucleic acids by molecular hybridization demonstrates the presence of viral genomes or of their RNA transcripts. (5) Group-specific antigen tests combined with the radioimmune competition technique have a very high sensitivity and specificity and can be used for the detection of viral antigens in cells. (6) Tissue culture plaque assays can be used for the quantitation of biologically active viruses. The XC test is based on the observation that cells releasing murine leukemia virus form syncytia when placed in contact with XC cells (RSV-transformed rat cells) (ROWE et al., 1970). The S+L−-test (BASSIN et al., 1971) uses an MSV-infected nonfocus-forming 3T3 cell line (S+L−-cells), which responds

to superinfection by murine leukemia viruses with the formation of MSV-type foci proportional to the concentration of the MuLV. (7) Biological activity is demonstrated by infection or transformation tests in tissue culture, or by inoculation experiments in animals. This is the most stringent and ultimate assay for viruses. Particles that have not been shown to possess biological activity are referred to as virus-like and are not called viruses.

B. Morphology

The morphologic structure of the RNA tumor viruses has been studied in detail with the electron microscope (see reviews by Bernhard, 1958 and 1960, and by Gross, 1970). By the thin-sectioning technique and by negative staining, the virus particles appear as almost spherical particles with an electron-dense nucleoid surrounded by an envelope consisting of one or two electron-dense membranes (Bernhard, 1960; Dmochowski, 1960). The viral surface is sometimes covered by projections. On the basis of their morphologic fine structure, Bernhard (1960) subclassified the RNA tumor viruses into A-, B-, and C-type particles. A-type particles (Fig. 1) are intracellular and consist of two concentric electron-dense shells with an electron-lucent center. Dalton (1972) distinguishes intracytoplasmic A-type particles, which possibly become the nucleoids of budding B-type particles, (Smith and Wivel, 1973) from intracisternal A-type particles, which lack, thus far, any demonstrable biological activity. Their average diameter is 70 nm. B-type particles (Fig. 1) possess an eccentric, electron-dense nucleoid, are extracellular, and average 105 nm in diameter. C-type particles (Fig. 2) possess a central electron-dense nucleoid, have a diameter of approximately 100 nm, and are also extracellular.

Contrary to some viruses, the RNA tumor viruses are assembled in the cytoplasm. B- and C-type viruses characteristically bud from the surface of their host cells, and this common property is generally accepted as an identifying morphologic criterion. C-type viruses are the causative agents of animal leukemias, lymphomas, and sarcomas, whereas B-type particles have been identified only in association with mammary tumors of mice, and possibly, in human milk.

C. Physical and Chemical Properties of RNA Tumor Viruses

By physical and chemical characterization, RNA tumor viruses are medium-sized viral particles with an overall physical density of approximately 1.16 g/cm^3 (see review, Beard, 1963). They consist, as determined by Quigley et al. (1971) for RSV, of approximately 64% protein, 31% lipid, 6% hexose, and 1.9% RNA. (Recovery for each experiment ranged from 92 to 107%.) In addition, glycoproteins, phospholipids, and also glycolipids have been reported (Bolognesi et al., 1972). On treatment with surfactants or ether, the

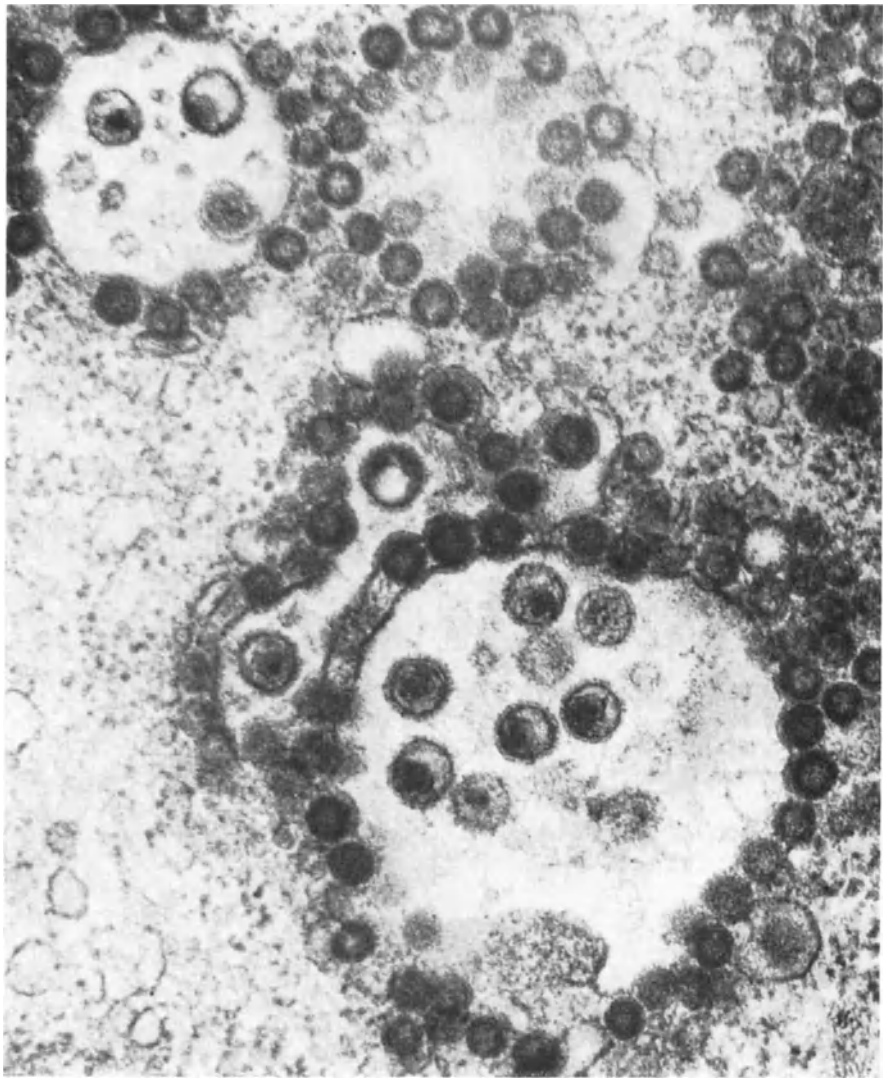

Fig. 1. A- and B-type particles by thin sectioning electron microscopy, magnification approximately 80,000. Note the eccentric nucleoids in the larger B-type particles. The outer membrane carries the characteristic projections (*spikes*). In addition, note the smaller A-type particles with electron-lucent centers. These intracytoplasmic A-type particles are assumed to be the precursors of mature B-type particles. (Courtesy of Dr. A. GOLDFEDER, New York)

virions release a core structure that contains RNA and varying amounts of proteins. The core may possess densities from 1.23 to 1.35 g/cm^3 depending on the amount of protein associated with the RNA. Core structures have been isolated from avian leukosis (BADER et al., 1970; STROMBERG, 1972), from murine leukemia (O'CONNOR et al., 1966; LANGE et al., 1973), and from murine mammary tumor (SARKAR et al., 1971) viruses.

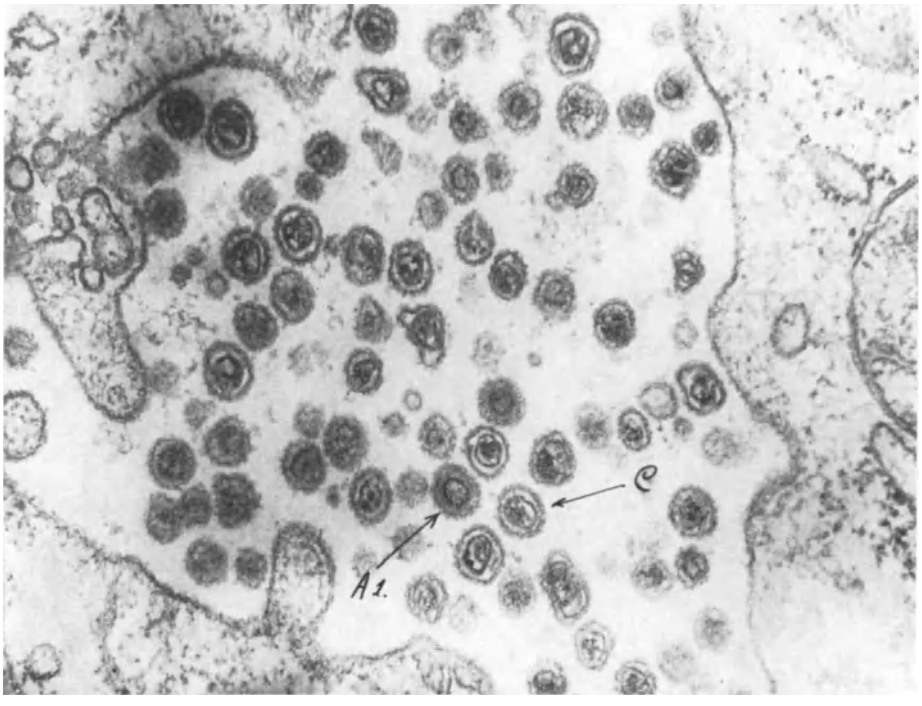

Fig. 2. C-type particles by thin sectioning electron microscopy, magnification approximately 50,000. Note the centrally located electron-dense nucleoid within an inner and outer shell. Note also occasional A-type particles with electron-lucent centers. (Courtesy of Dr. A. Goldfeder, New York)

1. Viral RNA

Crawford and Crawford (1961) isolated RSV particles, estimated their number by electron microscopy, and found by chemical analysis that their genetic material is RNA with a molecular weight of 9.5×10^6 daltons. Robinson and Baluda (1965) determined the size of RSV RNA in the ultracentrifuge as 71S corresponding to a molecular weight of 12×10^6 daltons. Similar values were obtained for the RNA of Rauscher-MuLV (Duesberg and Robinson, 1966; Mora et al., 1966). This high molecular weight (60–70S) RNA can be dissociated into 35S molecules and some smaller RNA species by denaturation with heat or dimethylsulfoxide (Duesberg, 1968; Erikson and Erikson, 1971; Travnicek and Riman, 1973). Recent electron microscopic observations (Delius et al., 1974; Mangel et al., 1974; Jacobson and Bromley, 1975), genetic studies (Duesberg et al., 1974; Beemon et al., 1974), and nucleic acid reassociation kinetics (Baluda et al., 1974) accumulated definitive evidence that the 70S RNA genome of RNA tumor viruses consists of no more than two identical single-stranded 35S RNA molecules with an approximate molecular weight of 3×10^6. Evidence has been provided that this 35S RNA is a precursor of the 60–70S RNA: RNA in freshly harvested particles is subsequently,

after an incubation period, incorporated into 60–70S RNA (CANAANI et al., 1973). This agrees with observations by TSUCHIDA et al. (1972) and TSUCHIDA and GREEN (1974) who describe 35S RNA but no 60–70S RNA in murine sarcoma virus-transformed mouse, rat, and hamster cells.

An interesting difference in the genetic information of transforming and nontransforming avian tumor viruses was found by DUESBERG and VOGT (1970). These authors detected a size difference of the 35S RNA subunits in several transforming and nontransforming viruses. In experiments with cloned stocks (DUESBERG and VOGT, 1973), by oligonucleotide fingerprinting and by DNA-RNA hybridization (LAI et al., 1973), they found that transforming viruses contain a larger 35S subunit, termed class *a*, whereas nontransforming viruses have only a smaller 35S subunit, termed class *b*, missing a piece *x*. DUESBERG and VOGT (1973) suggest that the large *a* subunits comprising *x* may be the minimal genetic requirement for nondefective transforming viruses. The correlation of spontaneous loss of transforming ability with loss of the larger class *a* subunits supports this interpretation.

The low molecular weight RNA mainly consists of two species: a 4S RNA with some properties similar to transfer RNA (BISHOP et al., 1970a; ERIKSON and ERIKSON, 1971; CARNEGIE et al., 1969) and a 7S RNA (BISHOP et al., 1970b). Neither RNA was found in hybridization experiments to be complementary to the 70S RNA genome (BISHOP et al., 1970b). In addition, small amounts of DNA have been reported in the virions of RSV (LEVINSON et al. 1970), which probably are cellular contaminants. The 60–70S RNA of the oncornaviruses contains polyadenylic acid regions (LAI and DUESBERG, 1972; GREEN and CARTAS, 1972; GILLESPIE et al., 1972) that represent approximately 1.5 % of the viral genome. Hybridization of ^{3}H-polyU to these polyadenylic acid regions can be used as an assay for the quantitation of RNA tumor viruses (SCHLOM et al., 1973a).

2. Viral Proteins

As determined by polyacrylamide gel electrophoresis and gel filtration chromatography, there are at least seven major proteins in avian (FLEISSNER, 1971; BOLOGNESI and BAUER, 1970; DAVIS and RUECKERT, 1972) and at least six in mammalian (NOWINSKI et al., 1972a; SCHÄFER et al., 1972a and b; MORONI, 1972; GRAVES and VELICER, 1974) leukemia-sarcoma viruses. With appropriate techniques, at least 11 major proteins can be identified in avian leukosis viruses (AMV and RSV) and in their cores (DAVIS and RUECKERT, 1972; STROMBERG et al., 1974). NOWINSKI et al. (1971a) detected five major polypeptides in ether-disrupted mouse mammary tumor virus, the major internal protein possessing a molecular weight of 52000.

There are at least two glycoproteins on the surface of avian and mammalian RNA tumor virions (DUESBERG et al., 1970; FLEISSNER, 1971; NOWINSKI et al., 1972a). Their approximate molecular weights are 70000 and 32000 in the avian system (FLEISSNER) and may be similar in the mammalian systems

Table 2. Designation and characterization of viral proteins

Protein	Molecular weight	Location	Antigenicity	Designation by FLEISSNER (1971)	BOLOGNESI and BAUER (1970)
Avian leukosis viruses					
p 10	10000			p 5	
p 12	12000	Internal	Group	gs 3	CF 1
p 15	15000	Internal	Group	gs 4	CF 2
p 19	19000	Internal	Group	gs 2	CF 3
p 27	27000	Internal	Group	gs 1	CF 4
gp 35	35000	Surface	Type	m 1	G I
gp 85	85000	Surface	Type	m 2	G II
				NOWINSKI et al. (1972)	GEERING et al. (1970)
Mammalian (murine) leukemia viruses					
p 10	10000	Internal	Group	p 4	
p 12	12000	Internal	Group, type	p 3	
p 15	15000	Internal	Type, group, interspecies	p 2	
p 30	30000	Internal	Group, interspecies, type	p 1	gs1/gs3
gp 45	45000	Surface		m1	
gp 71	71000	Surface	Type, interspecies, group	m2	

Modified according to AUGUST et al. (1974).

