Tumor Virus-Host Cell Interaction

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SPRINGER SCIENCE+BUSINESS MEDIA, LLC

Library of Congress Cataloging in Publication Data

Nato Advanced Study Institute on Tumor Virus–Host Cell Interaction, Monte Carlo, 1973.
Tumor virus–host cell interaction.

(NATO advanced study institutes series: Series A, life sciences; v. 5)
Includes bibliographies and index.
1. Host–virus relationships–Congresses. 2. Oncogenic viruses–Congresses. I. Kolber, Alan R. II. North Atlantic Treaty Organization. III. Title. IV. Series. [DNLM: 1. Cell transformation, Neoplastic–Congresses. 2. Oncogenic viruses–Congresses. QZ200 N105t 1973]
QR482.N37 1973 616.9'94'071 75-16203

ISBN 978-1-4684-2768-4 ISBN 978-1-4684-2766-0 (eBook)
DOI 10.1007/978-1-4684-2766-0

Lectures presented at the 1973 NATO Advanced Study Institute on Tumor Virus–Host Cell Interaction, held in Monte Carlo, Monaco, September 1973

© 1975 Springer Science+Business Media New York
Originally published by Plenum Press, New York in 1975
Softcover reprint of the hardcover 1st edition 1975

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Tumor Virus-Host Cell Interaction

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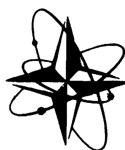

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Preface

The oncogenic virus can only be studied as a carcinogen when its relation to the host cell in which it resides is understood. The interaction between tumor virus and host cell was the subject of a recent North Atlantic Treaty Organization Advanced Study Institute. This volume is the edited proceedings of this study institute. One problem of fundamental importance in understanding malignant transformation is the mechanism by which the oncogenic vector promotes the aberrations in the host cell regulatory apparatus resulting in a cancerous cell. It is partly the purpose of this volume to help clarify this problem, and to stimulate the interest of others to continue the research necessary to this end.

The meeting from which this volume is compiled took place in the Principality of Monaco with the patronage of Her Serene Highness, Princess Grace and was supported principally by a grant from the North Atlantic Treaty Organization. The editor wishes to thank the North Atlantic Treaty Organization, Princess Grace of Monaco, and the Bureau de Tourism Monaco for their generous support. Additional financial assistance was provided by the Deutsches Studium Gesellschaft and I would like to thank Prof. Klaus Munk, Deutsches Krebsforschungszentrum, for his help in obtaining this aid. I would also like to thank the Verwaltung of the Deutsches Krebsforschungszentrum for their administrative help.

I would like to acknowledge the assistance of Jan McInroy (Professional Manuscript Service, Carrboro, North Carolina) and Janet Kolber for their competent preparation of the meeting proceedings into manuscript form. Lastly, I would like to thank the meeting participants for their contributions.

Carrboro, North Carolina Alan Kolber

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SIMIAN VIRUS 40
AND POLYOMA VIRUS

KNOWLEDGE AND IGNORANCE ABOUT THE MOLECULAR BIOLOGY OF SMALL ONCOGENIC DNA CONTAINING VIRUSES

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I intend first to examine what we know about the molecular biology of polyoma virus and SV40 and then to consider obscure points, speculating about their possible significance. What is known is summarized in Table 1. Let's now consider some outstanding questions relating to the various points.

PERMISSIVENESS

A generalization from work with both SV40 and polyoma virus is that cell types cannot be simply divided into permissive and non-permissive to wild-type virus, since there are intermediate types. An outstanding example is given by BHK cells in which polyoma virus elicits the synthesis of V antigen, and under special conditions may even produce infectious virus. Stable transformants of these cells are probably produced by defective genomes of polyoma virus, which lack functions detrimental for cell growth or survival.

The degree of permissiveness and how it affects the expression of the viral genome accounts for the various types of stably transformed cultures. They can be totally non-productive (PV-BHK); productive only upon fusion to permissive cells (SV-3T3); productive spontaneously but in a small proportion of cells (some PV-rat) and further inducible by fusion.

Probably, two main conditions determine permissiveness: how frequently the provirus is detached from the host chromosome and how efficiently the detached genome can be expressed. The latter point in turn may depend on the replication, transcription and translation of the genome.

TABLE 1. Main known properties of polyoma and SV40 viruses and of the infected cells

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1. The DNA is cyclic; its detailed anatomy is established using restriction enzymes.
2. Consequences of infection are cell-dependent. Cell Permissiveness.
3. New antigens appear in the cells after infection. U, T, V, and surface antigens (TSTA and fetal antigens).
4. Viral DNA aberrations are formed during replication. Oligomers, deletions.
5. The viral DNA is integrated into the cellular (or viral) DNA as provirus.
6. Cellular DNA is integrated in cyclic viral DNA molecules.
7. The provirus can be detached. Effect of fusion with permissive cells; effect of IUDR.
8. Hybrids (viral-cellular, or biviral) are formed and processed.
9. Transcription of the viral DNA follows a characteristic pattern.
10. Cellular functions are activated

    Cellular DNA replication
    Cellular mRNA transcription
    Enzymes
    Surface features (antigens, lectin sites, transports)
    Pre-existing proviruses may be activated
    Helper function?
11. Mutations. Ts: Late, affecting viral capsid proteins
    tsa, affecting viral DNA replication, perhaps integration
    ts3) affecting transformation, activation
    hr) of cellular functions
12. Transformation depends on the expression of both viral and cellular genes.

The role of cell permissiveness in the expression of viral mutations will be considered later.

NEW ANTIGENS

The questions are what they are molecularly and how they are specified. A molecular weight of about 80,000 has been assigned to the T antigen. If this is a monomer, it can marginally fit within the size of the viral genome specifying early functions, to which it belongs. Therefore, this part of the genome cannot also specify two additional molecules, corresponding to the U and TSTA antigens. The dilemma is solved if the three antigens are different determinants of the same molecule, or cellular molecules modified by the virus. A final solution requires the purification of the antigens and the determination of their sequences. The hypothesis of a single molecule with three determinants may have some validity for the following reasons.

1) The early SV40 region is transcribed by a single stable mRNA, which owing to the monocistronic nature of cellular messengers, will be probably transcribed into a single polypeptide chain.

2) Information obtained with non-defective adeno SV40 hybrids is compatible with the hypothesis that this polypeptide chain is the T antigen and that it evolves by cleavage. A first cleavage with elimination of the N-terminal fragment would generate the TSTA antigen, and a further cleavage would generate the U antigen. Furthermore, since the TSTA antigen appears at the cell surface, it may be further modified by glycosylation by host enzymes.

DNA ABERRATIONS

The question is the mechanism. These aberrations may be important for studying viral DNA recombination in the absence of conventional genetic recombination. Oligomers of complete as well as of defective molecules could be produced either by an accident of replication, i.e., lack of separation of daughter molecules, or by homologous recombination. Incomplete separation would yield dimers whose two parts are mirror images of each other, a feature that should be demonstrable by EM after denaturation and reannealing. Dimers in turn would generate tetramers, but not trimers. Trimers might derive from tetramers after excising a monomer; but then one would expect them to be rarer than tetramers. Present evidence on their proportions is ambiguous. Homologous recombination can also account for oligomer formation in cases where trimers are more frequent than tetramers, as in the tsa-3T3 cells. In these cells at high temperature recombination may proceed without DNA replication. The formation of monomers in cells infected by oligomers may also arise by the same mechanism. Deletions and

inversions probably arise by a rarer non-homologous recombination. Therefore the DNA aberrations suggest that recombination of the viral DNA occurs by the mechanisms recognized in the cellular DNA.

INTEGRATION

There are several questions concerning the mechanism of integration: the integration sites on the cellular chromosome (and on the DNA of other viruses, such as Adenoviruses for SV40 integration) --receptor sites; the sites of the viral chromosome that participate in integration--donor sites; and the enzymes involved. The problem of the receptor sites is now being attacked; methodology is developing rapidly, so that we can hope to have soon some answers. If either receptor and donor sites are non-specific, integration is likely to occur by non-homologous recombination, like the integration of the F episome in bacteria. The position of the donor site in the viral DNA can be inferred from the pattern of transcription of integrated genomes (see below), which suggests a position between 7 and 12 o'clock on the SV40 map. As to the enzymes, they may include the product of the viral A gene (identified by the tsa-type mutation) since the function of that gene is required transiently for the stable transformation of BHK cells by polyoma virus. However, since the normal role of this gene is in the replication of the viral DNA its requirement for integration may be indirect, through a requirement for replication.

The evaluation of the findings with polyoma virus and BHK cells depends also on an assessment of the role of integration in abortive transformation. If integration is required for stable but not for abortive transformation, it is likely that the gene A function is directly required for integration, since abortive transformation by the tsa mutant is temperature-independent. Therefore, it would be interesting to determine whether integration occurs in cells abortively transformed by the tsa mutant at the non-permissive temperature.