(STRAND and AUGUST, 1973; MOENNIG et al., 1974). The glycoproteins probably represent the small spikes at the outside of the lipid layer and carry the major type-specific antigenicity. The rest of the viral proteins seem to be localized inside the lipid layer, more or less closely associated with the viral core. The approximate molecular weights of these internal proteins are 27000, 15000, 12000, and 10000 (FLEISSNER, 1971; NOWINSKI et al., 1972a), and 19000 for the additional protein in avian viruses (FLEISSNER, 1971). The nomenclature used by the different groups for the viral proteins varies greatly and is confusing, and therefore a uniform nomenclature proposed at Memorial-Sloan Kettering Cancer Center in June 1973 (AUGUST et al., 1974) will be used here. Viral proteins and glycoproteins are designated according to their apparent molecular weights in thousands. Proteins will carry a small p and glycoproteins a small gp just before the number. The antigenic determinants of the viral proteins, characterized as type-specific, group-specific, and interspecies, will appear after the number (see Table 2).

The four major internal proteins p27, p19, p15, and p12 carry group-specific determinants in the avian system, whereas gp71 (69/71), p30, p12, and p10 possess group specificity in mammalian viruses. In the mammalian systems p30 and gp71 also carry interspecies cross antigenicity (GEERING et al., 1968; STRAND and AUGUST, 1973; HUNSMANN et al., 1974), and type-

specific determinants have been associated with p12 and p30 (STEPHENSON et al., 1974d; TRONICK et al., 1974b) and with gp71 (STRAND and AUGUST, 1974a; HUNSMANN et al., 1974). Recent evidence suggests that the mammalian p15 polypeptide contains besides type-specific also group and interspecies-specific reactivities (STRAND et al., 1974; SCHÄFER et al., 1975). Additional studies by IHLE et al. (1975) indicate that the mammalian p15 is principally involved in the virus neutralization reaction and is most probably localized on the virus surface. HUNSMANN et al. (1975) showed in the Friend murine leukemia system that gp71 and its corresponding antiserum can be used for active and passive immunization against Friend leukemia. Considering the known interspecies relationships of some viral proteins these results point to prophylactic possibilities in human leukemia.

3. Enzyme Activities

In addition to the RNA dependent DNA polymerase (see below), several nucleases (MIZUTANI et al., 1970, 1971; MÖLLING et al., 1971), one ligase (MIZUTANI et al., 1971), and other enzyme activities (MIZUTANI and TEMIN, 1971) have been reported. MIZUTANI et al. (1970) observed an endonuclease in virions of the Schmidt Ruppin strain of RSV and also (1971) a DNA-exonuclease (not detected by HURWITZ and LEIS, 1972) and a DNA ligase. Possible functions of this ligase could be the connection of small DNA pieces to larger molecules and the insertion of provirus DNA into the DNA of the host cell chromosome. MÖLLING et al., (1971) isolated from AMV virions a nuclease that selectively digests the RNA moiety of an RNA:DNA hybrid molecule. This activity, called ribonuclease H (STEIN and HAUSEN, 1969), is quite ubiquitous in RNA tumor viruses (GRANDGENETT et al., 1972) and appears to be closely associated with the viral DNA polymerase in the avian system (MÖLLING et al., 1971; WATSON et al., 1973; VERMA, 1975a).

In the murine system, RNase H activity has been separated from the polymerase in one study (WU et al., 1974a). In contrast, VERMA (1975b) finds RNase H activity associated with Moloney MuLV DNA polymerase on one major polypeptide. GRANDGENETT and GERARD (1975) identified two RNase H activities in lysates of Moloney murine sarcoma-leukemia virus: a larger enzyme, representing about 10% of the activity and copurifying with the DNA polymerase, and a smaller enzyme separable from the polymerase, thus explaining the results of both, WU et al. and VERMA.

4. Nucleic Acid Relatedness

The nucleic acid sequence homologies of the major RNA tumor virus groups are summarized in Table 3. Whereas extensive homology exists among the different avian leukemia-sarcoma viruses as a group, no such relationship can be detected with the avian reticuloendotheliosis viruses (KANG and TEMIN, 1973a; HALPERN et al., 1973; PURCHASE et al., 1973). The avian viruses are

Table 3. Homologies of RNA tumor virus genomes

	Viral RNA								
	Avian			Murine					
[^{3}H]DNA	ALV	REV	Rauscher MuLV	Kirsten MuLV	Moloney MuLV	AKR MuLV	Kirsten MuSV	S2 C13 Endogenous	MMTV
ALV	100.0	0–3			1.9				<1
REV	8.0	100							
R-MuLV	2.0		100	66.0	65.0	13.0	15	50.0	0
Ki-MuLV	1.6		58	100.0				97.0	
M-MuLV	0		60		100.0	14.0	6		
M-MuSV	1.0		53		100.0	35.0	34		
AKR-MuLV	0		8		16.0	100.0	100		0
S2 C13, Mouse, Endogenous	1.4		57	100.0				100.0	
R-FeLV	0		10	7.5	14.0	14.0	10	2.6	0
G-FeSV	1.0		14		14.0	11.0	12		
RD-114	<1.0			1.6				<1.0	
V-NRK, Rat, Endogenous				6.0				8.0	
CCL-38, Rat, Endogenous								7.0	
Visna	0		0				0		0
MPMV	3.0		3		2.0	4.0	3		0
GaLV	0		20	27.0				25.0	
SSV-1	0–2		16	22.0	6.0	10.0	85	15.0	
M7, Baboon, Endogenous			0						
Human Leukemic	0		5–19	0		5–14	30–50		

The data were collected from: Baxt et al., 1972; Benveniste and Todaro, 1973; Benveniste et al., 1974a; Gallo et al., 1973; Hehlmann et al., 1973a, b; Hehlmann and Spiegelman, 1974; Kang and Temin, 1973a; Miller et al., 1974; Quintrell et al., 1974; Larsen et al., 1975.

The studies were done with complementary DNA probes which in some cases may not reflect the entire viral genome; thus, homologies between some viruses may exist which have not yet been elucidated. In most instances, the amount of homology was determined by nuclease digestion (ribonuclease, nuclease S_1). The values obtained by Cs_2SO_4 density centrifugation are designated with an asterisk.

Abbreviations: ALV = avian leukosis virus, REV = reticuloendotheliosis virus, R-MuLV = Rauscher murine leukemia virus, Ki-MuLV = Kirsten murine leukemia virus, M-MuLV = Moloney murine leukemia virus, M-MuSV = Moloney murine sarcoma virus, R-FeLV = Rickard feline leukemia virus, G-FeSV = Gardner feline sarcoma virus, MPMV = Mason-Pfizer monkey virus, GaLV = Gibbon ape leukemia virus, SSV-1 = simian sarcoma virus 1 (woolly monkey), MMTV = mouse mammary tumor virus.

(by RNA-DNA hybridization, in percent hybridization)

Feline				Rat		Hamster	Pig		Primate				
Rickard FeLV	Gardner FeSV	RD-114	CCC	V-NRK Endogenous	CCL-38 Endogenous	CCL-14.1 Endogenous	PK-15 Endogenous	Visna	MPMV	GaLV	SSV-1	M7, Endogenous	Human leukemic particles
	3.8	2.9						2.9					0*
6.0	11.0				1.1	1.5	2.0	0	0	11.0	8.0		6*
<1.0		<1.0		4.0	6.0	6.0	1.8		1.2	26.0	22.0		
7.0	11.0	<1.0						0		10.0			
6.0	8.0									10.0	17.0		1.3–70
6.0	15.0							0	0	8.0	18.0		
2.5	3.0	2.0		4.0	6.5	5.0	2.5			24.0	25.0		
100.0	100.0	1.2	5.0	2.8	3.0	1.7	8.0	0	0	2.0	5.0		
70.0	100.0	2.8	4.4	1.7							4.0		
		100.0	75.0	1.4		1.0		1.4	<1.0	1.9	1.7		
				100.0						8.0			
				70.0	100.0								
		2.7	1.8					100.0	0				
1.6	4.0							1.0	100.0	2.0	4.0		
	1.7	0.7			2.5	1.8		3.0		100.0	49.0		
1.2	8.0	1.2		2.0	2.5	<1.0		<1.0	4.0	46.0	100.0		20
1.8		10.0				0				1.5	2.0	100	
0	5–10										30–50		20*

entirely unrelated to the mammalian RNA tumor viruses. Varying degrees of homology exist among the murine endogenous and leukemia-sarcoma viruses. No homology of these viruses to the murine mammary tumor virus can be detected. Surprisingly, almost no homology exists between the feline endogenous virus group (RD114, CCC) and the feline leukemia-sarcoma viruses. A similar situation evolved for the relation of C-type viruses in primates where no homology has been detected, thus far, between the endogenous baboon (M7, M28, etc.) and the primate leukemia-sarcoma viruses (gibbon ape, woolly monkey) (BENVENISTE et al., 1974a). No relationship has been detected between any of these and the Mason-Pfizer monkey viruses (MPMV). Amazingly, a considerable degree of homology, 20–30% in most tests (cf.

Table 3), between murine C-type viruses and the two primate leukemia-sarcoma viruses (gibbon ape, woolly monkey) has been found (BENVENISTE and TODARO, 1973; MILLER et al., 1974), whereas only little homology of these two groups is exhibited to cat, rat, hamster, or pig C-type viral RNAs. [^{3}H]DNA synthesized endogenously with the human leukemic viral-like particles (cf. Section III, 5) exhibits homology to murine and primate leukemia-sarcoma viral RNAs (BAXT et al., 1972; HEHLMANN et al., 1973a; GALLO et al., 1973) which is consistent with the nucleic acid relationship demonstrated by murine and primate leukemia-sarcoma viruses. Comparative studies on nucleic acid sequence homologies between the various C-type viruses have been conducted by HAAPALA and FISCHINGER (1973), BENVENISTE and TODARO (1973), QUINTRELL et al. (1974), MILLER et al. (1974), HARTER et al. (1973), CALLAHAN et al. (1974), and EAST et al. (1973, 1975).

D. Antigenicity and Host Range

1. Type Specificity

The avian leukosis viruses consist of seven subgroups (A–G) on the basis of: (1) type-specificity by fluorescent antibody and neutralization tests, (2) host range in cultured fibroblasts, and (3) interference, i.e., induction of resistance to infection, after preinfection with a leukosis virus of the same subgroup (ISHIZAKI and VOGT, 1966; DUFF and VOGT, 1969; FUJITA et al., 1974).

In contrast to the avian system, there is no direct correlation in the murine leukemia viruses between serotypes and host range. Two distinct antigenic types have been detected by AOKI et al. (1966). The G or Gross antigen is carried by leukemic cells infected with Gross passage A virus and by all leukemias of high incidence strains for spontaneous leukemia. The FMR or Friend-Moloney-Rauscher antigen is found in the cells of leukemias induced by the laboratory-derived Friend, Moloney, and Rauscher leukemia viruses. FMR antigen is absent in all leukemias of mice not experimentally infected with viruses of the FMR group (AOKI et al., 1966). By their ability to grow in genetically defined mouse cells, HARTLEY et al. (1970) reported three categories of murine-leukemia virus strains: N-tropic strains, like Friend and Gross passage A virus, have a 100–1 000-fold growth preference for NIH Swiss cells, whereas B-tropic strains grow 30–100 times better in Balb/c mouse embryo lines. Some virus strains such as Moloney and Rauscher grow equally well in both cell types and are called NB-tropic. Recently, a group of endogenous viruses has been isolated from tissues of NZB and NIH mice, which is growth-restricted in all homologous, i.e., mouse cells, but does grow "xenotropicly" in heterologous, e.g., rat and human cells (LEVY, 1973). LIEBER et al. (1974) and SHERR et al. (1974b) induced similar viruses from cell lines derived from disease-free mice that grow best in the rabbit corneal cell line SIRC ("S-tropic") (BENVENISTE et al., 1974b) and are restricted in mouse cells. Their presence in normal tissue and the failure to detect this class of

viruses in preparations of viruses with known leukemogenic potential was interpreted by LIEBER et al. (1974) as suggestive of a normal physiologic role of these viruses (see below).

In addition, type-specific radioimmunoassays for a low molecular weight polypeptide (p12) have been developed to discriminate between C-type viruses that cannot be well distinguished otherwise (STEPHENSON et al., 1974d; TRONICK et al., 1974b).

2. Group-Specific Determinants

Group-specific antigens in the avian leukosis virus system were first reported by HUEBNER et al. (1964). The detection in virions and in virus-infected tissues of antigenic determinants specific for distinct groups of RNA tumor viruses was soon found to provide a comparatively rapid and simple assay for subinfectious expression of RNA tumor viruses. Based on the detection of group-specific avian leukosis antibodies (HUEBNER et al., 1964) in hamster sera, SARMA et al. (1964) developed a complement-fixation test for avian leukosis (COFAL). These group-specific viral antigens are immunologically identical to those found in infected cells (BAUER and JANDA, 1967) and represent internal components of the virions (BAUER and SCHÄFER, 1965). At present at least four group-specific determinants have been identified in the avian leukosis system.

Group-specific antigens of the murine leukemia viruses were first defined by GEERING et al. (1966) by immunoprecipitation and by HUEBNER (1967) by complement fixation. The major group-specific antigen was isolated and characterized by GREGORIADES and OLD (1969) who termed it gs1. It was identified in neoplastic tissues of G^+ and G^--mouse strains, and was initially estimated to have a molecular weight of 35–40 thousand. These results were confirmed by SCHÄFER et al. (1969) who estimated for the group-specific antigen of Friend virus a molecular weight of 26000. Group-specific antigens were also detected in feline leukemia virus (FeLV) and in tissues of cats with lymphosarcoma (GEERING et al., 1968; HARDY et al., 1969), in rat C-type viruses (OROSZLAN et al., 1972a), in hamster C-type viruses (KELLOFF et al., 1970a, b; OROSZLAN et al., 1971a), and meanwhile in virtually every C-type virus isolated. The widespread occurrence of viral group-specific antigens under conditions where infectious virus or particles could not be found (PAYNE and CHUBB, 1968; GILDEN and OROSZLAN, 1972) have contributed significantly to the theory that viral genetic information is inherited through the germ line (TODARO and HUEBNER, 1972; GROSS, 1970; BENTVELZEN and DAAMS, 1969).

3. Interspecies Specificity

In 1968, GEERING et al. showed that a determinant of one of the MuLV group-specific antigens is shared by the feline leukemia virus (FeLV). This important observation was soon confirmed by SCHÄFER et al. (1970), and

extended by GEERING et al. (1970) who detected interspecies activity also in viruses obtained from peritoneal fluid of rats bearing the Novikoff ascites hepatoma and from a hamster lymphosarcoma. Though not group-specific but interspecies-specific, the antigen was termed gs3. No interspecies cross reactivity was found between mammalian and avian tumor viruses. Weak interspecies cross reactions of anti-Rauscher MuLV-gs3 rabbit serum were observed with two human tumor cell lines and one bovine leukotic line (SCHÄFER and DE NORONHA, 1971). FERRER (1972) did not find any such interspecies reactivity in bovine serum that contained antibodies to bovine C-type viruses. In 1971, SCHÄFER et al. and OROSZLAN et al. (1971b) reported the isolation of an interspecies determinant from feline leukemia virus and obtained a molecular weight of about 33000 (25000). GILDEN et al. (1971a) and OROSZLAN et al. (1971b) showed that the interspecies (gs3) determinants of murine and feline C-type viruses reside on the same major structural protein that also contains the group-specific (gs1) activity.

In 1972, PARKS and SCOLNICK and also OROSZLAN et al. (1972c) successfully applied radioimmunoprecipitation, a technique of high sensitivity, which measures the displacement of ^{125}iodine-labeled antigen by unlabeled antigen from an antigen-antibody complex, to the detection of MuLV gs1 and gs3. The radioimmunoprecipitation assay may provide a 500–10000-fold increase in sensitivity over standard immunodiffusion procedures and can detect as little as 5 ng of the MuLV group-specific antigen (SCOLNICK et al., 1972a). By radioimmunoassay, PARKS and SCOLNICK (1972) detected interspecies reactivity not only in the murine, feline, hamster, and rat leukemia viruses, but also in the primate woolly monkey (SSV-1) and gibbon ape C-type (GaLV) viruses, not, however, in the Mason-Pfizer monkey virus, in the feline and primate syncytium-forming viruses, or in visna virus. The woolly monkey and gibbon ape primate viruses, closely related to each other (PARKS et al., 1973b), could be distinguished from murine, hamster, rat, and feline C-type viruses on the basis of sensitivity and extent of displacement in the radioimmunoassay (PARKS and SCOLNICK, 1972). Mammalian interspecies-specific reactivity was also found in the major internal protein of RD-114 (OROSZLAN et al., 1972b) which does not show any species-specific reactivity with feline leukemia-sarcoma or any other mammalian C-type viruses. OROSZLAN et al. (1972c), and SCHÄFER et al. (1973) demonstrated that the interspecies antigens of woolly monkey virus (SSV-1), of RD-114, and of a pig virus (PV), are more closely related to each other than to those of the feline and murine leukemia-sarcoma viruses.

A second mammalian interspecies antigen (interspec II, gp69/71 interspecies) different from the major internal polypeptide has been found in two viral envelope glycoproteins (STEEVES et al., 1974) with apparent molecular weights of 69000 and 71000 (STRAND and AUGUST, 1973). By competition radioimmunoassay and by appropriate choice of antisera and competing proteins, typespecific, group-specific, and interspecies determinants were distinguished on each of the two viral glycoproteins (STRAND and AUGUST, 1974a).

Preliminary competition experiments failed to detect any cross reactivity of feline interspec II with RD-114, woolly monkey, gibbon ape, or Mason-Pfizer monkey viruses. There is suggestive evidence, however, that this is not due to the absence of antigens, but rather to a low affinity of antifeline antibodies to interspec II proteins of primate viruses (STRAND and AUGUST, 1974a). The presence in mammalian C-type viruses of shared viral antigens may have far-reaching implications for the detection of RNA tumor viral gene products in human tissues (cf. Chapter III, B).

4. Reverse Transcriptase

Serologic specificities similar to those reported by PARKS and SCOLNICK (1972) and SCHÄFER et al. (1973) evolve for the mammalian C-type viruses, if a third interspecies determinant, the viral DNA polymerase (see below), is used for immunologic characterization (SCOLNICK et al., 1972a). Natural antibody against the viral RNA-dependent DNA polymerase has been found by GERWIN et al. (1970) and AARONSON et al. (1971a) in sera from rats bearing murine C-type viruc-induced tumors. PARKS et al. (1972) prepared antisera against partially purified polymerases of avian and murine leukemia viruses and tested the sera with polymerases of a variety of mammalian and avian viruses for cross inhibition. They found cross reactions between the polymerases of the different avain leukosis and myeloblastosis strains and between the polymerases of murine, rat, hamster, and feline leukemia viruses. The polymerases from other mammalian viruses (MMTV, visna, and Mason-Pfizer monkey virus) were immunologically distinct from both avian and murine reverse transcriptases. When SCOLNICK et al. (1972a, b) tested primate C-type viral reverse transcriptases with antisera prepared against the polymerases of murine and feline leukemia viruses, they found two major groups: mouse, rat, hamster, and cat polymerases were inhibited by both, feline and mouse polymerase antisera; no inhibition was detected in the woolly monkey, gibbon ape, and RD-114 polymerases. DNA polymerase from ESP-1, a canditate human virus (PRIORI et al., 1971), was inhibited by MuLV-polymerase antiserum almost as well as the MuLV-polymerase, supporting a murine origin of ESP-1.

Two studies tried to determine the immunologic relationship between the polymerases of RD-114 and of woolly monkey and gibbon ape viruses. Although both studies (SCOLNICK et al., 1972b; LONG et al., 1973) confirm the close relationship of gibbon ape and woolly monkey polymerases, they differ in the degree of relationship of the RD-114 polymerase to the two primate viral polymerases. Both studies, however, seem to agree that RD-114 polymerase is more closely related to the polymerases of the two primate viruses than to those of lower mammals.

Antiserum prepared against MPMV-DNA polymerase neutralized the polymerases of MPMV and of X381, an agent recently isolated from a cell line of lactating mammary gland of a rhesus monkey (AHMED et al., 1973) but did

Table 4. RNA dependent DNA polymerases

Antibodies to polymerases	Avian			Reptilian	Mammalian				
	AMV	RSV (SR)	REV	Viper	MuLV	MuSV	HaLV	Rat LV	FeLV
AMV	+++	+++	− (+)	−	−				−
$RSV_{H(SR)}$	+++	+++	− (RAV-O)		−			−	−
$REV_{H(TDSNV)}$	− (+)	− (RAV-O)	+++						
MuLV	−	−		−	+++		+	+	+
MuSV	−	−			+	+	+		+
FeLV	−				++		+	+	+++
RD 114	−	−		−	−				−
MPMV	−				−				−
SSV-1	−								−
GaLV	−	−							−

Data from: Parks et al., 1972; Scolnick et al., 1972b, c; Nowinski et al., 1972b; Watson et al., 1972; Long et al., 1973; Todaro and Gallo, 1973; Gallagher et al., 1974; Todaro et al., 1974b; Yaniv et al., 1974; Mizutani and Temin, 1974; Sherr et al., 1974b.

+++ = Homologous reaction; ++ = less than homologous reaction; + some cross inhibition observed; − = no cross inhibition.

Abbreviations (see also text): SR = Schmidt-Ruppin strain of RSV (Rous sarcoma virus), TDSNV = Trager duck spleen necrosis virus of the reticuloendotheliosis virus (REV) group, SSV-1 = Simian sarcoma virus 1 (woolly monkey virus), GaLV = Gibbon ape leukemia virus, RAV-O = Rous ≠ associated virus-O of the avian leukosis virus group, MPMV = Mason-Pfizer monkey virus.

not inhibit the polymerases of any avian or mammalian RNA tumor virus including mouse mammary tumor virus and simian sarcoma virus (woolly monkey) (Yaniv et al., 1974). This immunologic unrelatedness of MPMV polymerase agrees with observations by Tronick et al. (1974b) and Nowinski et al. (1971b), who failed to detect any immunologic relationship between antigens of MPMV and those of other mammalian C-type viruses even by the sensitive competition radioimmunoassay (Tronick et al., 1974a).

The antigenic interrelationships among the various reverse transcriptases are summarized in Table 4.

E. Replication of RNA Tumor Viruses

Whereas the single-stranded (SS) RNA-containing bacterial and animal viruses replicate via a complementary RNA strand, the requirement of early DNA synthesis for the replication of the SS-RNA-containing tumor viruses was recognized as early as 1964 (Temin, 1964b; Bader, 1964). The inhibition

(antigens)

FeSV	RD-114	ESP-1	MMTV	Visna	MPMV	SSV-1	GaLV	Baboon	Human leukemia (AML)
			−	−	−		−		−
									−
	−	+	−	−	−	−	+	−	+
+			−		−				−
	−	+				−	−		−
	+++					++	+	++	−
			−		+++	−	−		−
						+++	+++	−	++
					−	+++	+++	-	++

of DNA synthesis by actinomycin D, amethopterin, cytosine arabinoside, or BUDR prevented growth of progeny Rous sarcoma virus (TEMIN, 1963, 1964b; BADER, 1964; BADER and BADER, 1970; MURRAY and TEMIN, 1970). Assuming that a viral-specific DNA intermediate might be involved in tumor viral RNA replication, TEMIN (1964a) postulated an intracellular DNA template for viral progeny RNA, which he termed, in analogy to the bacterial prophage, the provirus. Initial DNA synthesis would be necessary for formation of this provirus. DUESBERG and VOGT (1969) provided additional evidence for this hypothesis and showed that in cells preinfected with an avian tumor virus, early DNA synthesis remains a prerequisite for successful superinfection with another avian tumor virus. This also suggested that the DNA synthesized early after infection is rather viral than cell-specific. The first convincing evidence for the presence of viral DNA in infected cells came from hybridization studies by BALUDA and NAYAK in 1970.

1. RNA-Directed DNA Synthesis

The enzyme postulated for this "reverse transcription" of viral RNA into proviral DNA was first detected and described by TEMIN and MIZUTANI (1970) and BALTIMORE (1970). These authors described an RNA-dependent DNA polymerase activity in detergent-disrupted virions of Rous sarcoma and Rauscher mouse leukemia virus, which is dependent on Mg^{2+} or Mn^{2+} ions and on all four deoxyribonucleotides. The reaction is abolished by pretreatment with ribonuclease. The reaction product is resistant to ribonuclease and alkali treatment, but is sensitive to deoxyribonuclease.

The properties of this polymerase have been detailed previously in several fine review articles (TEMIN and BALTIMORE, 1972; SARIN and GALLO, 1973;

Green and Gerard, 1974) and will be summarized here only as far as it appears necessary in the context of RNA tumor viral association with human malignant tumors.

The RNA-dependent DNA synthesis of the viral enzyme, as measured by the incorporation of labeled deoxyribonucleotides (mostly [^{3}H]-labeled thymidine triphosphate) into an acid-insoluble DNA reaction product, depends: (1) on partial disruption of the virion by a nonionic detergent, (2) on the presence of all four deoxyribonucleotides, and (3) on the presence of magnesium or manganese (Temin and Mizutani, 1970; Baltimore, 1970; Spiegelman et al., 1970a). The enzyme accepts natural RNA and DNA as templates (Spiegelman et al., 1970b; Mizutani et al., 1970; McDonnell et al., 1970; Riman and Beaudreau, 1970), but also can use synthetic DNA-RNA hybrids and RNA-RNA duplexes, and though generally less specific than natural single-stranded RNA, some of these templates have been shown to be superior to natural templates in stimulating DNA synthesis by up to several orders of magnitude (Spiegelman et al., 1970c; Schlom et al., 1971a; Burny, 1970 and 1971, unpublished observations). The specificity of synthetic duplexes could be greatly enhanced by the use or ribohomopolymer templates combined with complementary deoxyribooligomer primers, e.g., poly(A) + oligo(dT) (Goodman and Spiegelman, 1971; Baltimore and Smoler, 1971) and poly(C) + oligo(dG) (Lewis et al., 1974), the latter being the most specific template-primer combination besides natural RNAs. Of the natural RNAs, 60–70S viral RNA was found to be the best template (Duesberg et al., 1971b). If heat-denatured viral RNA is used the reaction is somewhat reduced, but can be enhanced by the addition of oligodeoxyribonucleotide primers (Duesberg et al., 1971b). The effects of primers on the transcription of synthetic polyribonucleotides have been extensively described by Baltimore and Smoler (1971).

Viral reverse transcriptase appears to lack a precise template specificity (Spiegelman et al., 1971; Leis and Hurwitz, 1972) and can be used for copying of a variety of natural RNAs into complementary [^{3}H]DNA probes (Spiegelman et al., 1971; Ross et al., 1972; Verma et al., 1972; Kacian et al., 1972).

2. Reaction Product

The early formation of viral RNA-DNA hybrid molecules was first observed by Rokutanda et al. (1970). Manly et al. (1971), Fanshier et al. (1971) and Faras et al. (1971) confirmed the hybrid nature of the early reaction product and examined the time-course of DNA synthesis following infection by avian and murine RNA tumor viruses. In the avian systems, the initial DNA product is covatently linked to its RNA template (Verma et al., 1971; Leis et al., 1975). Whereas the initial product consists of nascent DNA chains attached to 60–70S RNA, at later times increasing amounts of single- and double-stranded DNA are found. These findings are consistent with the early synthesis of an RNA-DNA replicative intermediate with subsequent tran-

scription into a double-stranded DNA provirus as postulated by TEMIN (1964a). This DNA, then, could function as template for progeny RNA production, or could be integrated into the host cell genome as latent provirus, as has been described for DNA of DNA-containing tumor viruses. From there, for example, it could be activated for future virus production, or for cell transformation. Indeed, the demonstration by HILL and HILLOVA (1972) of infectious viral DNA in RSV-transformed mammalian cells provided evidence that the entire viral genome is transcribed into DNA. Infectious viral DNA was subsequently demonstrated also in the murine sarcoma (KARPAS and MILSTEIN, 1973) and in the reticuloendotheliosis (COOPER and TEMIN, 1974) virus systems. This viral DNA is probably synthesized in the cytoplasm (VARMUS et al., 1974c), transported into the nucleus, and subsequently found integrated in the cellular DNA (MARKHAM and BALUDA, 1973; VARMUS et al., 1973a; EVANS et al., 1974). Intermediate forms of the viral DNA include linear and nicked circular as well as closed circular supercoiled molecules (VERMA et al., 1974b, c; GIANNI et al., 1975). The inhibition of virus production and of the integration of viral DNA by ethidium bromide suggests that the circular molecule is the form that is integrated into the cellular DNA, and that integration is needed for virus production (GUNTAKA et al., 1975). This has become quite questionable since TEMIN and also WEINBERG have shown that linear DNA is more infectious than circular DNA (Cold Spring Harbor, 1975). Mistakes in the transcription process of this integrated viral DNA in some hosts could explain the host-induced alterations of tumor viral RNAs (SHOYAB et al., 1975).

The DNA-dependent DNA synthesis step, and the formation of double-stranded viral DNA can be selectively inhibited by actinomycin D (MCDONNEL et al., 1970; MANLY et al., 1971; RUPRECHT et al., 1973a).

Several groups confirmed by hybridization experiments that the in vitro DNA product of the endogenous reaction indeed represents a true copy of its RNA template (SPIEGELMAN et al., 1970a; ROKUTANDA et al., 1970; DUESBERG and CANAANI, 1970; GARAPIN et al., 1971), and the extent of transcription of the 60–70S RNA molecule into DNA seemed to be close to 100%, though particular portions of the RNA molecule apparently are copied preferentially (DUESBERG and CANAANI, 1970; VARMUS et al., 1971; GELB et al., 1971b; TAYLOR et al., 1973; GARAPIN et al., 1973). However, if single-stranded DNA is synthesized in the presence of actinomycin D, a complete and relatively uniform copy of the entire RSV genome is found (GARAPIN et al., 1973). TAVITIAN et al. (1974) reported the synthesis of single-stranded DNA that represents an almost complete and uniform copy of the entire 70S RNA genome using a conventional endogenous reverse transcriptase assay without actinomycin D in the Moloney murine leukemia-sarcoma virus system.