There are also some general questions concerning integration. For instance whether integration has other roles besides anchoring the viral to the cellular DNA, e.g. in transcription of the viral genome; or whether it causes transformation, by producing insertion mutations in cellular genes. I will return to these questions later.

Another general question is whether integration is required for stable transformation in all systems, since the alternative of a plasmidial state has been proposed. One wonders however whether the presence of an integrated genome in cells containing also a number of plasmidial forms has been excluded.

CELLULAR DNA IN VIRAL DNA

The presence of cellular DNA in viral DNA is interesting because it may allow the transduction of cellular genes, a possibility which has not yet been exploited. There are two main questions concerning this phenomenon: whether any part of the cellular DNA is preferentially integrated, and whether cellular DNA integrated in viral DNA plays any role in transformation. Since the cellular participation in different viral clones seems variable depending on their history, whereas all clones transform, it seems unlikely that the cellular DNA has an essential transforming role. The question whether there is preferential incorporation of certain parts of the cellular DNA is difficult to answer because the insertions we observe are those that do not confer any selective disadvantage on the viral DNA during serial passages in the presence of helper. Therefore, these molecules must be able to replicate as rapidly or more rapidly than the helper, and must be efficiently encapsidated. I suspect these limitations severely curtail the number of detectable insertions.

DETACHMENT OF THE VIRAL DNA

The main question is whether it requires any viral function. This question must be considered in view of the behavior of the tsa 3T3 line, which is stable at high temperature when the polyoma genome is defective, owing to the mutation in the A gene. When the temperature is lowered, reactivating the A gene, many cells initiate a lytic cycle. However this behavior is not decisive for supporting a role of the A gene in excision. In fact, it is not conclusively established that tsa-3T3 cells contain only integrated genomes. Furthermore, the behavior of tsa-3T3 cells is also compatible with the interpretation that the A gene causes the multiplication of spontaneously detached genomes.

A similar uncertainty concerns the events occurring after the fusion of SV3T3 cells with permissive cells. Since in these cells the whole early region of the genome is transcribed, the A gene is likely to be expressed, unless there is a post-transcriptional block. Nevertheless, no massive excision occurs, since the transformed cells are stable. Hence, it seems more likely that excision is not promoted by the A gene. A picture to be considered is that in SV3T3 cells excision promoted by non-viral factors occurs at a low continuous rate, and that owing to the lack of late viral proteins (since the cells are non-permissive) the DNA is then degraded. If so, the role of IUDR in considerably increasing the proportion of cells that yield virus after fusion might be to enhance excision rate. In trying to test this possibility one might consider that excision may be lethal for the cells since otherwise virus-free

SV3T3 derivatives should be found; but this is contrary to experience.

A general question concerning both excision and integration is whether they are carried out by the Campbell model, i.e., through a single act of recombination between specific sites. That a single recombination is involved seems likely, because the viral DNA is cyclic and native linear intermediates are unknown. Failure to isolate "cured" SV3T3 cells may indicate that the host chromosome is not resealed. Concerning the second point, whether there are specific viral sites for insertion and excision, we lack direct evidence and must look for hints. The inferences we can make depend on the structure of the provirus and on requirements for infectivity of the excised genome. We can consider three possibilities. If proviruses were always integrated in tandem pairs, the detachment of a complete viral DNA molecule could occur by homologous recombination within the two proviruses, without need for a specific site. But there is no evidence for tandem proviruses (it should be looked for). Secondly, if a single provirus is integrated, and the whole DNA, to the last nucleotide, is required for infectivity, then there must be specific sites. Finally, if a part of the genome is not required for infectivity, specific sites are not required because any crossing over in that region, provided it generates a molecule of the right length for encapsidation, would generate an infectious molecule. Under the latter model one would expect infectious molecules to be generated in a minority of excision, most being of aberrant sizes and composition. Since the direct product of excision is not known, this possibility cannot be ruled out.

PATTERN OF TRANSCRIPTION: FORMATION OF HYBRID MESSENGERS

The main question is what determines the pattern of transcription. The question is especially difficult because although we know lots about the stable transcripts, we do not know much about transcription itself. Since in some cases at least the primary product of transcription is processed--i.e., partly degraded to yield the stable transcripts--we cannot infer how transcription occurs from our knowledge of stable messengers.

Another complicating event is that in the cells there may be more than one kind of template. For instance, in late lytic infection the cells contain both free and integrated viral DNA. Since these DNAs have a different topology, it is unlikely that they are transcribed equally.

Let us look first at transcription <u>in transformed cells</u>, such as SV3T3, in which the viral DNA is integrated. There the direct

product of transcription is often quite long, including the early sequences, plus anti-late sequences, mostly to the left of early (i.e., clockwise), and cellular sequences. These probably transcribe the part of the cellular DNA adjacent to the provirus, suggesting that when the SV40 genome is integrated, the ring opens in the late region to the left (clockwise) of the early region. Transcription of these messages would begin in the cellular DNA, continuing on the late region of the viral DNA but on the wrong strand, generating anti-late sequences and then true early sequences. Transcription appears to terminate sometimes at the right end of the early region where there may be a normal terminator, but often, proceeds a little beyond it, indicating that termination is inefficient. In the nucleus the cellular and anti-late sequences are removed, implying that there are processing signals at both sides of the early sequences. The size distribution of the unprocessed nuclear messengers after a short pulse suggests that in SV3T3 cells many RNA molecules may contain just early sequences; these messengers would initiate at a viral promoter at the left end of the early region and terminate at the terminator at the right end of this region.

In late lytic infection in mouse kidney cells polyoma virus mRNA is also heterogeneous. The strand orientation of the long messengers is not determined; they also may contain cellular, anti-late, and early sequences. This finding has led to the suggestion that lytic transcription may come from integrated genomes. One important difference from the SV40 transformed cells is that the large lytic messengers seem not to be processed, since they continue to accumulate even after a 6 hour chase. Is it possible that the cells used in the experiments lacked the necessary processing enzymes? In late infection of SV40-infected BSC-1 cells most of the pulse-labeled RNA is similar in size to the stable messengers, suggesting that the transcription may come from free templates, beginning at the physiological promoter and ending at the physiological terminator. These RNAs may be subsequently processed to yield smaller species.

Early lytic transcription of SV40 genomes in BSC-1 cells produces a proportion of long messenger, again raising the question whether a proportion of early lytic transcription is from integrated genomes. However, it is not clear that these messengers contain cellular sequences; they might simply arise when transcription on free genomes fails to terminate. A possible way to solve this difficulty is to establish whether integration requires the A gene function. If so, clearly early transcription could not come from integrated genomes.

A reasonable position at the present time is that physiological lytic transcription comes from free genomes, starting at a viral promoter and ending at a viral terminator, whereas in transformed

cells transcription can originate either at a viral or at a cellular promoter, often but not always ending at a viral terminator. The efficiency of the viral initiation and termination signals would be greater when free DNA is template perhaps owing to its supercoiled state. Transcription from integrated genomes in lytic infection may be functionally irrelevant.

In this discussion I have so far avoided reference to the evidence that in late lytic infection the SV40 genome is transcribed symmetrically. The meaning of this observation is not clear. Since it has not yet been confirmed in work from other laboratories, it may depend on some special condition of the experiment (e.g., the state of the cells, the virus strain, or multiplicity of infection). An alternative interpretation of the observations should be considered, i.e., that extensive complementarity arises in systems in which termination is especially inefficient, possibly leading to complete transcription of one or both strands. If the extra sequences were not removed by processing, extensive messenger complementarity would result. Therefore, it is not clear at the present time that meaningful transcription must necessarily be symmetrical.

ACTIVATION OF CELLULAR FUNCTIONS

The main question here is how the cellular functions are activated. It may occur at the transcriptional level because SV40 induces a marked stimulation of cellular mRNA synthesis; however, it is not known whether any of the extra messengers is due to transcription of genes previously silent. Activation could also be post-transcriptional, but nothing is known about this possibility. As to the mechanism of activation, two main models can be considered: a functional model in which a viral protein interacts with a cellular regulatory system whose breakdown then activates the cellular functions; and a steric model in which the integration of the viral DNA inactivates a cellular function (controlling a negative regulator) from which the activation of cellular genes results. This point is clarified by the study of mutations.

MUTATIONS

The study of mutations appears to support the functional model, since a temperature-sensitive mutation (ts3) makes many aspects of transformation temperature-dependent. Temperature-dependence would be difficult to reconcile with the steric model. The ts3 mutant may be related to the host range (hr) mutants which Tom Benjamin will discuss in the meeting, and only multiply in transformed cells. As I will report later in this meeting, the ts3 mutant also has a

marked cell dependence at high temperature, similar to that of the hr mutants. However, the ts3 mutant has also some mysterious properties, which I will discuss later.