The size of the in vitro reaction product in the endogenous system is about 4S or 50–100 nucleotides (TAYLOR et al., 1972). Using natural mRNA, e.g., globin RNA as template, a sedimentation value of 8S has been determined (ROSS et al., 1972; VERMA et al., 1972; KACIAN et al., 1972), representing

about 500 nucleotides. At optimal detergent concentration, JUNGHANS et al. (1975) report full length (2.5×10^6 daltons or greater) DNA transcripts of RSV-RNA up to 10% of the total DNA yield.

The direction of synthesis for the enzyme has been determined as $5' \rightarrow 3'$ with addition of nucleotides to the 3'-OH group of primer (SMOLER et al., 1971) and agrees with the direction of synthesis of other known DNA polymerases.

F. RNA-Directed DNA Polymerase

1. In Viruses

After initial reports by BALTIMORE (1970) and TEMIN and MIZUTANI (1970), reverse transcriptase activity has been detected in virtually all known oncogenic RNA viruses (SPIEGELMAN et al., 1970a; GERWIN et al., 1970; GREEN et al., 1970; HATANAKA et al., 1970; SCOLNICK et al., 1970) including mouse mammary tumor virus (SPIEGELMAN et al., 1970a; SCOLNICK et al., 1970), leukemia and sarcoma viruses of mice, cats, hamsters, and primates (SPIEGELMAN et al., 1970a; GREEN et al., 1970; SCOLNICK et al., 1972a; KAWAKAMI et al., 1972), Mason-Pfizer monkey virus (MPMV) (SCHLOM and SPIEGELMAN, 1971a) and a reptilian virus (HATANAKA et al., 1970). Later, RNA-dependent DNA activity was discovered in a class of "slow" viruses (Visna virus: SCHLOM et al., 1971b; STONE et al., 1971a; Maedi virus: LIN and THORMAR, 1972; STONE et al., 1971b), which cause slow progressive neurologic and pulmonary disease in sheep and transform mouse cells in culture (TAKEMOTO and STONE, 1971), in "foamy" viruses (PARKS et al., 1971), and in the so-called endogenous viruses, for which oncogenicity has been established only in some instances (ROWE, 1973; STEPHENSON et al., 1974c). The preliminary communication of an RNA-directed DNA polymerase in preparations containing Australian antigen (HIRSCHMAN et al., 1971) has not been confirmed by others. No characteristics of reverse transcriptase were shown.

Reverse transcriptase has been purified and characterized from avian, mammalian, and reptilian systems (KACIAN et al., 1971; ROSS et al., 1971; DUESBERG et al., 1971a, b; FARAS et al., 1972; TWARDZIK et al., 1974). The best characterized enzyme stems from avian myeloblastosis virus (KACIAN et al., 1971) and consists of two subunits with 69000 and 110000 molecular weights. The combined enzyme, however, sediments with about 6S corresponding to a molecular weight of 110000, a value also reported by DUESBERG et al. (1971a) and FARAS et al. (1972). In contrast, the DNA polymerase isolated from murine leukemia virus consists of a single polypeptide with an estimated molecular weight of 70000 (ROSS et al., 1971; HURWITZ and LEIS, 1972). A similar value has been obtained for the DNA polymerase of woolly monkey virus (ABRELL and GALLO, 1973), whereas the Mason-Pfizer monkey virus polymerase, from an RNA tumor virus neither B- nor C-type, possesses an apparent molecular weight of 110000 (ABRELL and GALLO, 1973). The DNA polymerase of the B-type mammary tumor virus with a molecular

weight of approximately 100000 (DION et al., 1974) also seems to be different from the DNA polymerases of mammalian C-type viruses. This is consistent with the detection of two viral RNA-dependent DNA polymerases with molecular weights of 70000 and somewhat larger than 70000 in mouse milk, which is known to contain B- and C-type particles (HOWK et al., 1973). The reptilian C-type viral polymerase has an apparent molecular weight of 109000 as estimated by glycerol gradient centrifugation (TWARDZIK et al., 1974).

An RNA-dependent DNA polymerase and a 60–70S RNA have recently been reported also in intracisternal A-type particles from a mouse plasma cell tumor and a neuroblastoma cell line (YANG and WIVEL, 1973 and 1974).

2. In Cells

The expected presence of RNA-directed DNA polymerase activity in tumor viral-infected cells and in certain malignant tissues of animals has been demonstrated by many groups (ROSS et al., 1971; COFFIN and TEMIN, 1971; BURNY et al.: avian myeloblasts, 1971 unpublished; HEHLMANN et al.: ascites cells of rat chloroleukemia, 1971 unpublished). Reverse transcriptase activity detected in human tissues will be detailed further below.

Of interest is the detection by some groups of an RNA-directed DNA polymerase even in apparently normal, uninfected cells. COFFIN and TEMIN (1971 and 1972) observed in high-speed pellets from detergent-disrupted uninfected rat cells a ribonuclease-sensitive DNA polymerase activity, similar to that observed in a preparation from B77 virus-infected rat cells. The endogenous template was reported unrelated to avian leukosis virus RNA and to RNA from a rat C-type virus. The findings were extended by the observation of similar reverse transcriptase-containing particles in uninfected avian leukosis virus group-specific negative chicken embryos (KANG and TEMIN, 1972). The DNA product of the endogenous reaction did not hybridize with RNA of Rous sarcoma virus or of reticuloendotheliosis virus, but hybridized to RNA from the same chicken cell fraction. The reaction was sensitive to ribonuclease and partially resistent to actinomycin D. The same authors supported their findings by the detection of early DNA-RNA hybrids in the endogenous reaction (KANG and TEMIN, 1973b). Further, this DNA polymerase was serologically distinct from those of avian leukosis-sarcoma viruses and of reticuloendotheliosis viruses (MIZUTANI and TEMIN, 1973, 1974). TEMIN interprets these results as consistent with his hypothesis that RNA viruses with a DNA-polymerase evolve from normal cellular components (TEMIN, 1971).

Reverse transcriptase has been isolated also from normal rhesus monkey placenta, 1st trimester (MAYER et al., 1974), but never from any normal adult tissue. Evidence suggesting a reverse transcriptase in oocytes of *Xenopus laevis* (CRIPPA and TOCCHINI-VALENTINI, 1971; BROWN and TOCCHINI-VALENTINI, 1972; MAHDAVI and CRIPPA, 1972), however, has been challenged on technical grounds (BIRD et al., 1973).

The observation of reverse transcriptase in apparently normal embryonic cells can be expected, as endogenous viruses would be anticipated to be expressed at some stage of embryonic life. It suggests an interesting aspect for an understanding of origin and natural history of RNA tumor viruses and their possible role in normal cell differentiation (see also Chapter IV, 4). Reverse transcriptase of an exogenous RNA tumor virus, as shown by immunology, would be of diagnostic value for the detection of RNA tumor viruses in malignant cells.

Some uncertainty as to the diagnostic use of the reverse transcriptase came from reports that *E. coli* polymerase I, under certain conditions, would transcribe heteropolymeric RNA regions (Cavalieri and Carrol, 1970; Loeb et al., 1973; Modak et al., 1973, 1974; Gulati et al., 1974; Sarin et al., 1974). *E. coli* polymerase I, however, is 10–200-fold less efficient than reverse transcriptase, prefers poly-A regions for transcription, and no activity of this kind has been observed in vivo. Further, no eukaryotic DNA polymerase has been shown capable of transcribing heteropolymeric single-stranded RNA, only reverse transcriptase (see below).

3. Biological Significance

Convincing evidence for the biological role of viral reverse transcriptase comes form two lines of experiments: Hanafusa and Hanafusa (1968) isolated a variant of the Bryan strain of RSV, called RSV (α), which yielded physically intact but noninfectious particles, requiring for their recovery the superinfection with avian leukosis viruses. Hanafusa and Hanafusa (1971) reported that this noninfectious RSV is deficient in DNA polymerase, both by enzyme assay even with the most sensitive synthetic template-primer complexes, and by a neutralization blocking test against antibody to the DNA polymerase (Hanafusa et al., 1972). The second line makes use of two temperature-sensitive mutants of RSV (ts 335 and ts 337), which are defective early in their growth cycles (Wyke, 1973) and lack a function necessary for initiation of viral replication and cell transformation (Linial and Mason, 1973). Verma et al. (1974) report that these mutants possess a thermolabile DNA polymerase, and genetic studies (Mason et al., 1974; Friis et al., 1975) indicate that the enzyme is necessary for initiation of infection.

4. Inhibitors

Because of the apparent key role of reverse transcriptase in the process of infection and transformation of cells by tumor viruses, specific inhibitors of its activity appeared to provide potentially useful drugs for the prevention of cancer. Gurgo et al. (1971) reported that certain derivatives of the antibiotic rifamycin (2,5-dimethyl-4-N-benzyldemethylirifampicin and demethylrifampicin) inhibit the DNA polymerase of several RNA tumor viruses at concentrations of about 100 μg/ml. Yang et al. (1972) and Gurgo et al. (1972) tested more than 200 rifamycin derivatives and examined selected

compounds as to their specificity for reverse transcriptase versus other normal or leukemic polymerases. TING et al. (1972) could correlate inhibition of focus formation and of reverse transcriptase activity of murine leukemia-sarcoma viruses by some derivatives of rifamycin SV, and SMITH et al. (1972) found that certain rifamycin derivatives are more cytotoxic for fresh human leukemic cells than for normal white blood cells. WU et al. (1973) further correlated prevention by rifamycin SV derivatives of leukemogenic activity of Rauscher leukemia virus in mice with the magnitude of enzyme inhibition. The degree of splenomegaly of the infected animals served as a quantitative index. The relatively high concentration needed and a low drug tolerance seem to limit the present therapeutic value of the rifamycins.

Other inhibitors have been described by several groups. The most potent of these appears to be ethidium bromide (MÜLLER et al., 1971; HIRSCHMAN, 1971), but also streptovaricins (BROCKMAN et al., 1971), daunomycin, acridines, and olivomycin (MÜLLER et al., 1971) inhibit reverse transcriptase to varying degrees. A problem is very often selectivity as well as specificity, and the inhibition of RLV-DNA polymerase by single-stranded polyribonucleotides reported by TUOMINEN and KENNEY (1971) was found by others (ABRELL et al., 1972) to be similar for viral and for cellular DNA polymerases. WU et al. (1972) reported inhibition of virus production by cordycepin (= 3′-deoxyadenosin), and recently, WU et al. (1974b) found an inhibitory effect also by interferon. This was confirmed by FRIEDMAN and RAMSEUR (1974) who reported inhibition of virus release from AKR virus-producer cells by interferon. The interferon effect can be partially competed for by stimulation with dexamethasone (PARAN et al., 1973).

III. Association of RNA Tumor Viruses with Human Cancer

The similarity of some malignancies in man with certain tumors in animals, for which a viral etiology has been demonstrated, has caused an intensive search for analogous agents in human cancer. Initial electron microscopic and serologic data encouraged later biochemical studies.

An etiologic relationship between the viral-like particles and any human neoplasm has not been established, and Koch's postulates have not been fulfilled in a single case of a putative viral cancer in man. The regularity of their association with some human cancers, however, suggests at least a contributory role. In addition, Koch's postulates might not at all be adequate for viral involvement in cancer, as latent periods may be long, and the virus may not be present at the height of the disease.

A. Electron Microscopic Evidence

1. Leukemias and Lymphomas

In 1957, DMOCHOWSKI and GREY first demonstrated the presence in the lymph nodes of two leukemic patients of particles resembling those found in

some murine leukemias (DALTON et al., 1961a and b). DMOCHOWSKI et al. (1958/1959) extended these observations to other patients including one case of lymphosarcoma, and soon similar particles were detected by many in cell material (BRAUNSTEINER et al., 1960; DMOCHOWSKI et al., 1967; SEMAN and SEMAN, 1968), in plasma (ALMEIDA et al., 1963; BENYESH-MELNICK et al., 1964a, b; SMITH et al., 1964; BURGER et al., 1964; VIOLA et al., 1967; PORTER et al., 1964; SEMAN and SEMAN, 1968), and in the urine (AMES et al., 1966) from patients with leukemia. The electron microscopic techniques used in these studies were either ultra-thin sectioning or negative staining with phosphotungstic acid (BRENNER and HORN, 1959). Though the negative staining method initially was thought to have a higher sensitivity and to reveal more of the fine structure of the particles observed (BENYESH-MELNICK, 1964a, b), it was soon abandoned as a technique for the identification of C-type particles, as many products of cytolysis and also mycoplasmas may have similar appearance. Other sources of confusion were certain platelet fragments and lysosomes in some preparations from leukemic patients (PORTER et al., 1964; BRYAN et al., 1965; PRINCE and ADAMS, 1966). With care, these ambiguities of identification can be eliminated.

In order to get statistically significant information, studies of large series were undertaken to compare the frequency of particles in leukemic patients and normal controls (PORTER et al., 1964; LEVINE et al., 1967; DMOCHOWSKI et al., 1967; SEMAN and SEMAN, 1968). Together, by thin-section electron microscopy, these authors screened blood cells, biopsies, and plasma pellets of a total of 382 cases with leukemia or lymphoma. Approximately 30% of the blood cells, 34% of the biopsies, and 12% of the plasma pellets were designated as positive by the authors for the presence of viruslike particles. Particles in normal control specimens were rarely observed. PORTER et al. (1964) identified virallike particles in none of 51 random controls and in 1 of 36 age- and sex-matched normal controls. Other large-scale studies (NEWELL et al., 1968; ROSS and HARNDEN, 1969) failed to detect significant differences between leukemic and normal plasma and blood cell preparations, and the frequency of viral particles in human leukemias, if present at all, is felt to be considerably lower than in animal leukemias.

2. Breast Cancer and Human Milk

The presence of a milk factor (BITTNER, 1936) in high cancer strains of mice, capable of initiating mammary tumors, suggested the search for a similar agent in human milk and breast cancer. Early electron microscopic studies by GROSS et al. (1950) revealed small spherical particles measuring 20–200 nm in diameter in all milk specimens from women with a high familial incidence of breast cancer. It is doubtful, whether these particles represented virus. LUNGER et al. (1964) described distinct differences between milk from normal and from breast cancer patients and reported spherical structures of 60–90 nm

in diameter. Particles were also seen by JENSEN and SCHIDLOVSKY (1964) in two samples of presumably normal human milk. Using thin-section electron microscopy, FELLER et al. (1967) and FELLER and CHOPRA (1968) observed "small particles" of approximately 30 nm in diameter more frequently in breast cancer milk and biopsies than in normal controls. The nature of these "small particles" is unclear. In 1969, the same group extended their observations and described three types of viruslike specimens in milk. In addition to the "small particles", they found herpes viruslike particles and B-type-like particles of approximately 100 nm in diameter (CHOPRA and FELLER, 1969; FELLER and CHOPRA, 1969). SEMAN et al. (1969) and DMOCHOWSKI et al. (1969) examined breast cancer biopsy specimens of 44 patients and reported particles resembling murine B-type and C-type viruses and "small particles". B-type particles were found in 8 cases, C-type particles in 4 cases, and "small particles" in 13 cases. Up to 1971, this group carried out ultrastructural studies on biopsies of 84 breast cancers, 13 metastatic lymph nodes, and 3 fibroadenomas (SEMAN et al., 1971) with particles resembling B-type and/or C-type viruses in 34 out of the 100 breast tumor biopsies, in 2 out of 33 specimens of breast cancer pleural effusions, and in 4 out of 21 cancer milk specimens.