The dependence of transformation on cellular functions can be explained if a transforming virus activates a specific part of the genome, including that corresponding to these functions. Especially interesting is the recent demonstration that a cellular function required for the expression of transformation by SV40 is not required by murine sarcoma virus. This finding suggests that different transforming viruses activate different parts of the host genome.

STUDIES OF SV40 DNA REPLICATION

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The genome of Simian Virus 40 (SV40) is a covalently closed duplex DNA molecule of 3.6 x 10^6 daltons. We have been concerned with defining the mechanism by which SV40 DNA is replicated during the permissive infection. The small size of SV40 DNA and its replicative intermediates has permitted both the biophysical and biochemical studies which we shall summarize in this communication.

COVALENTLY CLOSED TEMPLATE STRANDS AND SUPERHELICAL STRUCTURE

Replicating DNA molecules of SV40 have been found to contain newly synthesized strands noncovalently associated with covalently closed template strands (Sebring *et al.*, 1971; Jaenisch, Mayer, and Levine, 1971). In electron micrographs, the majority of replicating molecules observed have a superhelical, unreplicated branch and two untwisted, replicated branches. When pulse-labeled replicating molecules are banded in cesium chloride density gradients containing ethidium bromide or propidium diiodide, a fraction of the labeled molecules bands at the same density as DNA II (interrupted, circular SV40 DNA) and the remaining labeled DNA bands at densities greater than that of DNA II (Sebring *et al.*, 1971). While "mature" replicating molecules (those near to completion of replication) band with DNA II in dye-density gradients, partially

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replicated molecules band at densities between those of DNA I and DNA II. This assessment was made by analyzing the sizes of the newly synthesized strands from molecules banding at different densities in the gradient. An inverse relationship was observed between the extent of replication and the banding density (Sebring et al., 1971). Replicating molecules of SV40 DNA can thus be fractionated simply by nonartifactual means.

The unwinding of the template strands in replicating SV40 DNA has been demonstrated (Sebring et al., 1971; Mayer and Levine, 1972; Salzman, Sebring, and Radonovich, 1973) by alkaline sucrose gradient sedimentation. The closed circular template strands (radio-labeled) sediment with progressively lower S values as the extent of replication increases. The mechanism by which the template strands of SV40 DNA separate as replication proceeds has not yet been elucidated. An activity capable of removing negative superhelical turns from closed circular template strands may be involved. Such an activity was identified in extracts of E. coli by Wang (1971) and a similar one has also been detected in extracts of mouse embryo cells using superhelical, polyoma DNA as substrate (Champoux and Dulbecco, 1972). It has previously been proposed that SV40 DNA replication may be intermittent in individual molecules (Sebring et al., 1971). A rate-limiting, diffusable factor such as a swivel protein may act successively on several molecules which have undergone different extents of replication and a given molecule may be blocked in further replication until the factor(s) binds to it.

ORIGIN AND DIRECTION OF DNA SYNTHESIS

SV40 DNA replication has been shown to start at a specific site on the viral genome (Nathans and Danna, 1972; Thoren, Sebring, and Salzman, 1972). In order to determine the direction of SV40 DNA replication and also to substantiate that the origin for replication was specific, we utilized restriction endonuclease from E. coli, endo R Eco RI, to cleave fractionated replicating molecules. Endo R Eco RI creates one double-strand break in SV40 DNA at a specific site (Morrow and Berg, 1972; Mulder and Delius, 1972; Fareed, Garon, and Salzman, 1972). When replicating molecules of SV40 DNA were cleaved by this enzyme, examination of the cleavage products by electron microscopy and sedimentation in sucrose gradients showed the Eco RI cleavage site to be 33% of the genome length from the origin of replication (Fareed, Garon, and Salzman, 1972). Replication was observed to be bidirectional. Similar results were obtained independently using a different method by Danna and Nathans (1972).

INVOLVEMENT OF DNA II IN REPLICATION

The replication of covalently closed, cyclic DNA molecules has been studied for a number of different viruses, extrachromosomal genetic elements and microorganisms (for reference, see Helinski and Clewell, 1971). In most of these systems, a mechanism must be present to allow for separation of duplicated molecules from nonsegregated, circular diploids. The mechanism must also insure that circularity of the progeny molecules is maintained after segregation.

In order to learn more about the segregation of circular molecules, we have examined SV40 DNA II obtained after pulse-labeling cells that are actively synthesizing viral DNA (Fareed and Salzman, 1972a; Fareed, McKerlie, and Salzman, 1973). Both physical and enzymatic techniques were used to evaluate the distribution of the pulse-label in DNA II. After a short period of labeling with ^{3}H-thymidine, greater than 90% of the pulse-labeled DNA in DNA II sediments at 16S in alkaline sucrose gradient and a similar amount is susceptible to degradation by exonuclease III of E. coli (Richardson, Lehman, and Kornberg, 1964). Therefore, the pulse-labeled DNA is contained in the interrupted strand of DNA II. In order to demonstrate that the interruption in DNA II is specifically located, pulse-labeled DNA II molecules were cleaved by the R_I restriction endonuclease. As already discussed, the cleavage site for the R_I enzyme is about 33% of the genome length from the origin. Cleavage of DNA II by the R_I nuclease produces two specific fragments of the newly synthesized linear strand. Since the small fragment corresponds to 17.7% of the genome length and the termination point for DNA replication is 50% of the genome length from the origin (Fareed, Garon, and Salzman, 1972), the interruption in DNA II is situated at the termination point.

In Figure 1, a schematic diagram is used to summarize the different steps in SV40 DNA replication. It should be emphasized that major uncertainties still exist in the understanding of (1) the primary initiation of DNA replication, (2) the mechanism by which the template strands are separated and by which segregation of daughter molecules takes place, and (3) the formation of superhelical progeny molecules.

DISCONTINUOUS DNA CHAIN GROWTH

In contrast to the process of strand separation, the mechanism of SV40 DNA chain growth appears to be better understood. We have investigated this mechanism by pulse-labeling cells infected with SV40 for different intervals using ^{3}H-thymidine. Very brief periods

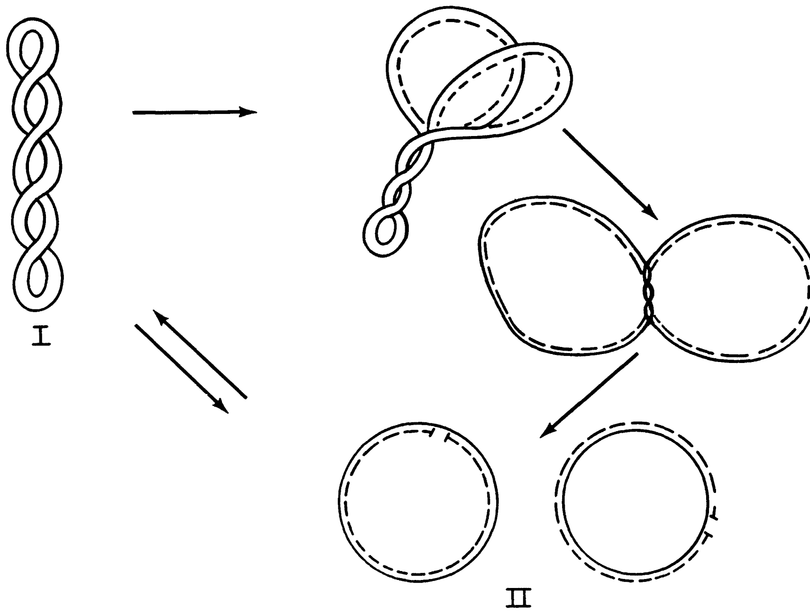

Fig. 1. Scheme for SV40 DNA replication.

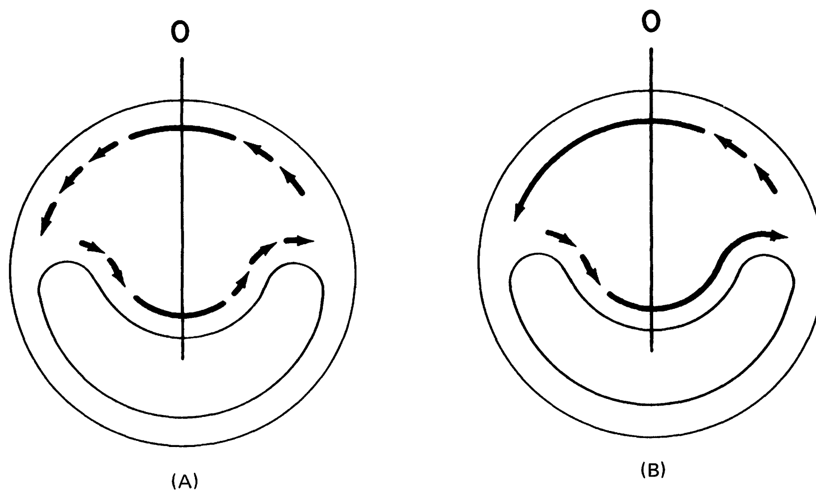

Fig. 2. Two models for SV40 DNA chain elongation.

of pulse labeling from 15 to 45 seconds at 37°C were carried out in order to assess whether a short-lived chain growth intermediate existed. Nascent short DNA chains were identified sedimenting at 4S in alkaline sucrose gradients under these conditions and were shown to be precursors of elongating strands (Fareed and Salzman, 1972b. Replicating molecules, fractionated according to extent of replication, were examined and the 4S DNA was found to be associated with molecules at all stages of replication prior to segregation of daughter molecules. Except for its small size (about 150 nucleotides), the 4S DNA appeared to be analogous to Okazaki fragments (about 1,000 nucleotides in length) which are precursors of growing chains in *Escherichia coli* (Okazaki *et al.*, 1968; Oishi, 1968). We, therefore, postulated a discontinuous mechanism for SV40 DNA chain growth in which 4S chains are formed at the replication forks and, subsequently, linked through phosphodiester bonds to the growing chains finally to yield full length (16S) strands. Recently, a similar process has been elucidated for the *in vitro* replication of polyoma DNA (Magnusson *et al.*, 1973). Furthermore, these investigators have identified ribonucleotides linked to the 5' termini.

The two most probable models depicting SV40 DNA chain elongation are illustrated in Figure 2 (models A and B). In model A, the 4S chains are formed on both template strands at each growing point and, therefore, should contain complementary sequences. Purified 4S DNA from molecules of this type would be capable of extensive renaturation. In model B, chain growth in the 5' to 3' direction occurs in a continuous manner while the other daughter strand, which would require a polymerase with a 3' to 5' activity if it were to be synthesized continuously, is formed discontinuously. In contrast to model A, the short chains formed on only one template strand at each form (model B) should not be able to renature when separated from the template strands. This would be the case regardless of the extent of replication since the origin for replication is the same in all molecules. For these models we have assumed that 4S chains are synthesized in the 5' to 3' direction (Kornberg, 1960; Mitra and Kornberg, 1966) as are the nascent short chains of phage T4 DNA (Okazaki and Okazaki, 1969; Sugino and Okazaki, 1972).

In order to test these two alternative possibilities, labeled 4S chains have been purified from partially fractionated and unfractionated replicating SV40 molecules (Fareed, Khoury, and Salzman, 1973). The purified 4S DNA was first shown to anneal exclusively with denatured SV40 DNA immobilized on nitrocellulose filter discs. Next, the labeled 4S chains from both fractionated (Sebring *et al.*, 1971) and unfractionated replicating SV40 molecules were allowed to renature at 68°C in 1 *M* NaCl solution. After the renaturation process, however, the labeled strands were

76 to 90% resistant to the N. crassa nuclease; 70 to 92% acted as double helical DNA on hydroxyapatite. This extensive self-annealing of the 4S strands demonstrates that complementary genetic sequences are present and provides evidence for discontinuous synthesis of both daughter strands at each replication form in SV40 DNA (model A in Figure 2). In agreement with these results, brief pulse labeling of infected cells at room temperature has been observed to introduce greater than 50% of the radioactivity into 4S chains (Fareed and Salzman, unpublished results). Furthermore, when the infected cells are treated with FUDR to inhibit DNA synthesis and then briefly pulse-labeled with ^{3}H-thymidine to reverse the inhibition greater than 90% of the label can be located in 4S strands (Salzman and Thoren, 1973). The primer for synthesis of a 4S strand is, presumably, a short RNA segment annealed to the template strand. This initiation reaction must be contrasted to the primary initiation of DNA replication which occurs at a specific site in all molecules. Further research will be needed to determine if specific signals (genetic sequences) are required for the initiation of synthesis of each 4S DNA strand.

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AN ANALYSIS OF THE STRUCTURE OF THE REPLICATING FORK DURING DISCONTINUOUS SYNTHESIS OF SV40 DNA AND THE DETECTION OF GAP CIRCLE INTERMEDIATES

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Polyoma and SV40 are small DNA tumor viruses whose well-defined properties have made them ideal subjects for intensive investigation over the last ten years (Black, 1968; Eckhart, 1968; Crawford, 1969; Green, 1970; Sambrook, 1972; Butel, Tevethia and Melnick, 1972). The DNA of these viruses has been extensively characterized by Vinograd and his collaborators (Vinograd and Lebowitz, 1966; Bauer and Vinograd, 1968; Radloff, Bauer and Vinograd, 1967) and by Crawford (Crawford and Black, 1964; Crawford, 1969). They have shown that the genome of SV40 is a single closed-circular molecule of double-stranded DNA containing about 12-15 negative superhelical turns and has a molecular weight of about 3×10^6 daltons. Over the last four years experiments from a number of laboratories working on the mechanism of SV40 DNA replication have yielded a clear outline of the events of viral DNA replication (for a recent review see Levine, 1973). These events may be conveniently divided into four stages: 1) initiation, 2) polynucleotide chain propagation, 3) segregation of the two newly-made daughter molecules, and 4) maturation of the progeny molecules.

Initiation of SV40 DNA replication takes place at an unique site on the SV40 genome (Nathans and Danna, 1972; Thoren, Sebring, and Salzman, 1972; Fareed, Garon, and Salzman, 1972). Protein synthesis is required for the start of each round of DNA replication (Kit and Nakajima, 1971; Kang _et al_., 1971), and at least one of these proteins is coded for by the tsA gene product of SV40 (Tegtmeyer, 1972). Once DNA synthesis has begun, replication

proceeds bidirectionally from the unique origin (Danna and Nathans, 1972; Fareed et al., 1972; Jaenisch, Mayer, and Levine, 1971; Bourgaux and Bourgaux-Ramoisy, 1971). When the replicating molecules are labeled for very short periods of time (15-45 seconds at 37°C) with ^{3}H-thymidine, nascent short (4S) DNA chains (about 100-150 nucleotides long) are found associated with the SV40 replicating forms (Fareed and Salzman, 1972). These fragments can be chased into the growing progeny polynucleotide strands of SV40 DNA and are therefore a likely intermediate in the polynucleotide chain propagation step. Similar oligonucleotide intermediates have been detected by Magnusson et al. (1973) in an in vitro nuclei system replicating polyoma DNA. Furthermore, these investigators have demonstrated the existence of ribonucleotides linked to the 5'-terminus of the 4S DNA chains of polyoma DNA. These results are similar to the polynucleotide chain propagation steps previously described for E. coli (Okazaki et al., 1968; Sugino, Hirose, and Okazaki, 1972). They suggest that synthesis of each 4S fragment is primed by oligoribonucleotides at the replication fork.

The discontinuous synthesis of 4S fragments on SV40 or polyoma DNA can be exaggerated in the presence of inhibitors of DNA synthesis such as hydroxyurea (HU) (Magnusson et al., 1973; Magnusson, 1973; Laipis and Levine, 1973) or 5-fluorodeoxyuridine (Salzman and Thoren, 1973). These agents slow the rate of polynucleotide chain propagation by severely limiting the pool of one of the deoxyribonucleoside triphosphates in the cell (Skoog and Nordenskjold, 1971). Under these conditions the 4S oligonucleotide fragments are readily detected in association with the replicating molecules even when the labeling times are longer than 15-45 seconds. These 4S fragments observed in the presence of HU are readily chased into the longer progeny strands of SV40 DNA and less efficiently into mature closed-circular molecules (Laipis and Levine, 1973).

Polynucleotide chain propagation proceeds until the two replication forks meet (or almost meet) at the terminus of DNA replication. Since this terminus is about 180 degrees around the circular genome from the origin (Danna and Nathans, 1972) it is likely that the two forks traverse each half of the DNA molecule at about the same rate. This observation is supported by the electron micrographs of replicating SV40 DNA (Jaenisch et al., 1971; Sebring et al., 1971) where the two newly replicated branches of the molecule are always the same length. The two interlocked replicating circles then separate, generating two progeny molecules that sediment at 16S. Failure to separate the replicating daughter molecules apparently results in the formation of a catenated dimer (Jaenisch and Levine, 1973). These interlocked dimers are unstable and decay, probably via a recombination event, to either a concatenated dimer or two monomers (Jaenisch and Levine, 1973). When cycloheximide is used to inhibit protein synthesis there is a two- to fourfold increase

in the percentage of viral DNA synthesized in the form of catenated dimers (Jaenisch and Levine, 1972 and 1973). These data suggest that protein synthesis is also required for an efficient segregation of the two newly-synthesized daughter molecules.

The two newly-segregated progeny molecules sediment at 16S and are composed of a circular template strand and a linear newly-synthesized progeny strand containing a polynucleotide chain interruption located specifically at the terminus of replication (Fareed, McKerlie, and Salzman, 1973). These molecules must be processed to form the closed-circular and superhelical mature viral DNA.

The experiments presented in this paper deal with two of the four stages of SV40 DNA replication: the polynucleotide chain propagation step and the maturation or processing of the post-segregational 16S intermediate to form mature viral DNA.