The most extensive study on milk specimens from women with breast cancer and from women with or without a high family record of breast malignancies was carried out by MOORE and associates (MOORE et al., 1969; MOORE et al., 1971 a, b). Using the negative staining technique they examined 101 milks of "high risk" American families, 46 milks from women of the Parsis in Bombay, who, compared to their lower incidence of other cancers, have a relatively higher incidence of breast cancer than the rest of the Bombay population, and 181 "control" milks. Particles resembling murine B-type viruses have been originally reported in 39% of the Parsi milks, in 31% of the "high risk" milks, and in 12% of the normal controls. After a more stringent re-evaluation of their electron microscopic data, SARKAR and MOORE (1972) are willing to call only 13 out of a total of 381 milk specimens positive for B-type particles with only one or two particles detected in each case. Particles resembling B-type viruses were present in 41 specimens, and particles resembling C-type viruses were seen in 134 specimens. CALAFAT and HAGEMAN (1973) are challenging even these data on technical grounds, pointing out that cell debris may assume morphologic characteristics similar to C-type viruses in negatively stained preparations. By the more specific thin sectioning technique, CALAFAT and HAGEMAN (1973) could not detect any intact B- or C-type particles in milk samples from 43 Dutch women. This, however, contrasts (1) with data by FELLER and CHOPRA (1971) who also used the thin-section technique and observed viruslike particles resembling various stages of the murine B- and C-type RNA tumor viruses in milk samples from 9 out of 59 women; and (2) with biochemical tests by SCHLOM et al. (1971 a), who correlated, in part, the detectability of viruslike particles in the electron microscope with the presence of reverse transcriptase, a characteristic feature of the RNA tumor viruses (see below).

3. Other Human Tumors and Cell Lines

Though many human tumors have been screened, no evidence of viruses has been found in most instances. In some cases, viruslike particles have been observed. MORTON et al. (1969b) reported intracisternal A-type particles in a human chondrosarcoma. ELLIOTT et al. (1973a and b) detected C-type particles in three papillary cancers of the human renal pelvis and in four transitional tumors of the human urinary bladder. Particle-producing cultures were obtained from the three papillary tumors. KALTER et al. (1973a) reported the detection of budding and mature C-type particles in 4 of 6 normal human placentas, demonstrating the presence of C-type particles in a nonpathologic tissue. Electron microscopic evidence for human C-type particles also has been obtained in cell lines, e.g., of a cultured human liposarcoma (MORTON et al., 1969a, b; HALL et al., 1970; MALMGREN and MORTON, 1971); of a cultured human brain biopsy from a patient with Creutzfeldt-Jakob disease (HOOKS et al., 1972, 1973); and of a human rhabdomyosarcoma and metastatic adenocarcinoma after treatment with 5-iododeoxyuridine and dimethylsulfoxide (STEWART et al., 1972a, b). MPMV-like viruses, neither B- or C-type, have been observed in HeLa cells (GELDERBLOM et al., 1974), in a human amniotic cell line, AO (PARKS et al., 1973c), and in several other human lines (ILYIN et al., 1973; BUKRINSKAYA et al., 1974; ZHDANOV et al., 1972, 1973). In several instances, C-type particles, originally identified by electron microscopy in cell lines derived from a human rhabdomyosarcoma and from an American Burkitt's lymphoma and thought to be of human origin (MCALLISTER et al., 1972; PRIORI et al., 1971), turned out later to be rather of non-human (feline and murine) origin.

The fact that even in the most extensive series published only up to 35% of the patients with leukemias, lymphomas, and breast cancers were positive for B- or C-type particles points to the difficulties of the electron microscopic approach. The limiting factors include: (1) that the virus may be present in titers below the detection threshold of the electron microscope; (2) that the tumor may be caused by a virus that does not produce electron microscopically visible particles as observed in some cell lines (BASSIN et al., 1971), and (3) that even if a viruslike structure has been observed, its biological relatedness to the disease remains undetermined.

Further, in the search for virus structures in human tumors, the difference in animals between the number of C-type particles encountered in spontaneous tumors and laboratory-induced neoplasms of inbred strains should be kept in mind. While in most laboratory-induced animal tumors, C-type particles are detected in large numbers and without difficulty (DALTON et al., 1961a, b), only few particles are seen with considerable effort in spontaneous animal neoplasms (DMOCHOWSKI, 1970). In this respect, the search for viral particles in malignant disease of man probably resembles the situation in spontaneous tumors of outbred animals as compared with tumors of inbred animals such as mice of some strains.

B. Serologic Evidence

Three prinicpal techniques have been applied to identify immunologically RNA tumor virusrelated structures in human cancer. (1) Animal RNA tumor viruses and viruslike particles from patients' plasma are used to produce antibodies, which in turn are tested for reactivity with the same patient's or with other patients' tumor cells. (2) Antibodies already present in the patient's serum and assumed to be viral-specific are tested against tumor cells of the same or of other patients. In either case, if cross-reactivity is detected, this is interpreted as suggestive of a viral agent. (3) Antibodies synthesized against interspecies-specific antigens of mammalian RNA tumor viruses are used for the search of cross-reactive antigens in human tumor cells.

The first technique was used by producing an antiserum in rabbits against viruslike particles in plasma pellets of individuals with leukemia. In subsequent immunofluorescent assays, this serum reacted specifically with various cellular elements of the bone marrow and peripheral blood of several patients with leukemia and lymphoma and failed to react with leukocytes from normal donors (FINK et al., 1964; BATES et al., 1969; BANKOLE et al., 1972). A similar antibody prepared against the murine Rauscher leukemia virus (RLV) cross-reacted with the bone marrow and blood cells of some human leukemias (FINK et al., 1964, 1965; IOANNIDES et al., 1968; BATES et al., 1969). Reversely, neutralizing antibody against RLV was detected in rare instances of human leukemia (FINK et al., 1964), and CHARNEY and MOORE (1971) reported in the sera of breast cancer patients neutralizing antibodies against mouse mammary tumor virus.

The results, in addition to supporting an association of a viral agent with some human malignancies, also indicate a possible immunologic relationship of the agents associated with the corresponding neoplasms in mice and men. No evidence exists in human leukemias of neutralizing antibodies against avian (SOLOMON et al., 1969) or feline leukemia viruses (HARDY et al., 1969).

The second technique was applied in an immunofluorescent study on human osteosarcomas by MORTON and MALMGREN (1968). These authors tested sera from patients with osteosarcoma, from their relatives and close associates, and from normal donors against sarcoma cells of the same and of other patients. Positive reactions were obtained in 100% with tumor cells of the same patient and in 80% with tumor cells from other patients. Sera from family members and close associates reacted positively with about 90%, whereas sera from normal blood bank donors showed a positive immunofluorescent reaction in only 29% of cases. The complete cross-reactivity of the sera suggests similar, if not identical, antigens in the different sarcomas. The high reactivity in normal sera suggests considerable ubiquity of the antigen. However, evidence that the reactive antigen(s) are viral is not available. Similar results were obtained by the detection in the serum of a patient with liposarcoma of antibodies cross-reacting with antigens both in the original liposarcoma and in the cultured liposarcoma cells producing C-type particles

(MORTON et al., 1969a and b; HALL et al., 1970). PRITCHARD et al. (1971) induced osteosarcomas in Syrian hamsters by inoculating them at birth with cell-free extracts of human osteosarcomas, and demonstrated sarcoma-specific antigen in human osteosarcomas and in four of the human osteosarcoma-induced hamster sarcomas. Similar though somewhat less convincing studies link human malignant melanoma to a common cytoplasmic antigen (LEWIS et al., 1969; MUNA et al., 1969; MORTON et al., 1968). Whether this common antigen is of viral nature remains to be determined, but it is of interest that electron microscopic (BIRKMAYER et al., 1972) and biochemical evidence (BIRKMAYER et al., 1974; HEHLMANN et al., 1975; BALDA et al., 1975) indicate the presence of RNA tumor viruslike particles in human malignant melanomas and other skin cancers.

The third approach, i.e., to detect RNA tumor viruses and some of their structures by immunologic interspecies cross-reactions became possible through the detection by GEERING et al. (1968) of an interspecies-cross-reacting antigen in mammalian RNA tumor viruses. SCHÄFER et al. (1971) were the first to demonstrate weak cross-reactivity between anti-Rauscher MuLV-gs3 antibodies and two human malignant cell lines. OLSEN and YOHN (1972) and SUTHERLAND and MARDINEY (1973) reported antibodies to mammalian RNA tumor virus interspecies antigen (p30) in the sera and renal immune complexes of cancer patients. CHARMAN et al. (1974) failed to detect RLV-related antibodies in the sera of more than 100 cancer patients and controls. These authors did not examine cytoplasmic fractions for RLV-related antigens.

Additional reports on interspecies specific viral related proteins in human tissues (SHERR and TODARO, 1974b; STRAND and AUGUST, 1974b) appear, in spite of their potential importance, still preliminary and should be discussed at a later time. In this context and in the context of results to be reported below, the detection by SHERR and TODARO (1975), in the peripheral white blood cells of five patients with acute leukemia, of antigens related to the p30 structural proteins of the gibbon ape leukemia and simian sarcoma viruses (GALV and SSV-1) deserves mention. This finding agrees with the earlier reports of nucleic acid (GALLO et al., 1973; MAK et al., 1975) and of reverse transcriptase relatedness (TODARO and GALLO, 1973; GALLAGHER et al., 1974) between human acute myelogenous leukemic cells and certain primate type-C viruses.

C. Reverse Transcriptase in Human Neoplasms

The specific association of an RNA-dependent DNA polymerase with RNA tumor viruses offered a new powerful approach for the detection of viral structures in tumor tissue and body fluids of patients with malignant disease. Again the search concentrated on the mesenchymal leukemias, lymphomas, and sarcomas, and on breast cancer.

1. Leukemias

Less than 6 months after the initial reports by TEMIN and MIZUTANI (1970) and BALTIMORE (1970) of an RNA-dependent DNA polymerase in RNA tumor viruses, GALLO et al. (1970) described an enzyme in leukocytes of three patients with acute lymphatic leukemia (ALL), which stimulated in a ribonuclease-sensitive reaction the incorporation of [^{3}H]-TMP into an acid-precipitable polymerization product. The reaction was dependent on all four deoxyribonucleotides and on magnesium. The DNA reaction product, however, was not further characterized. Based on studies with synthetic templates, SPIEGELMAN, KEYDAR, BURNY, and HEHLMANN (Paris, 1970) reported interesting differences between the DNA polymerase activities of a large series of leukemic white blood cells, embryonic tissues, and normal controls, but these results were never published. ACKERMAN et al. (1971) reported endogenous RNA-dependent DNA synthesis in cell-free preparations from human leukemic cells in tissue culture. Failure to characterize the reaction product again left doubt as to the nature of the reaction. Some initial confusion was also caused by the identification of a reverse transcriptase solely on the basis of synthetic templates. Synthetic duplexes may serve as superior templates for the detection of the enzyme, but, in most instances, are less specific than natural heteropolymeric RNA (SCHLOM et al., 1971a). Thus, initial studies by PENNER et al., (1971a, b) and by SCOLNICK et al. (1971) remained inconclusive, as either a reaction dependent on a heteropolymeric RNA was not included in the study (SCOLNICK et al., 1971), or the template RNA remained uncharacterized (PENNER et al., 1971a, b). The importance of product characterization in RNase-sensitive reverse transcriptase-like reactions was stressed by BOBROW et al. (1972) who describe an RNase-sensitive reaction in a cytoplasmic fraction of normal human lymphocytes stimulated by phytohemagglutinin (PHA), which fails to transcribe heteropolymeric regions of viral 70S RNA and to form DNA-RNA hybrids. This reaction, therefore, was interpreted as RNA-primed rather than RNA-directed.

An easy source of confusion also was the presence of other "normal" cellular DNA polymerases in normal and leukemic white blood cells and much effort was devoted by Gallo's group and by others to the distinction of a true reverse transcriptase from other cellular DNA polymerases. A specific distinction was obtained on the basis of the preferential stimulation of reverse transcriptase reactions by the ribohomopolymer-deoxyhomooligomer combinations poly(A)-oligo(dT) (GOODMAN and SPIEGELMAN, 1971) and poly(C)-oligo(dG) (LEWIS et al., 1974a) and by viral heteropolymeric RNAs. Three distinct cellular DNA polymerases have been described previously (ROBERT et al., 1972; SMITH and GALLO, 1972; LEWIS et al., 1974a, b; WEISSBACH et al., 1971; FRIDLENDER et al., 1972; BOLDEN et al., 1972; MCCAFFREY et al., 1973). The nomenclature used by these groups is confusing. Essentially, the enzymes are (1) a high molecular weight polymerase with a sedimentation constant of 6–8S found in the cytoplasm of proliferating cells. This enzyme is called

DNA-polymerase I by Smith and Gallo (1972), DNA-polymerase C by McCaffrey et al., (1973), and DNA polymerase α according to a new nomenclature proposed for general use in 1974 (Gallo, personal communication). (2) A low molecular weight enzyme with a sedimentation value of 3.3 S found in the nucleus and cytoplasm of resting and dividing cells (called DNA polymerase II by Smith and Gallo, 1972; DNA-polymerase-N by McCaffrey et al., 1973; DNA-polymerase β according to the new nomenclature). Both polymerases prefer poly(dA)-oligo(dT) over poly(A)-oligo(dT) and cannot transcribe heteropolymeric RNA; (3) Bolden et al. (1972) characterized a third polymerase in HeLa cells and PHA-stimulated lymphocytes, which had been described earlier by Weissbach et al. (1971) and Fridlender et al. (1972). This enzyme is stimulated by poly(A)-oligo(dT) similar to reverse transcriptase, but is distinct by its inability to transcribe heteropolymeric RNA (Bolden et al., 1972) or oligo(dG)-primed poly (C) (Lewis et al., 1974a). It was designated R-DNA polymerase by Fridlender et al. (1972), DNA polymerase III by Lewis et al. (1974a, b), and DNA polymerase γ by the 1974 convention. Its size ranges from 5.4 s to 6.3 s depending on the source of the enzyme (Lewis et al., 1974b). A fourth DNA polymerase has been characterized on immunologic and kinetic grounds (Livingston et al., 1974).