The experiments to be described attempt to elucidate the detailed structure of the nascent 4S fragments and the longer progeny strand found at the replication fork of SV40 DNA. Two methods are available to obtain SV40 replicative intermediates with 4S fragments hydrogen bonded at the replication forks: 1) short pulses with ^{3}H-thymidine (15-45 seconds) of SV40 infected African green monkey kidney cells (AGMK) yield replicating molecules labeled preferentially in the 4S fragments (Fareed and Salzman, 1972) and 2) longer labeling times in the presence of hydroxyurea (Magnusson et al., 1973; Laipis and Levine, 1973) yield replicating molecules with the same properties. This latter procedure has the advantage of yielding a large quantity of labeled replicating DNA and was therefore employed in the studies described here. The disadvantage of using an inhibitor like HU is that it may induce some aberrant form of DNA replication.

To isolate and characterize the viral replicative intermediates synthesized in the presence of HU, the following experiment was performed. Monolayer cultures of AGMK cells were infected with SV40 (moi 50-100 PFU/cell) and 36 hours later HU (10 mM) was added to half the cultures. Five minutes after the addition of this drug, ^{3}H-thymidine (430 μc/ml) was added to the HU-treated and untreated cultures for 15 minutes at 37°C. The viral DNA was selectively extracted by the procedure of Hirt (1967) and banded to equilibrium in ethidium bromide-cesium chloride (EtBr-CsCl) density gradients. Under these conditions replicating viral DNA is fractionated into a graded series of molecules just beginning replication (at a density adjacent to form I DNA) all the way to molecules that have almost completed their replication (lighter density regions) (Jaenisch et al., 1971; Sebring et al., 1971; Mayer and Levine, 1972). The replicating intermediates isolated in this manner were divided into three classes: 1) molecules just beginning replication or

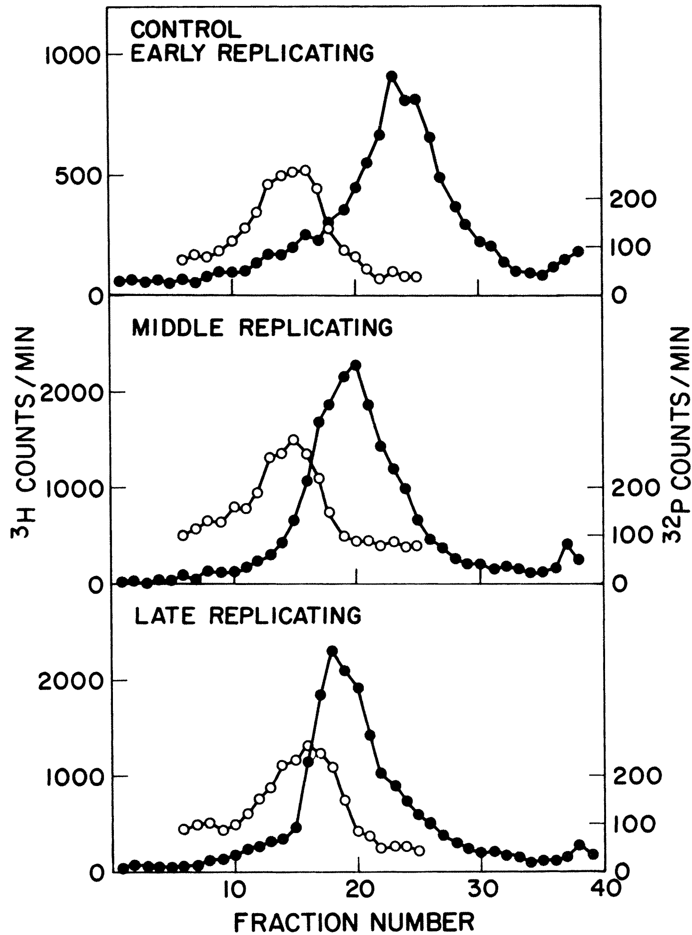


FIG. 1. Alkaline sucrose gradient sedimentation analysis of the replicative intermediates of SV40 DNA at different stages of replication (early, middle and late).

SV40 infected AGMK cells were labeled with ^{3}H-thymidine for 15 minutes at 36 hrs. after infection and the viral DNA was selectively extracted by the procedure of Hirt (1967). The DNA was centrifuged to equilibrium in a CsCl-EtBr gradient (Jaenisch *et al.*, 1971). This procedure separates viral DNA that has just begun to replicate (early), DNA in the middle of replication (middle), or DNA completing replication (late). Each one of these three fractions was then sedimented in a 10-30% alkaline sucrose gradient (0.75M NaCl, 0.25M NaOH) resting on top of a 60% sucrose cushion (0.75M NaCl, 0.25M NaOH). Fractions from the gradient were analyzed as described previously (Laipis and Levine, 1973).

o——o ^{32}P-labeled SV40 form II DNA marker

●——● ^{3}H-labeled SV40 replicating DNA

early replicating forms, 2) molecules about half completed in their replication or middle replicating forms and 3) molecules almost completed replication (late replicating forms). Each of these fractions from the HU-treated or untreated cultures was then sedimented through an alkaline sucrose gradient in order to size the newly replicated strands of DNA. Figure 1 presents the alkaline sucrose gradient sedimentation profiles of labeled viral DNA synthesized in the absence of HU. As replication proceeds (from early to late replicating molecules) the average size of the growing progeny strands increases. This can be seen in the increased sedimentation rate of these DNA molecules compared to a ^{32}P-labeled form II sedimentation marker. There is little evidence of any 4S fragments associated with the replicative intermediates because of the longer length of time of this pulse-labeling period (15 minutes) and the rapidity with which these oligonucleotides are joined to the longer growing strands. During a 15 minute labeling period the parental or template strands of the replicative intermediate are not labeled with ^{3}H-thymidine and therefore are not detected in these gradients. When the early, middle and late replicative intermediates obtained from HU-treated cells were sedimented through alkaline sucrose gradients (Figure 2), 4-5S oligonucleotide fragments were observed in addition to the longer growing progeny strands of SV40 DNA. These oligonucleotides were associated with molecules in all stages of replication (early, middle and late), eliminating the possibility that the 4-5S fragments were peculiar to the initiation event (only the early replicating DNA) or the segregation process (only the late DNA). That the 4S fragments were intermediates in polynucleotide chain propagation could be demonstrated by pulse-labeling them in the presence of HU followed by a chase in the presence or absence of HU (Laipis and Levine, 1973). When the 4S fragments were labeled with ^{3}H-thymidine in the presence of HU for 10 minutes and chased in the presence of unlabeled thymidine for 60 minutes essentially all the 4S fragments were incorporated into the larger polynucleotide growing strand and 20-30% of these fragments could be chased into mature closed-circular SV40 DNA. These observations were independent of whether the chase was performed in the presence or absence of HU (Laipis and Levine, 1973).

Under the conditions of these experiments HU inhibits the rate of viral DNA synthesis between 85-95% (Laipis and Levine, 1973). Viral DNA synthesis never completely stops. The rate of polynucleotide chain propagation is slower, however. Since the 4S fragments do appear to be synthesized in HU, albeit at a slower rate, the step in polynucleotide chain propagation most sensitive to HU is the joining of the 4S fragments to form the longer growing progeny strand of viral DNA.

Two general structures might be considered for these HU-treated replicating molecules. These possibilities are reviewed in

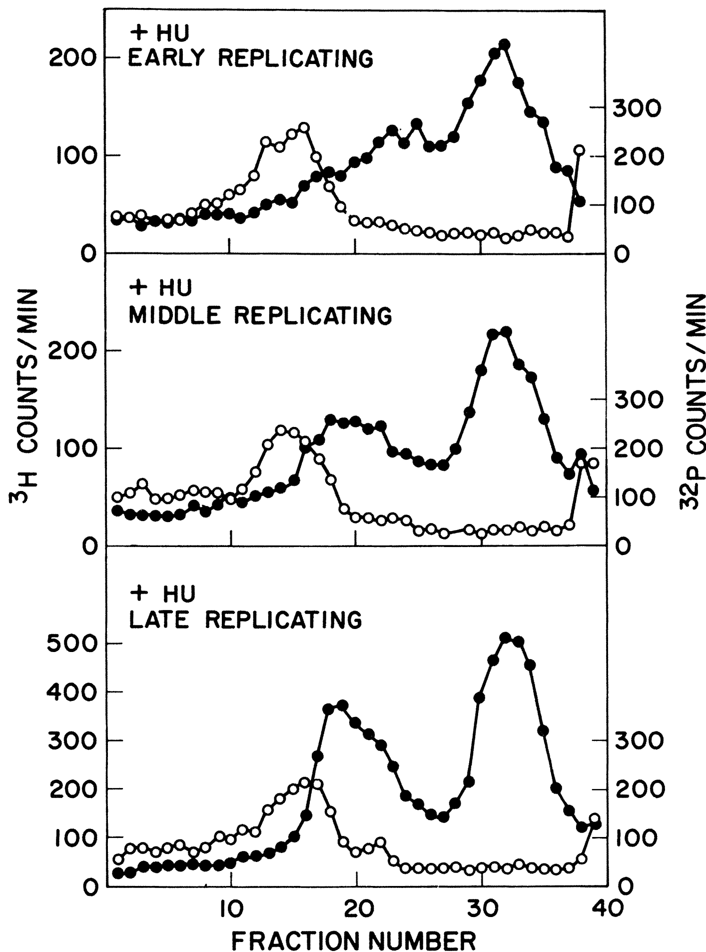


FIG. 2. Alkaline sucrose gradient sedimentation analysis of the SV40 replicative intermediates synthesized in the presence of hydroxyurea.