After proper distinction from cellular DNA polymerases and purification Sarngadharan et al. (1972) isolated reverse transcriptase from human leukemic cells with template preferences and reaction conditions similar to the viral enzyme. The AMV-70S RNA-stimulated reaction product was identified, by back-hybridization, to be a true copy of the AMV-70S RNA template. Similar results were obtained by Baxt et al. (1972) and Hehlmann et al. (1973a) who described reverse transcriptase activity associated with a high molecular weight RNA in a particulate fraction of human leukemic cells (see below). Further evidence for the viral nature of this enzyme was provided by immunologic comparison: Todaro and Gallo (1973) and Gallagher et al. (1974) prepared antibodies to purified RNA-directed DNA polymerases of primate C-type viruses (SSV-1, gibbon ape virus), and demonstrated cross-reactivity between human leukemic (AML) and primate C-type viral DNA polymerases. Reverse transcriptase has been purified from a patient with leukemia to a single band of 70000 daltons in a gel electrophoresis (Witkin et al., 1975).

2. Human Milk

Based upon the electron microscopic observations by Moore et al. (1971a,b) of particles in human milk similar to murine B-type particles, Schlom et al. (1971a) isolated a particulate fraction from human milks with density characteristics of RNA tumor viruses ($\varrho = 1.16$–1.19 g/cm^3) and demonstrated endogenous reverse transcriptase activity in this fraction. The reaction was abolished by ribonuclease and depended on the presence of all four deoxyribonucleotides and magnesium. No characterization of the reaction product

was included, but the authors, in later publications (see below) were able to demonstrate that this reverse transcriptase is associated with a high molecular weight RNA (SCHLOM et al., 1972) and that the particles containing the two can be converted to cores with a density of 1.26–1.27 g/cm^3 analogous to other known RNA tumor viruses (FELDMAN et al., 1973). Initially, the authors hoped to correlate their findings of reverse transcriptase with breast cancer. Later, DNA polymerase activity in human milk was found by GERWIN et al. (1973) and by ROY-BURMAN et al. (1973), but no correlation with the presence of breast cancer or the donors' family histories of cancer could be made.

In addition, reverse transcriptase has been isolated from normal rhesus monkey placenta (MAYER et al., 1974). C-type particles have been found in normal baboon placentas (KALTER et al., 1973b; BENVENISTE et al., 1974a) and in 4 of 6 apparently normal human placentas (KALTER et al., 1973b). The detection of C-type particles and reverse transcriptase in apparently normal embryonic tissues (cf. II, 6) suggests some role for reverse transcriptase and/or endogenous C-type viruses in normal differentiation (see IV, 4).

D. Tumor Viral RNA in Human Malignancies

Another powerful approach in the search for an association of tumor viral structures and of tumor viruslike particles with human cancer was the application of the technique of molecular hybridization to the detection of viral-specific nucleic acids in virus-infected animal systems and in human malignant tissues. In principle, radioactively labeled viral-specific single-stranded DNA or viral RNA were used to detect complementary RNA or DNA sequences in cytoplasm or nucleus of animal and human cells. The subsequent identification and isolation of the hybrid structures were accomplished by three techniques, each with clear advantages and disadvantages. (1) Cs_2SO_4 density equilibrium centrifugation (HALL and SPIEGELMAN, 1961) separates viral DNA pieces complexed to large RNA molecules from uncomplexed DNA. The method is very sensitive because of low backgrounds, but the least specific because also poorly matched hybrid structures ("loops" and "tails") will be registered as hybrids. (2) Hydroxyapatite chromatography (BERNARDI, 1965; BRITTEN and KOHNE, 1968; KOHNE and BRITTEN, 1971) separates single-stranded DNA from double-stranded molecules by elution with phosphate buffer, and distinguishes well-matched duplexes from poorly matched hybrids on the basis of their thermal stability. This method is specific and convenient, because large amounts of reactions can be screened rapidly. (3) Single-strand-specific nucleases (most popular are the easily purified nuclease S-1 from *Aspergillus oryzae* (LEONG et al., 1972; VOGT, 1973) and the commercially available micrococcal nuclease from *Staphylococcus aureus* (KACIAN and SPIEGELMAN, 1974) selectively degrade single-stranded DNA while leaving intact DNA-DNA and DNA-RNA hybrids. This method is stringent (non-base paired structures are destroyed) and rapid for multiple assays. Recently,

virus-specific DNA has been detected, in mouse and rat cells, by in situ hybridization and subsequent autoradiography (LONI and GREEN, 1974, 1975).

Using Cs_2SO_4 density centrifugation, SPIEGELMAN and associates first reported RNA sequences in human leukemias, sarcomas, lymphomas, and breast cancer homologous to RNAs of known animal RNA tumor viruses and suspected human candidate viruses (AXEL et al., 1972b; HEHLMANN et al., 1972a, b; KUFE et al., 1972, 1973a). The approach was to use animal tumor viruses of known oncogenicity and, if possible, putative human RNA tumor viruses to generate, in an endogenous reverse transcriptase reaction, a radioactively labeled complementary DNA probe (c-DNA) that could detect, by RNA-DNA hybridization, the presence of viral-specific RNA in human tumors.

The assumption of a possible cross relationship between animal tumor viruses and their putative counterparts in the analogous human diseases was supported indirectly by the histologic and pathologic similarities between the corresponding human and animal malignancies as well as by the occasional detection of immunologic cross-reactivity between the known mouse leukemia or mammary tumor viruses and cell or serum components from human leukemias and breast cancer patients (FINK and RAUSCHER, 1964; FINK et al., 1965; BATES et al., 1969; IOANNIDES et al., 1968; CHARNEY and MOORE, 1971; PRIORI et al., 1972).

RNA-DNA hybridization was successfully applied by GREEN et al. (1971) to detect viral-specific RNA both in the nuclear and cytoplasmic fractions of mouse cells infected with the Moloney sarcoma–leukemia virus complex. With a similar approach, AXEL et al. (1972a) demonstrated MMTV sequences in a polysomal RNA fraction from mouse mammary tumors and showed the feasibility of detecting, by DNA-RNA hybridization, tumor virus-related RNA in tumor tissue. With this technique, AXEL et al. (1972b) then detected in human breast cancer, RNA homologous to mouse mammary tumor virus RNA. Nineteen out of 29 human breast tumors (67%) were shown by the authors to contain MMTV-related RNA sequences in the cytoplasm, whereas 14 normal or nonmalignant breast tissue specimens, including fibrocystic disease and fibroadenomas, did not show any homology, Parallel investigations with human leukemias (HEHLMANN et al., 1972a), sarcomas (KUFE et al., 1972), and lymphomas (HEHLMANN et al., 1972b) revealed RNA in these malignancies related specifically to RNA of the Rauscher murine leukemia virus, whereas no homology to [^{3}H]-MMTV-DNA was detected. In the case of the human leukemias, more than 90% were reported to give this kind of specific reaction (HEHLMANN et al., 1972a, 1973b), while more than 50 normal control tissues remained negative. None of the above malignancies was found to react with [^{3}H]-DNA synthesized with the avian myeloblastosis virus (AMV). DAS et al. (1972a, b) further reported RNA in human breast cancers that hybridized to [^{3}H]-DNA synthesized endogenously with the human milk particles described by MOORE et al. (1971a) and SCHLOM et al. (1971a). KUFE et al. (1973a, b) extended the findings of RLV-related sequences in human leukemias and lymphomas to Burkitt's lymphomas and to the epithelial

nasopharyngeal carcinomas. Extensive previous evidence including molecular hybridization had linked these malignancies to the herpes-like DNA containing Epstein Barr virus (EBV) (EPSTEIN et al., 1964 and 1965; WRIGHT, 1967; HENLE et al., 1968, 1969 and 1970; GUNVEN et al., 1970; ZUR HAUSEN et al., 1970; NONOYAMA et al., 1973; WOLF et al., 1973; KLEIN et al., 1974). The relationship between EBV and RLV-related sequences in these malignancies is unclear.

In spite of the experimental precautions (HEHLMANN et al., 1972b), taken to insure interpretability of the hybridization results, the authors initially did not convince their peers because of low amounts of radioactivity involved and lack of hybridization kinetics. The studies remained unconfirmed for 2 years, until several laboratories (GALLO et al., 1973; VAIDYA et al., 1974; MAK et al., 1975; LARSEN et al., 1975) confirmed and extended these results: In addition to hybridization with RLV-RNA, GALLO et al. (1973) detected extensive homology, in two cases with AML, to RNA isolated from type C simian sarcoma virus (SSV-1). About 50% of the DNA, synthesized in an endogenous reverse transcriptase reaction with a particulate fraction of the leukemic leukocytes, hybridized to SSV-1 RNA, whereas only 5–27% hybridization was observed with RLV-RNA and little above background hybridization with FeSV-RNA, the hybridization value with RNA from AMV serving as a background (= 0%). VAIDYA et al. (1974) confirmed the presence of MMTV-related RNA in human breast cancer using quantitative hybridization conditions. RNA sequences homologous to MMTV-RNA were detected in 5 out of 17 breast tumors. The extent of the homologies ranged from 18–76%. The thermal stability of the hybrids was comparable to that in the homologous MMTV system. However, 12 out of 17 tumors were negative. MAK et al. (1975) synthesized complementary [^{3}H]-DNA with particles released from leukemic bone marrow cells in culture. Up to 60% homology to simian sarcoma viral RNA and 10–30% homology to murine leukemia and sarcoma viruses including strains Kirsten, Rauscher, and Gross were reported. LARSEN et al. (1975) detected, by S1 nuclease assay, viral related RNA in 22 out of 46 leukemics using c-DNA synthesized with Moloney-MSV. COLCHER et al. (1974) found RNA in human breast cancer related to that of the primate virus MPMV.

The results imply the presence in 48–91% of human leukemias, in 69–75% of human lymphomas and sarcomas, and in 30–67% of human breast cancers of information specifically homologous to some RNA sequences of viruses causing analogous diseases in mice and primates. The relevance of these findings to the etiology of human cancer remains still unknown.

Since murine mammary cancer can result from a variety of circumstances including hormonal stimulation, chemical carcinogens, radiation, and infection with B-type viruses, SCHLOM et al. (1973b) undertook a comparative study of the biologic and molecular basis of a variety of mouse mammary tumors. These authors detected MMTV-specific RNA in virus-producing and spontaneous nonproducing tumors, in tumors appearing at a later age, and in those induced by nonviral carcinogens. The latter observation was also

made by HEHLMANN et al. (1974), who detected complete MMTV-particles in urethan-induced mouse mammary tumors. VARMUS et al. (1973c), in a more quantitative study with digestion of unhybridized single-stranded [^{3}H]-DNA probe by single-strand-specific nuclease, found differences in the amounts of viral-specific RNA in high incidence, virus-producing (GR, RIII, C3H/an, DBA/2), and nonproducer strains (Balb/c), suggesting, in view of similar amounts of MMTV-specific DNA sequences in high and low tumor incidence strains (VARMUS et al., 1972b), transcriptional regulation. The results of the studies by SCHOLM et al. (1973b) and by VARMUS et al. (1973a), however, agree that in spite of quite dissimilar etiologic circumstances, similar viral RNA sequences are detected in all mouse mammary tumors tested encouraging the assumption that a similar situation might exist for human breast cancer.

E. Simultaneous Detection of Reverse Transcriptase and 70S RNA in Human Malignancies

Another line of evidence for the presence of RNA tumor viruses in human tumors, which confirms the detection of RNA-tumor viral related nucleic acid sequences, comes from the simultaneous detection in human neoplasms of two structures characteristic of the RNA tumor viruses: a high molecular weight (HMW) RNA and a reverse transcriptase associated with it. The technique (SCHLOM and SPIEGELMAN, 1971b; SPIEGELMAN et al., 1961) stems from the early observations by SPIEGELMAN et al. (1970a), ROKUTANDA et al. (1970), and BISHOP et al. (1971), that the initial DNA product is found complexed to its 70S RNA template. If early DNA product is found on sedimentation analysis in a 70S position, and if evidence is provided that its apparent size is due to its being complexed to a 70S RNA, evidence is provided for the presence of reverse transcriptase that uses a 70S RNA template. The assay conditions were standardized by SCHLOM and SPIEGELMAN (1971b) with purified preparations of avian and mammalian RNA tumor viruses including the primate virus MPMV, and were successfully applied to the particles observed in human milks (SCHLOM et al., 1972). Milk specimens from 10 out of 20 women showed reverse transcriptase activity associated with HMW RNA in one series (SCHLOM et al., 1972); 77% of 40 samples was positive in another series (GERWIN et al., 1973). No correlation could be made in either study between the presence of particles and the donor's family history of cancer. SCHLOM et al. (1972) and MCCORMICK et al. (1974) point out that specific inhibitors, e.g., ribonuclease, may obscure positive results. The sensitivity of this test could be further increased by the isolation of cores or nucleoids from the milk particles (FELDMAN et al., 1973). This procedure avoids the interference of cell debris present at the density of intact virions (1.16–1.19 g/cm^3), but not present at densities characteristic of viral cores (1.24–1.27 g/cm^3). DION and MOORE (1972) applied polyacrylamide gel electrophoresis for the analysis of simultaneous detection tests.

The simultaneous detection technique was soon applied to malignant tissues. After its applicability had been tested successfully in tissues known to contain tumor viruses (RLV-infected mouse spleens: HEHLMANN, 1972, unpublished results; HEHLMANN et al., 1973a; mouse mammary tumors: GULATI et al., 1972), BAXT et al. (1972) found reverse transcriptase associated with a high molecular weight RNA in the peripheral white blood cells of 22 out of 23 patients with leukemia. Contamination with cytoplasmic, enzymatically active proteins, ribosomes, and nucleic acids required the inclusion of additional controls, and besides ribonuclease sensitivity and dependence on all four deoxyribonucleotides, sequence homology of the reaction product with RLV-70S RNA was demonstrated by the authors (BAXT et al., 1972). No such activity was found in 18 normal white blood cell samples including those from donors with reactive leukocytosis. The amount of the reaction product generated was increased by prior density fractionation of the cytoplasmic structures (HEHLMANN et al., 1973a, b; HEHLMANN and SPIEGELMAN, 1974), and virtually no leukemic cell preparation was reported devoid of RNA tumor viruslike particles in this series. KOTLER et al. (1972, 1973) detected in arginine-deprived human leukemic, but not in normal cells, particles resembling C-type RNA tumor viruses as observed by electron microscopic criteria, and containing a 60–70S RNA-associated reverse transcriptase.

The demonstration of viruslike particles in human leukemias was soon extended to other human malignancies. AXEL et al. (1972c) found 70S-RNA-containing particles in 79% of 38 breast adenocarcinomas; KUFE et al. (1973b) detected particles containing an RNA-instructed DNA polymerase and a high molecular weight RNA in 87% of Burkitt's tumors; and SPIEGELMAN et al. (1973) and KUFE et al. (1973c) reported similar particles in other human lymphomas including Hodgkin's disease. In these tumors, specific homologies of the [^{3}H]-DNA, generated with the human particles, to the RNAs of the murine mammary tumor or leukemia viruses were reported, but in most studies only 10–20% of the products were demonstrated to be hybridized.

CUATICO et al. (1973 and 1974) found 70S RNA and reverse transcriptase containing particles in 59% of 51 human brain tumors, in 72% of 25 human gastrointestinal malignancies, and in 7 out of 10 lung cancers. No homology was found among the RNAs of the particles in the different neoplasms, and no homology was detected between these human particles and a variety of animal viruses including RLV and visna virus. Studies by YANIV et al. (1973) have extended the detection of tumor viruslike particles to human leukemic plasmas in spite of reports that human sera lyse RNA tumor viruses (WELSH et al., 1975). Similar particles were described in human malignant melanomas (BIRKMAYER et al., 1974; BALDA et al., 1975; HEHLMANN et al., 1975).

The preparation of cores from human mammary adenocarcinomas increased the sensitivity of the simultaneous detection test (MICHALIDES et al., 1975) similarly to what previously has been observed in human milks (FELDMAN et al., 1973): viral cores, because of their higher density, band in regions comparatively free of cellular contaminants and nucleases.

The detection of RNA tumor viruslike particles in Burkitt's tumors, which thus far have been linked to the DNA containing Epstein Barr virus (EBV), has raised the question of the etiologic roles of these viruses in this malignancy. Of interest in this context is the observation by PETERS et al. (1973) that a line of "isolator-derived, barrier-sustained, specific pathogen-free" chicken requires exposure to both the DNA containing Marek's disease virus and the RNA containing avian leukosis virus for development of Marek's disease. But it also should be considered that, while 87% of the actual Burkitt tumors were positive in the simultaneous detection test, all of six cultured Burkitt cell lines gave negative results (SPIEGELMANN and HENLE, unpublished observations).

Attempts to cultivate the particles discovered in human malignancies failed in most instances. However, KEYDAR et al. (1973), e.g., reported, as determined by [^{3}H]-uridine incorporation and by the simultaneous detection test, the production of virus by human embryonic cells cultivated with human breast cancer cells or infected with milk from breast cancer patients. FURMANSKI et al. (1974) similarly describe RNA tumor viruslike particles in cultured normal human mammary cells, and MCGRATH et al. (1974) report an RNA tumor viruslike particle in the human breast cancer line, MC-7. PARSONS et al. (1974) detected particles in human melanoma cell lines possessing some properties of RNA tumor viruses. MAK et al. (1974a and b) cultured bone marrow cells from leukemic patients in remission and relapse and found particulate structures morphologically and biochemically resembling RNA tumor viruses. Marrow specimens from two patients without malignant disease failed to give evidence of RNA tumor viruslike particles before or after culture. GALLAGHER and GALLO (1975) report the production of budding C-type virus by cultured human acute myelogenous leukemia cells possessing a reverse transcriptase immunologically related to the primate woolly monkey and gibbon ape C-type viruses. It appears wise not to be too enthusiastic about each new human tumor viral isolate, as previous, highly publicized claims for the isolation of human tumor viruses turned out later to be contaminating animal viruses. Animal contamination might be responsible for Gallo's isolate inspite of the facts that (1) the same virus was isolated in three separate culturings involving no animal passage, and (2) all three viral isolates possess the same immunologic characteristics as the original white blood cells from other patients with AML. If the isolates are really of human origin, it would appear that an infectious virus closely associated with acute human leukemia possesses striking similarity with C-type viruses isolated from a woolly monkey sarcoma and from gibbon ape myelogenous leukemia (TEICH et al., 1975). C-type viruses with similar immunologic properties have been isolated from a cultured human lymphosarcoma (NOOTER et al., 1975) and from a normal human fibroblast strain (PANEM et al., 1975). Also here, the question of contamination arises. Transforming viruses containing a 70S-RNA and a reverse transcriptase have recently been described in a human adenocarcinoma cell line (BALABANOVA et al., 1975).

The reasons for the difficulties encountered in growing the putative human viral particles may include unique growth requirements as well as the possibility that these particles are highly defective.

F. Tumor Viral DNA in Human Leukemias and Lymphomas

Experimental approaches to detect tumor viral DNA sequences in virus-infected, malignant, or apparently normal cells are of considerable interest because of the obvious implications for origin and mode of transmission of RNA-tumor viruses and of their malignant potential. The provirus theory of TEMIN (1964a) stipulates that the RNA tumor viruses are added to the cell by an infectious process and subsequently exist as a DNA provirus incorporated in the DNA of the transformed cells. It thus is predicted that the DNA of the transformed cells acquires sequences not present in the normal uninfected cell. The virogene-oncogene theory of HUEBNER and TODARO (1969) and TODARO and HUEBNER (1972), in contrast, postulates that all viral information required for malignant transformation is present in the DNA of all cells, transmitted vertically through the germ lines through thousands of generations. Cancer, according to this theory, is not normally caused by infection with an external virus but rather by activation of the inherited endogenous virogene or that part of the virogene responsible for malignant transformation. According to the provirus theory, only the infected or transformed cells would contain a complete set of viral sequences, whereas according to the oncogene hypothesis, infected as well as normal cells would harbor the complete information for the generation of viruses and for malignant transformation. Investigations on animal systems, therefore, were designed along the following lines: (1) Do cells, after infection, acquire viral-specific DNA sequences, i.e., does a DNA provirus exist? (2) Do normal, uninfected cells contain viral DNA sequences and if they do, is there a quantitative difference between infected and uninfected cells? (3) Do malignant or viral-infected cells contain DNA sequences not found in their normal counterparts?

In hybridization experiments with labeled viral RNA and immobilized cellular DNA, viral-specific DNA was observed both in infected and in normal cells. Whereas some groups found in infected cells 1.16 to 4 times more viral DNA than in uninfected cells (TEMIN, 1964c; BALUDA and NAYAK, 1970; ROSENTHAL et al., 1971; BALUDA, 1972; BALUDA and DROHAN, 1972), others could not detect any difference (WILSON and BAUER, 1967; YOSHIKAWA-FUKADA and EBERT, 1969).

The studies agreed, however, in that all normal and infected cells contained at least one viral DNA equivalent per genome, in this respect supporting the oncogene hypothesis. Initial studies using the acceleration of reassociation kinetics of labeled double-stranded viral DNA product by unlabeled cellular DNA (GELB et al., 1971a) failed to detect differences in the viral DNA content between normal and transformed mouse and chicken cells (GELB

et al., 1971b; VARMUS et al., 1972a). Using the same technique, VARMUS et al. (1972b) and GELB et al. (1973) failed to detect differences in the amounts of viral DNA in tissues from mice with a high and with a low incidence of leukemias and mammary tumors.

In hybridization experiments with labeled RSV-RNA and cellular DNA in large excess (MELLI et al., 1971), NEIMAN (1972) first clearly distinguished normal chick embryo DNA from DNA from RSV-induced sarcomas. In a later publication, NEIMAN (1973a) proposed that viral-specific sequences in normal avian embryos reflect the presence of low levels of endogenous leukosis viruses partially homologous with RSV. Of considerable importance for the specific detection of viral sequences in transformed cells proved to be the observation by BALUDA (1972) that rat embryo cells transformed by an avian sarcoma virus acquire 3.4 viral DNA equivalents. Though reported without comment at the time, this finding clearly demonstrates the acquisition of new viral information with transformation, since uninfected rat embryo cells contain less than 0.1 viral DNA equivalents. This specific detection of viral DNA sequences in transformed cells was soon confirmed in other heterologous systems. VARMUS et al. (1973b) obtained similar results for B-77 and RSV-transformed normal rat kidney and 3T3 cells; GOODMAN et al. (1973) extended this observation to marmoset monkey fibroblasts transformed by RSV or FeSV. VARMUS et al. (1973c) further documented in homologous and heterologous systems, the incorporation of viral DNA into host cell DNA after infection. These results establish that in certain identifiable situations the provirus mechanism is operative, and new viral information is added to the cell from the outside.

In order to decide which mechanism might be applicable to human neoplasms, SPIEGELMAN and his colleagues designed experiments to answer the question whether "the DNA of transformed cells contains viral-related sequences that are not found in the DNA of normal cells." BAXT and SPIEGELMAN (1972) provided evidence that human leukemic cells contain unique sequences in their DNA not present in the DNA of normal white blood cells. [^{3}H]DNA synthesized in an endogenous reverse transcriptase reaction with the particles detected in human leukemic cells (BAXT et al., 1972; HEHLMANN et al., 1973a, b) was extensively hybridized, in a precycling step, to DNA from normal white blood cells, in order to remove sequences shared by the RNA of the human particles and the DNA of normal cells. The precycled human leukemic [^{3}H]DNA product (as shown for eight leukemic patients) then hybridized extensively to human leukemic DNA but not to normal white blood cell DNA.

In a subsequent study, BAXT et al. (1973) confirmed these results with two pairs of identical twins. The leukemic member of each pair contained again DNA sequences in his cells that were not present in the white blood cells of his healthy sibling. BAXT (1974) showed further that the leukemia-specific sequences are related to the RNA of the Rauscher murine leukemia virus. The authors interpret these results to support the provirus theory,

since viral-related information must have been acquired subsequent to fertilization.

Additional support for an infectious component in human leukemogenesis comes (1) from the observation by THOMAS et al. (1972) of leukemic transformation of bone marrow cells from a male donor engrafted into his total-body-irradiated leukemic sister, (2) from the earlier reports by GOH and SWISHER (1965) and GOH et al. (1967) who find, by chromosomal studies on chronic myelocytic leukemia in two pairs of identical twins, the Philadelphia (Ph') chromosome only in the leukemic twins whereas their healthy identical siblings are Ph' chromosome–negative, and (3) from epidemiological studies (for literature see SCHIMPFF et al., 1975).

Possible criticisms of the leukemic DNA hybridization studies include that the relationship between unique sequences and the disease is not known, and also that normal white blood cells might have lost, during their differentiation process, some sequences. The latter possibility, however, is contrary to the common assumption that human white blood cells contain a complete set of the human genome.

Analogous data were obtained by KUFE et al. (1973d) for Hodgkin's and Burkitt's lymphomas. The nuclear DNA of both types of lymphomas is reported by the authors to contain unique human lymphoma-particle-related sequences that are not detectable in normal cellular DNA. The study further suggests that the particle-related sequences in Hodgkin's and Burkitt's lymphomas possess sequences in common indicating a relationship between their respective particles.

The results obtained with precycled [^{3}H]DNA probes in human leukemias and lymphomas were soon extended to the classical murine and avian model systems. SWEET et al. (1974) showed, using extensively precycled, single-stranded Rauscher leukemia virus (RLV) [^{3}H]DNA, that splenic DNA of RLV-infected Balb/c mice contained leukemia-specific sequences absent in normal Balb/c DNA. And SHOYAB et al. (1974b), using [^{3}H]35S RNA from avian myeloblastosis virus (AMV) exhaustively prehybridized to normal chicken DNA, demonstrated AMV sequences in leukemic cells not present in normal chicken cells. Identical results were reported in RNA-DNA hybridizations by SHOYAB et al. (1974a) and in DNA-DNA annealing studies by VARMUS et al. (1974a). Further support comes from data of CHATTOPADHYAY et al. (1974), who showed, with a single-stranded [^{3}H]-DNA probe prepared endogenously with AKR murine leukemia virus in the presence of actinomycin D, that DNA of the high leukemia incidence AKR mouse strain contains viral-specific sequences not present in the DNA of the nonvirus-yielding low incidence NIH strain. LOMY et al. (1974) extended this observation and reported a good correlation between the frequency of virus-specific DNA sequences and virus yield. Nonvirus-yielding strains contain only part of the viral genome.

Of diagnostic and prognostic value might be the determination whether progression and remission of a malignant disease can be correlated with presence and absence of viral particles and of viral genetic information. By

Table 5. Association of RNA tumor

Detection of virus by:	Mesenchymal origin		
	Leukemias	Lymphomas	Sarcomas
EM	+1 +2 +3 +4 +81	+1 +2 +3 +4	+5 +6
Viral antigens	+21 +22 +23 +24 +25 +26 +27 +28 +78 +83 +84	+27 +29	+5 +6 +30 +31 +82
Viral related RNA	+34 +35 +36 +52 +76 +86	+37 +38	+69
Viral related DNA	+43 +44 +45	+46	
Reverse transcriptase	+47 +48 +49 +50 +35 +52 +53 +54 +79 +83 +84 +85 +87 +88	+70 +71	
HMW-RNA and reverse transcriptase	+35 +52 +53 +54 +74 +75 +76 +77 +87 +88	+70 +71	

A+ sign means that the respective viral parameter has been detected. The number refers to the accompanying references.

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molecular hybridization and by simultaneous detection of a high molecular weight RNA associated with a reverse transcriptase, HEHLMANN et al. (1974) showed in the XGF mouse mammary tumor system (GOLDFEDER, 1972) that viral particles are present in growing and absent in regressing tumors. Examining white blood cells of human leukemias, however, VIOLA et al. (1976) detected leukemia-specific DNA sequences and particles both in the acute phase of the disease and during clinical remission, suggesting that even after

viruses with human malignancies

Histogenetic origin unclear	Epithelial origin				
Melanomas	Human milk	Breast cancer	Gastro-intestinal cancers	Lung cancer	Brain tumors
+12	+14 +15 +16 +17 +18 +19 +20	+7 +8 +9 +10 +11			+13
+66 +67 +68		+32 +33			
+42		+39 +40 +41			
+63 +64 +65	+51 +55 +56 +57 +59 +60 +62	+58 +61 +62 +80	+73	+73	+72
+63 +64 +65	+55 +56 +57 +59 +60 +62	+58 +61 +62 +80	+73	+73	+72

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successful induction of a remission, e.g., by chemotherapy, viral particles and the genetic potential for a relapse continue to be present in the cell genome. Mak et al. (1974a, b) detected by the simultaneous detection test, RNA tumor viruslike particles in cultures of bone marrow cells derived from leukemic patients in relapse and in remission.

A summary of the associations between RNA tumor viruses and human malignancies is presented in Table 5.

IV. Viral Oncogenesis and Origin of RNA Tumor Viruses

No conclusive evidence exists to date that the particles identified in human neoplasms are causative or contributory agents in these malignancies. Nevertheless, questions for their origin, for the mode of their transmission, and for their relation to carcinogenesis have become central issues particularly under the aspects of therapy and prophylaxis. Several theories try to account for the origin of RNA tumor viruses and attempt to unify our present knowledge on viral replication and carcinogenesis.

A. Provirus Theory

The provirus theory as proposed by TEMIN (1964a) suggested replication of the RNA tumor viruses via a DNA intermediate called "provirus." After infection with an exogenous RNA tumor virus, the proviral genetic information would be newly added to the host cell genome. Activation of this proviral DNA could result in the production of progeny virus and in malignant transformation. No attempt was made at that time to explain the mechanism of malignant transformation or the incorporation of proviral DNA into host cell DNA. The theory solely implies the addition of viral information not previously there by an infectious process (horizontal transmission).

The provirus hypothesis is supported, as far as extraneous addition of viral information by an infectious process is concerned, by the detection in virus infected and transformed cells of unique viral sequences not present in uninfected normal cells, as documented in several animal and human systems (BALUDA, 1972; NEIMAN, 1972; GOODMAN et al., 1973; BAXT and SPIEGELMAN, 1972; BAXT et al., 1973; KUFE et al., 1973d; VARMUS et al., 1973a, b; CHATTOPADHYAY et al., 1974; SWEET et al., 1974) and by horizontal transmission experiments in animals (RUBIN et al., 1961; HARDY et al., 1973). The presence, however, of RNA tumor viral sequences in normal, uninfected C-type particles, and the apparent vertical transmission of some RNA tumor viruses (MMTV), AKR) suggest an alternate mechanism, at least in certain instances.