SV40 infected AGMK cells were labeled with ^{3}H-thymidine five minutes after the addition of HU (10 mM) for 15 minutes (at 36 hours after infection). The replicative intermediates were isolated and analyzed as described in Figure 1.

o——o ^{32}P-labeled SV40 form II DNA

●——● ^{3}H-labeled SV40 replicating DNA synthesized in the presence of HU

Figure 3. The short fragments, hydrogen bonded to the parental strands of SV40 DNA at the replication forks, could be separated from themselves or from the longer progeny strands by a simple 3'OH, $5'PO_4$ single stranded nick (Figure 3A). Alternatively, these fragments might be separated by a gap of one or more nucelotides (Figure 3B). Several variations of these two basic themes might be imagined, such as the absence of a $5'PO_4$ group or the presence of ribonucleotides linked to the 5' end of the deoxyribonucleotide fragments.

To distinguish between these alternatives, ^{3}H-thymidine-labeled replicating SV40 DNA synthesized in HU-treated cells was isolated by EtBr-CsCl density centrifugation as described previously. This DNA was employed as a template in an *in vitro* DNA synthesizing system using purified *E. coli* polynucleotide ligase and T-4 DNA polymerase. These enzymes were chosen because *E. coli* ligase, unlike T-4 ligase, will not join DNA to oligoribonucleotides (Olivera and Lehman, 1968), and T-4 DNA polymerase, unlike *E. coli* DNA polymerase I, does not possess a 5'exonuclease activity and cannot nick translate (Cozzarelli, Kelly, and Kornberg, 1969; Kelly *et al.*, 1969). If the 4-5S fragments were indeed hydrogen bonded to the template strands of SV40 DNA at the replication forks (as in Figure 3) then one can distinguish whether there is a nick (Figure 3A) between the 4-5S fragment and the adjacent longer polynucleotide strand or a gap (Figure 3B). If a simple break in the phosphodiester linkage exists between the oligonucleotides and the growing progeny DNA and if this break is bounded by $5'PO_4$ and 3'OH groups, *E. coli* polynucleotide ligase alone would join the 4S fragment to the longer DNA strand. If, however, a gap is present between these DNA molecules (Figure 3B), both DNA polymerase and polynucleotide ligase would be required to join the longer progeny strand to the 4S fragment. The absence of a $5'PO_4$, a blocked 3'OH group or a ribonucleotide at the 5' end of a fragment would prevent the joining of the 4S fragment in the presence of both *E. coli* ligase and T-4 polymerase.

To test these alternative hypotheses, SV40 replicative intermediates (the late fraction) labeled and synthesized in HU-treated cells, were isolated by EtBr-CsCl density gradient centrifugation. These templates were incubated with unlabeled deoxyribonucleoside triphosphates and cofactors (Laipis and Levine, 1973) in 1) the absence of any added enzymes, 2) the presence of E. coli DNA ligase, 3) the presence of T-4 DNA polymerase, and 4) the presence of both ligase and polymerase. DNA synthesis and/or ligation was allowed to proceed for 20 minutes at 30°C. At that time each sample was analyzed to determine if the 4S fragments were joined to the longer polynucleotide chains by sedimentation through an alkaline sucrose gradient. These results are presented in Figure 4. In Figure 4A the SV40 late replicative intermediate, incubated in the absence

FIG. 3. Possible structure for the hydrogen bonding of the 4-5S fragments in the replicative intermediates of HU-inhibited SV40 DNA.

Model A: The 4S fragments are not separated from each other and the longer progeny strand. There is a break or a nick in the phosphodiester backbone of adjacent nucleotides bounded by a 3'-OH and a 5'-PO_4 group. This nick is repairable by E. coli polynucleotide ligase alone.

Model B: The 4S fragments are separated from each other and the longer progeny strands by gaps of one or more nucleotides (single-stranded regions). These gaps are repairable by both T-4 DNA polymerase and E. coli polynucleotide ligase but not by DNA ligase alone.

FIG. 4. Alkaline sucrose gradient analysis of HU-treated SV40 replicative intermediates incubated in the presence or absence of T-4 DNA polymerase and E. coli DNA ligase.

SV40-infected AGMK cells (36 hours after infection) were treated with 10 mM HU and the viral replicating DNA was labeled with ^{3}H-thymidine 5 minutes later for a period of 15 minutes. The selectively extracted viral DNA (Hirt, 1967) was purified in EtBr-CsCl gradients (Jaenisch et al., 1971), and the late replicative intermediate was employed as a template for in vitro repair with E. coli DNA ligase (10 units, 18,000 units/mg protein, 1.8 mg/ml) and/or T-4 DNA polymerase (0.75-1.5 units, 1500 units/mg protein, 0.05 mg/ml). The reaction mixture of 0.2 ml contained 66 mM Tris, pH 8.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.33 mM of each of the four deoxytriphosphates, 15 μM DPN and 150 μg/ml of bovine serum albumin. DNA synthesis was allowed to proceed for 20 minutes at 30°C. The products were analyzed by alkaline sucrose gradients as in Figure 1.

o——o ^{32}P-SV40 form I and II markers

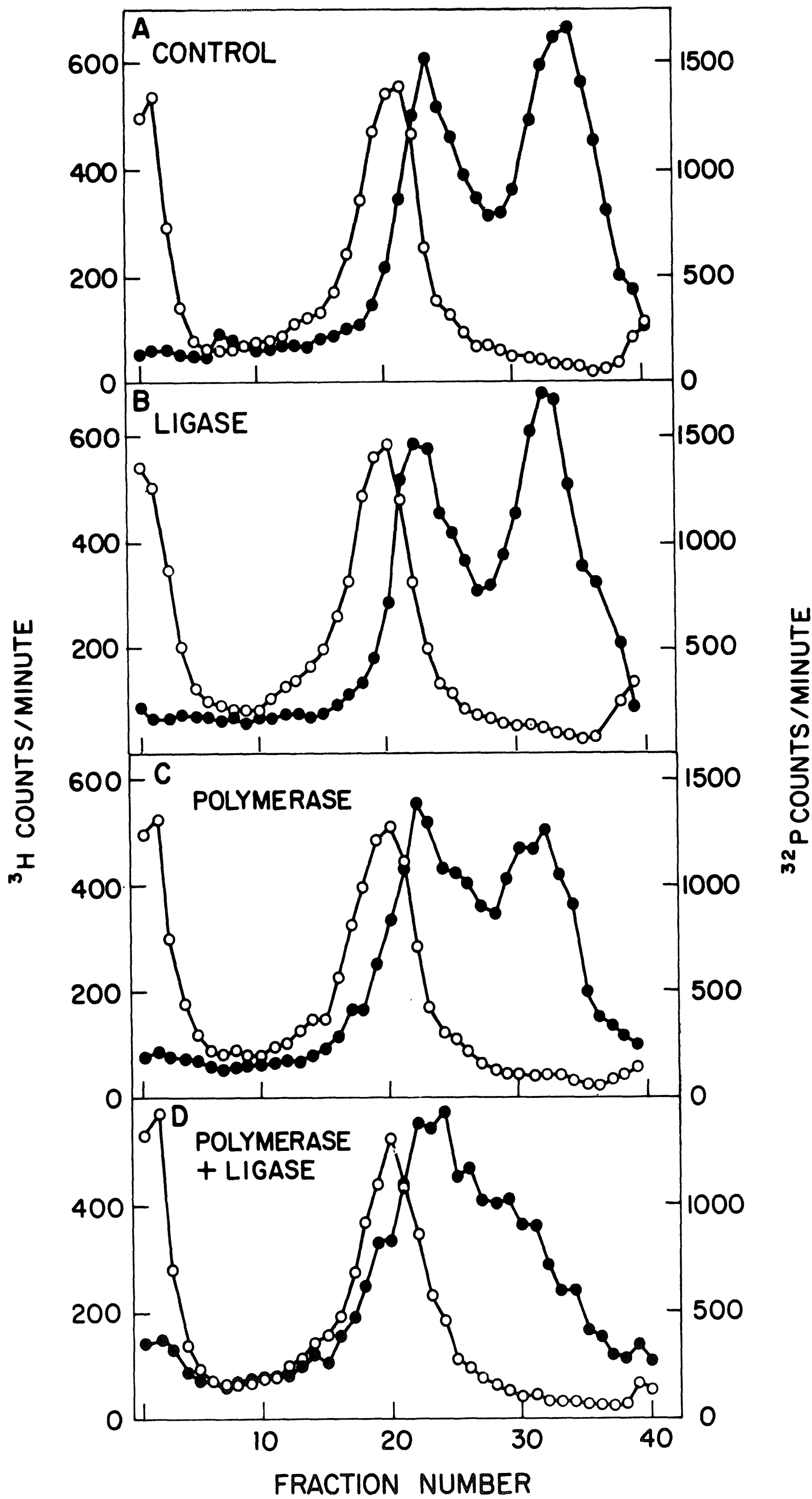
●——● ^{3}H-SV40 HU replicative intermediate

A) SV40 template alone, no enzymes

B) SV40 template plus DNA ligase

C) SV40 template plus DNA polymerase

D) SV40 template plus DNA ligase and polymerase


A CONTROL
B LIGASE
C POLYMERASE
D POLYMERASE + LIGASE
^{3}H COUNTS/MINUTE
^{32}P COUNTS/MINUTE
FRACTION NUMBER
0
200
400
600
500
1000
1500
10
20
30
40

of any enzymes, contains both the 4S fragments and the longer polynucleotide strands of DNA. Incubation of this template with either E. coli ligase alone (Figure 4B) or T-4 polymerase alone (Figure 4C) yielded essentially the same result. When the same template was treated with both enzymes, however, (Figure 4D) about 50% of the short oligonucleotide fragments now sedimented as if they were covalently attached to the longer progeny DNA.

In order to improve the efficiency of joining the 4S fragments to the longer polynucleotide strands the template DNA for this reaction was further purified before incubation with these enzymes. The ^{3}H-labeled SV40 replicative intermediate synthesized in HU-treated cells, as described previously, was purified by EtBr-CsCl density gradient centrifugation. The late replicating molecules were then sedimented through a neutral sucrose gradient where the replicating DNA sediments at 25S (Levine, Kang, and Billheimer, 1970). In addition to the further purification of the replicating DNA, this procedure separates the 25S replicative intermediate from the 16S intermediate produced by the segregation of the two daughter molecules.

When this purified replicating template was incubated with both DNA polymerase and DNA ligase 80% of the 4S fragments were joined to the larger progeny strands (Figure 5C) as demonstrated by the sedimentation of the labeled replicative form through an alkaline sucrose gradient. Incubation of the HU-treated replicative intermediate with ligase alone does not join the 4S fragments to the longer viral DNA strand (Figures 5A and 5B). These data demonstrate that at least 80% of the 4S fragments at the replication fork of HU-treated SV40 DNA are separated from themselves and from the progeny molecules by gaps of one or more nucleotides. Most of these gaps are bounded by 3'OH and $5'PO_4$ groups. A small proportion, about 20%, of the gaps are not repairable in vitro.

When the HU late replicative intermediates, purified by EtBr-CsCl density gradients, were repaired with both DNA polymerase and DNA ligase (Figure 4D) a small percentage (1-4%) of the labeled DNA sedimented in an alkaline sucrose gradient as mature closed-circular DNA. When the EtBr-CsCl late replicating fraction was further purified by a neutral sucrose gradient two species of DNA were observed. In addition to the 25S replicative DNA, a 16S post segregational intermediate formed by the separation of the two replicating daughter molecules (Fareed et al., 1973) was present in this gradient. This 16S DNA contained a circular parental strand and a linear progeny strand with a polynucleotide chain interruption. This interruption could be a nick bounded by a 3'OH and a $5'PO_4$ group or a gap as was found in the replicative intermediate. To distinguish between these alternatives the 16S

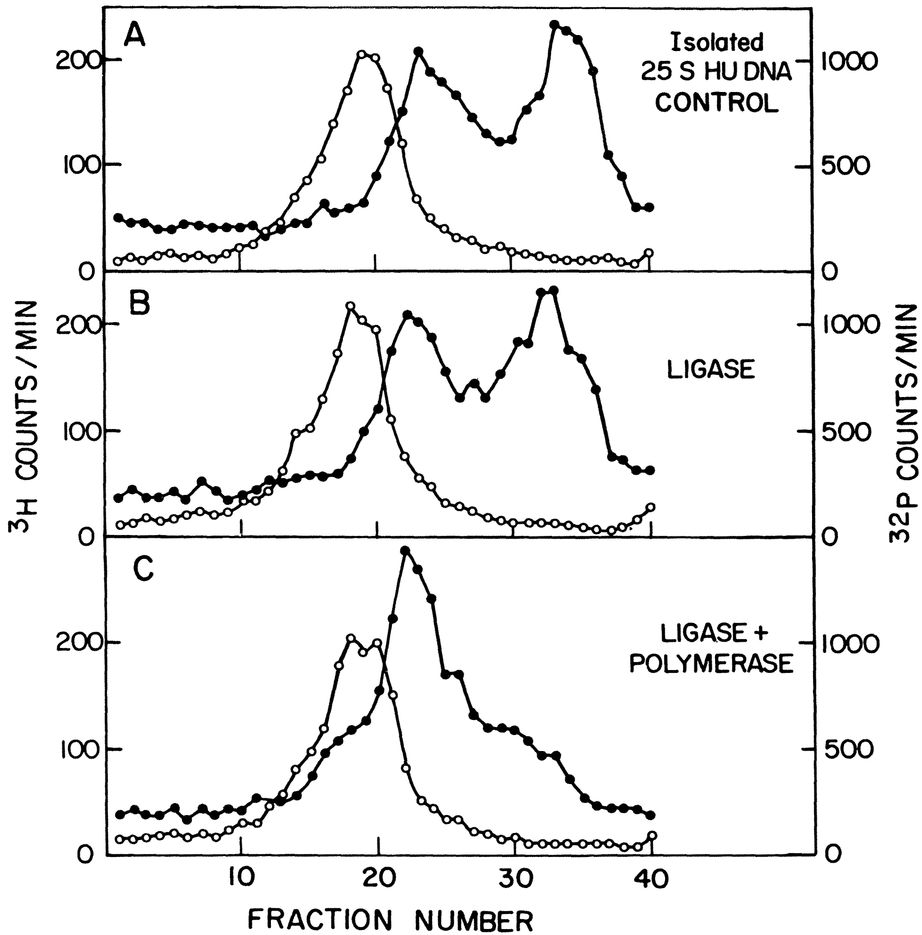


FIG. 5. Alkaline sucrose gradient of HU-treated SV40 replicative intermediate repaired by DNA polymerase and/or DNA ligase.

The conditions of the experiment and enzymes reactions are identical to Figure 4 except that the HU-treated SV40 replicative intermediate was purified first in an EtBr-CsCl gradient followed by a neutral sucrose gradient where it sediments at 25S.

o——o ^{32}P-SV40 form II marker

●——● ^{3}H-labeled SV40 HU-treated replicative intermediate

A) 25S replicative intermediate alone

B) 25S replicative intermediate treated with DNA ligase alone

C) 25S replicative intermediate treated with DNA polymerase plus DNA ligase

post segregational intermediate was incubated with E. coli DNA ligase alone, with E. coli DNA ligase plus T-4 DNA polymerase or in the absence of these enzymes. The product of these reactions was analyzed on alkaline sucrose gradients as before. The 16S template incubated in the absence of enzymes (Figure 6, top panel) contains a long linear strand and a small level of 4-5S fragments. Treatment of this template with polynucleotide ligase alone does not alter this result (Figure 6, middle panel). Incubation of the 16S molecules with DNA polymerase plus DNA ligase converted about 60% of these molecules to the closed-circular form. These experiments demonstrate that the HU-treated 16S post segregational intermediate contains a gap of one or more nucleotides in the linear newly-synthesized progeny strand.

DISCUSSION

The addition of HU to SV40-infected AGMK cells results in an 85-95% decrease in the rate of incorporation of ^{3}H-thymidine into SV40 replicating DNA. The residual viral DNA synthesized is initially in the form of 4-5S fragments. Similar oligonucleotide fragments have been observed when short pulse-labeling times with ^{3}H-thymidine (15-45 seconds) were performed during normal SV40 DNA replication (Fareed and Salzman, 1972). These short DNA fragments are hydrogen bonded to SV40 replicating molecules in all stages of duplication. Pulse-chase experiments indicated that these oligonucleotides are likely a normal intermediate in polynucleotide chain propagation.

The in vitro DNA synthesis experiments described in this paper demonstrate that the 4S fragments, hydrogen-bonded to the template strands at the replication forks, are separated from themselves and from progeny molecules by gaps of one or more nucleotides. These gaps are bounded by 3'OH and 5'PO_4 groups. A small proportion of the 4S fragments (about 20%) were not repaired (joined to the longer polynucleotide strand) in vitro. One obvious possibility is that the 5' end of these DNA chains may contain a ribonucleotide which could not be sealed with E. coli DNA ligase. A short sequence of ribonucleotides has been suggested as the primer for 4S DNA fragments in polyoma replicating DNA (Magnusson et al., 1973).