B. Oncogene-Virogene Theory

The oncogene-virogene hypothesis, as proposed by HUEBNER and TODARO (1969) and TODARO and HUEBNER (1972), postulates that the cells of most or all vertebrates contain RNA tumor viral genomes in their DNA since early in evolution, and that the viral genomes are vertically transmitted (inherited) like regular cellular genes. Horizontal transmission is thought to be relatively unimportant, but may have played a role thousands of generations ago in the original acquisition of the virogene by the cell. The theory further postulates that the virogene, including the portion responsible for oncogenesis (the oncogene), remains in an unexpressed form in normal cells with the help of repressors. Destruction of the repressor system, e.g., by carcinogens, irradia-

tion, or by the normal aging process and, perhaps, even by exogenous viruses, may "switch on" the endogenous oncogenic information and thus lead to cell transformation. Vertical transmission (inheritance) from cell generation to cell generation and through the germ line has been proposed independently by BENTVELZEN et al. (1968, 1970) and BENTVELZEN and DAAMS (1969) for the transmission of mouse mammary tumor viruses.

The oncogene theory and the concept of the vertical transmission of the RNA tumor viruses is supported: (1) by the presence of viral gene products and of genetic information in normal, uninfected cells (DOUGHERTY and DISTEFANO, 1966; PAYNE and CHUBB, 1968; HUEBNER et al., 1970; PETERS et al., 1972; PARKS et al., 1973a; ROSENTHAL et al., 1971; BALUDA, 1972; VARMUS et al., 1972a, b; GELB et al., 1971b, 1973); (2) by the inducibility of C-type viruses from many vertebrate cell clones; and (3) by the known vertical transmission of some RNA-tumor viruses (MMTV, AKR). The limitations of this hypothesis come, in part, from the failure to demonstrate the presence of oncogenes. In addition, oncogenicity of induced endogenous viruses has been demonstrated only in some instances (ROWE et al., 1971; STEPHENSON et al., 1974c), and their role in natural carcinogenesis therefore remains in doubt. Finally, the documented horizontal transmission mechanism in some systems and the presence of unique tumor viral sequences after transformation in some other systems limits the universality of the oncogene hypothesis.

Since both vertical and horizontal transmission have been quite well documented in several instances, it appears likely that both modes of transmission take part in natural carcinogenesis.

C. Protovirus Theory

The protovirus theory (TEMIN, 1971) is not supported by clear experimental evidence. It also proposes vertical transmission of the information for cancer, but not the viral or oncogenic information as such is inherited, but rather the potential for the *de novo* formation of carcinogenic information. RNA tumor viral genomes are thought to evolve from cell genes. RNA-directed DNA synthesis is of central importance in this theory for the stabilization of genetic variation both in normal development and for carcinogenesis. The protovirus theory suggests further that information is exchanged between cells. This might lead to the acquisition of new genetic information with subsequent integration of this material into the cellular DNA. By some random event, the assembly in one cell of sufficient appropriate genetic information, including some DNA specifying an RNA-dependent DNA polymerase and some regulatory functions, could give rise to a virus. The virus, by infection, could make new DNA that has all the information necessary for neoplastic transformation. Only in extreme cases, the germ line would be infected, accounting for inheritance of some viral features (e.g., endogenous viruses), playing no part in the etiology of spontaneous tumors.

D. RNA Paraprocessing

An alternative idea on the origin of RNA tumor viral genomes comes from evidence concerning the involvement of RNA tumor viruses in human cancer and from molecular hybridization studies between the RNAs of two distinct classes of RNA tumor viruses and normal cellular DNA (GILLESPIE and GALLO, 1975).

Class I viruses, apparently generated more recently in evolution, are closely related in nucleotide sequence to certain cell genes (class I genes), whereas class II viruses are more distantly related to normal cellular DNA (GALLO et al., 1974; GILLESPIE et al., 1975; see below). Because of similarities in size and in degree of polyadenylation between high molecular weight viral RNA and nuclear RNA, viral RNA is felt to originate from nuclear RNA transcripts of class I genes. But instead of the usual processing mode of RNA transcripts by cleavage into smaller pieces, some large RNA transcript of a class I gene may escape complete cleavage due to environmental or hormonal changes. This incompletely cleaved, "paraprocessed" RNA would then be polyadenylated in the cytoplasm. If this RNA contains the information for a reverse transcriptase, the progenitor cell could produce replicating, infectious virus. If also some regulatory proteins are coded for by this RNA, cell transformation might occur. As far as the role of reverse transcriptase is concerned, the model resembles Temin's protovirus theory. With regard to the paraprocessing of RNA transcripts of normal cellular (Class I) genes, however, this new theory is quite distinct and puts together comprehensively what is known at present about cell metabolism, and structure and replication of RNA tumor viruses.

E. Endogenous Viruses

The presence of a "latent" endogenous RNA tumor virus in the tissue of otherwise normal, low tumor incidence strains of mice was first suggested by irradiation experiments which induced lymphatic leukemia and thymic lymphomas in healthy C57Bl or C3H mice. Cell free preparations of these tumors contained an agent which could induce *de novo* the same type of neoplasm in non-irradiated C57Bl or C3H mice inoculated at birth (LIEBERMANN and KAPLAN, 1959; GROSS, 1959; KAPLAN, 1967). The C57Bl agent was later referred to as radiation leukemia virus (Rad LV). Viral particles and avian leukosis group-specific (gs) antigenic activity in apparently normal chicken embryos were reported by DOUGHERTY and DISTEFANO in 1966 and by DOUGHERTY et al. in 1967. PAYNE and CHUBB (1968) showed mendelian inheritance of this antigen. Similar tests by HUEBNER et al. (1970) and TAYLOR et al. (1971, 1973) showed that mouse embryos with no infectious virus detectable revealed the presence of group-specific antigens in some organs at some stage of their development. BENTVELZEN et al. (1970) showed genetic transmission of mouse mammary tumor viruses. WEISS et al. (1971) induced avian RNA tumor viruses in uninfected gs^+ and gs^- cells by radiation and chemical carcinogens and

demonstrated the presence of complete viral genomes in normal cells. ROWE et al. (1971) cloned noninfectious AKR mouse embryo lines and demonstrated that each cell has the capacity, after activation by radiation or by SV40-viral superinfection, to produce infectious murine leukemia virus. The spontaneous release of mouse leukemia virus has been reported by AARONSON et al. (1969) in previously virus-free Balb/c mouse embryo cell lines and by TODARO (1972) in clonal lines of Balb/3T3 cells.

The inducibility of C-type viruses from cultured normal cells was greatly enhanced by exposure to 5-iododeoxyuridine (IUDR) and 5-bromodeoxyuridine (BUDR) (LOWY et al., 1971; TEICH et al., 1973; ROWE et al., 1972). Each clone of normal AKR (LOWY et al., 1971) or Balb/c mouse embryo cells (AARONSON et al., 1971 b) that has been tested by these authors could be induced to form C-type virus. In transformed mouse cells, the number of released viruses increased 5- to 15-foldafter treatment with IUDR or BUDR (LIEBER et al., 1973 a). Also from nonproducer sarcomavirus-transformed rat cells focus-forming virus (KLEMENT et al.,1971) and budding C-type virus (KLEMENT et al., 1972) were induced with BUDR. Spontaneous and chemical induction of C-type viruses have been demonstrated subsequently in clonal lines of cats (LIVINGSTON and TODARO, 1973; SARMA et al., 1973; FISCHINGER et al., 1973), of Chinese hamsters (LIEBER et al., 1973 b), and of guinea pigs (NAYAK and MURRAY, 1973), and in a porcine cell line (PK15) derived from pig kidney cells (ARMSTRONG et al., 1971; LIEBER et al., 1973 b; TODARO et al., 1974a). The expression of B- and C-type viruses can be activated by hormones (MÜHLBOCK, 1972; HELLMAN and FOWLER, 1971). However, a sarcoma virus has never been induced from untransformed cells by any of the above methods.

Antigenic and genetic evidence suggests that genetic information for more than one distinct endogenous C-type virus may be present in mammalian cells (AOKI and TODARO, 1973; STEPHENSON and AARONSON, 1973; AARONSON and STEPHENSON, 1973; AARONSON and DUNN, 1974; STEPHENSON et al., 1974a, b, and e; BENVENISTE and TODARO, 1974a). The search for endogenous viruses is complicated by the presence in some of these viruses of xenotropic properties (LEVY, 1973) that restrict their growth in homologous cells, but allow their propagation in heterologous cell lines (LEVY, 1973; TODARO et al. 1973; STEPHENSON et al., 1974a, b). Presumably endogenous C-type viruses have been isolated also from normal baboon placentas and cell lines (KALTER et al., 1973 a; BENVENISTE et al., 1974a) and from normal human placentas (KALTER et al., 1973 b). The induction of endogenous viruses in many cells of vertebrates is in agreement with the oncogene-virogene hypothesis. Oncogenicity of these induced endogenous viruses has only infrequently been demonstrated (ROWE et al., 1971; STEPHENSON et al., 1974c).

The earlier assumption (OLD et al., 1968) that hosts are immunologically tolerant to their endogenous viruses is contrary to recent evidence (AARONSON and STEPHENSON, 1974; NOWINSKI and KÄHLER, 1974), which demonstrates

widespread occurrence of neutralizing antibodies to endogenous C-type viruses in inbred mice. Antibodies to simian C-type viruses were demonstrated earlier in sera of gibbons from colonies with an incidence of leukemia or lymphoma (Kawakami et al., 1973). It is of interest in this context that Hersh et al. (1974a, b) found humoral and cell-mediated immunoresponsiveness to immunization with Rauscher MuLV in patients with leukemia and other malignancies.

F. Evolution of RNA Tumor Viruses

Interesting aspects on the origin of RNA tumor viruses, on their evolution, and on their possible role in the evolution of their animal hosts evolve from nucleic acid and antigenic comparisons of vertebrates by endogenous C-type viral probes. Of particular interest turned out to be the endogenous viruses of cats (McAllister et al., 1972; Livingstone and Todaro, 1973; Sarma et al., 1973; Fischinger et al., 1973) and of baboons, a class of Old World monkeys (Kalter et al., 1973b; Benveniste et al., 1974a; Todaro et al., 1974b). Nucleic acid hybridization and immunologic characterization revealed that these two groups of viruses are genetically different from previously studied feline or primate leukemia virus (Ruprecht et al., 1973b; Benveniste et al., 1974a) but possess significant nucleic acid homology and immunologic relatedness among each other (Sherr et al., 1974a, b; Sherr and Todaro, 1974a; Hellman et al., 1974; Todaro et al., 1974c). The endogenous viruses of the cat (RD-114, CCC) show extensive homology of their RNA to the DNA of the domestic cat (Ruprecht et al., 1973b; Neiman, 1973b; Baluda and Roy-Burman, 1973; Gillespie et al., 1973; Okabe et al., 1973; Sarma et al., 1973), of the European wild cat, and of other cats originating from the Mediterranean area (Benveniste and Todaro, 1974c), but not to DNA of the African, South East Asian, and South American large cats. [^{3}H]-DNA synthesized with the endogenous viruses of the baboon (M7, M28, etc.) exhibits, by extent of hybridization and by thermal stability of the hybrids, extensive homology to baboon DNA, less homology to the DNA of several Old World monkeys (patas, African green, rhesus), and some homology to the DNA of New World monkeys, apes, and man (Benveniste et al., 1974a; Benveniste and Todaro, 1974b). No homologies were detected between baboon virus RNA and the DNA of nonprimates. These findings correlate well with the phylogenetic relationships determined on the basis of anatomical and fossil comparisons. The data essentially mean that C-type viral genes have evolved with the animal species, over millions of years, showing a remarkable preservation of their genetic information. The fact that all primates (Benveniste and Todaro, 1974b) carry this viral information in their DNA, suggests that the presence of the viral genome may have provided selective advantage for these animals in evolution. This interesting possibility might be useful for the future interpretation of RNA tumor viral information in normal and malignant human tissues.

The partial nucleic acid homology between endogenous baboon and cat viruses, and between primate DNA and the DNA from cats originating from the Mediterranean area, suggests that horizontal transmission of C-type viruses has occurred in the past between vertebrate species not closely related phylogenetically, and that subsequently these viruses have been transmitted vertically through the germ line as cellular genes ever since (BENVENISTE and TODARO, 1974c). Since all primates show at least some homology of their DNA to the baboon virus RNA, but only few geographically defined species of cat, BENVENISTE and TODARO (1974c) conclude that an endogenous primate virus infected an ancestor of the domestic cat.

A second example of trans-species infection of RNA tumor viruses may be the infectious primate C-type viruses SSV-1 (woolly monkey virus) and GALV (gibbon ape leukemia virus) which show partial sequence homology to the RNAs of laboratory mice (BENVENISTE and TODARO, 1973) and strongly cross-reactive reverse transcriptase and p30 antigens with endogenous xenotropic C-type viruses from the asian feral mous *mus caroli* (LIEBER et al., 1975). Since sequences homologous to the RNAs of SSV and GALV have not been detected in the cellular DNAs of normal primates (BENVENISTE et al., 1974c), LIEBER and associates conclude that these viruses are transmitted horizontally among primates and, most probably, originate from transspecies infection with an endogenous xenotropic murine virus, e.g. from *M. caroli*. With the virus genome not yet incorporated into the host cell genome, this infection might represent a recent, and perhaps contemporary event.

The observation of extensive homology between the RNAs of endogenous viruses and the DNAs of their indigenous hosts, and the lack of such homology in other viruses, e.g., in gibbon ape and woolly monkey viruses, which show partial homology of their RNAs to normal mouse DNA, but not to any primate DNA (SCOLNICK et al., 1974), led GALLO et al. (1974) and GILLESPIE and GALLO (1975) to a new classification of RNA tumor viruses into class I and class II viruses. Class I viruses show extensive homology of their RNA to the DNA of their indigenous hosts ($>70\%$), are rarely oncogenic, are transmitted vertically, and include essentially all induced viruses as well as the mouse mammary tumor virus (MMTV) and the AKR-leukemia virus. Class II viruses exhibit only partial homology ($<30\%$) to their respective host DNAs, are probably transmitted horizontally, are usually oncogenic and include the avian tumor viruses (AMV, RSV) and the murine (e.g., strains Rauscher, Moloney), feline (e.g., strains Rickard, Gardner) and primate (gibbon ape, woolly monkey) leukemia-sarcoma viruses. It is of interest in this context that the RNA-dependent DNA polymerase isolated from human myelogenous leukemias exhibits strong cross reactivity with antisera against the polymerases of the primate gibbon ape and woolly monkey viruses, but not with antisera against the polymerases from the endogenous primate baboon and rhesus monkey viruses (GALLO et al., 1974). The presence of these two distinct classes of viruses also in human malignancies seems probable and would

explain the detection of C-type viruses in normal embryonic tissues (KALTER et al., 1973a, b).

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