HU inhibits DNA replication by inhibiting ribonucleotide reductase, resulting in a drastically reduced deoxyriboguanosine triphosphate pool (Moore, 1969; Skoog and Nordenskjold, 1971). The question remains why this should result in a gap between 4S fragments and the longer growing progeny strands while the 4S fragments themselves are synthesized, albeit at a slower rate. One possibility is that two DNA polymerases are involved in SV40 DNA

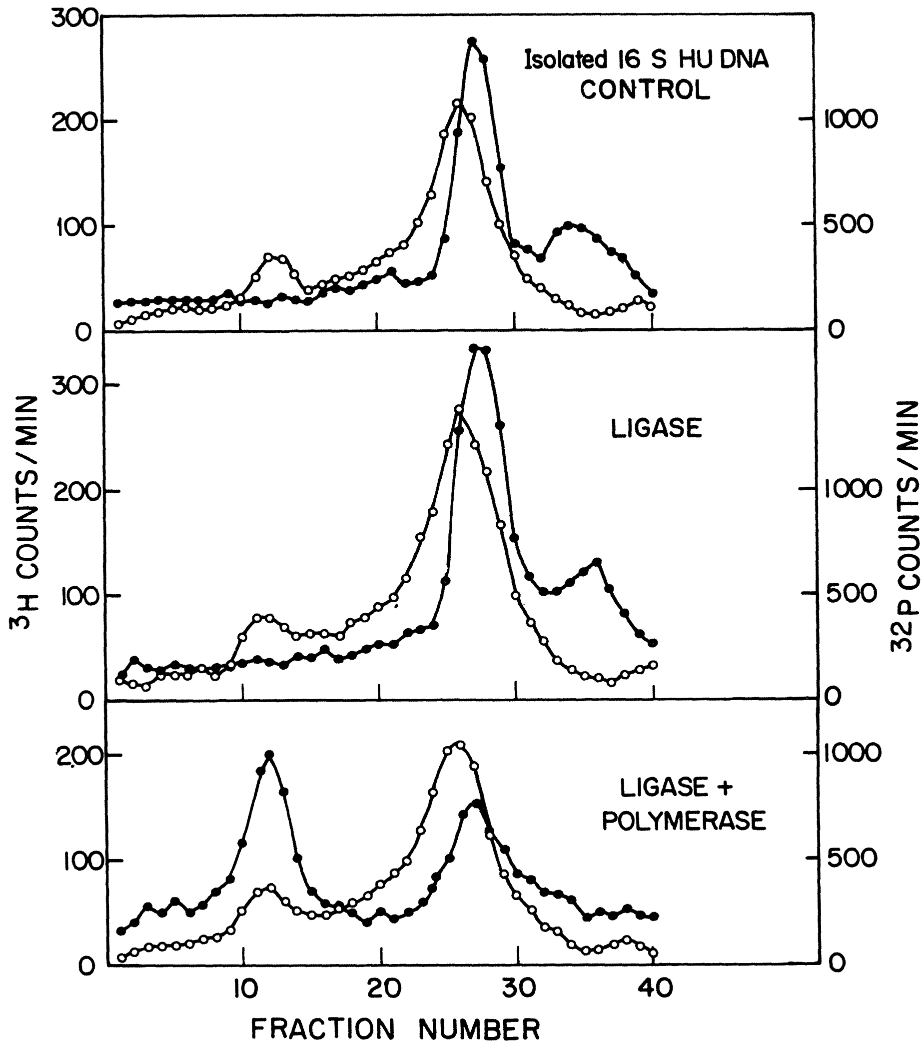


FIG. 6. Alkaline sucrose gradient analysis of the HU-treated post segregational 16S intermediate during SV40 DNA replication. Repair synthesis with DNA ligase and DNA ligase plus DNA polymerase.

The 16S post segregational intermediate was isolated from HU-treated cells as described in Figures 4 and 5. It was treated with ligase and/or polymerase in vitro and the product was analyzed as described in Figure 1.

o——o ^{32}P-labeled form I and II SV40 DNA

●——● ^{3}H-labeled 16S post segregational intermediate of SV40

replication. One of these polymerases synthesizes the short oligodeoxynucleotides (4S fragments) that are primed by the oligoribonucleotides. The second polymerase would then be responsible for filling in the gaps left between fragments. The "gap-filling polymerase" would then be expected to have a higher K_m for deoxyribonucleoside triphosphates and therefore be more sensitive to low levels of these nucleotides in the cellular pools.

There are at least three different polymerases found in mammalian cells (Weissbach et al., 1971; Chang and Bollum, 1971; Fridlender et al., 1972; Sedwick, Wang, and Korn, 1972; see this volume). Polymerase I is found in the cytoplasmic fraction (C or maxi polymerase) and shows quantitative alterations with different stages of the cell cycle and with cell growth (Iwamura, Ono, and Morris, 1968; Chang and Bollum, 1972a; Chang, Brown, and Bollum, 1973). Polymerase II can be found in the nucleus (N or mini polymerase), and its activity remains relatively constant despite changes in cell growth rate or cell cycle alterations (Chang and Bollum, 1972a; Chang et al., 1973). Polymerase III (also called the R or A polymerase) is the only one of these polymerases that can use a polyriboadenylate:oligodeoxythymidylate template efficiently in the presence of Mg^{++} (Fridlender et al., 1972). Polymerase I carries out considerable synthesis from a polydeoxythymidylate: oligoriboadenylate (dT_{600}:rA_{10}) template while polymerase II cannot employ this type of primer effectively (Chang and Bollum, 1972b). These data suggest that polymerase I would be a good candidate for the synthesis of the 4S fragments primed by ribonucleotides. In addition, polymerase II has a five to eight fold higher K_m for deoxyribonucleoside triphosphates than polymerase I, suggesting it might be preferentially inhibited by a low nucleoside triphosphate pool and therefore be the "gap-filling polymerase." The role of polymerase III (if any) is at present unclear.

There are two alternative explanations for the presence of gaps between the 4S fragments and the longer progeny strands of viral DNA. 1) When the ribonucleotides that prime the 4S fragments are removed, perhaps by ribonuclease H, this should leave a single-stranded region between the 4S oligonucleotide and the progeny DNA. 2) The initiation of new 4S fragments may not occur at the nucleotide adjacent to the primer strand. Either mechanism would result in a gap during normal SV40 DNA replication. Similar models suggesting the involvement of two DNA polymerases in polynucleotide chain propagation have been proposed for bacteria (Kuempel and Veomett, 1970; Okazaki, Arisawa, and Sugino, 1971), SV40 (Salzman and Thoren, 1973) and polyoma virus DNA replication (Magnusson, 1973).

ACKNOWLEDGEMENTS

We wish to thank Drs. M. Bessman and P. Modrich for generous gifts of purified enzymes. The excellent technical assistance of A. Teresky and C. Kaminski is gratefully acknowledged. This research was supported by National Cancer Institute Grants CA11049-05 and CA12068-03 and American Cancer Society Grant E591. One of us (PJL) is a postdoctoral fellow of the National Cancer Institute.

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THE REPEATED SEQUENCES IN SERIALLY PASSAGED SV40 DNA

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ABSTRACT

Three populations of altered SV40 DNA molecules, independently derived by serial passage of plaque-purified virus at high multiplicity of infection, were found to contain reiterated viral sequences, as determined by reassociation kinetic experiments. In each case, the reiterated sequences corresponded to some of those contained in _Hin_-fragments C or D, obtained by digesting plaque-purified virus DNA with _Hemophilus influenzae_ restriction endonuclease. It is suggested that the reiteration of sequences in _Hin_-fragment C, which is known (Danna and Nathans, 1972; Fareed, Ganon, and Salzman, 1972) to contain the origin of SV40 DNA replication, is responsible for the advantageous replication rate of altered viral genomes during serial passage.

A lack of uniformity was observed in the reassociation rates of different _Hin_-fragments (cleaved from plaque-purified SV40 DNA) incubated in the presence of increasing concentrations of plaque-purified virus DNA. The possibility that this finding is an indication of sequence duplication in the plaque-purified SV40 genome is discussed.

INTRODUCTION

High multiplicity serial passage of SV40 in monkey cells results in the emergence of defective virus with altered genomes. One such alteration consists of the insertion of host DNA sequences (substituted DNA) (Frenkel, Lavi, and Winocour, 1974; Lavi and Winocour,

1972; Lavi et al. 1972; Rozenblatt et al., 1972); other alterations involve viral sequence deletion (Tai et al., 1972; Yoshiki, 1968a and 1968b) and reiteration (Brockman, Lee, and Nathans, 1973; Martin et al., 1973; Winocour et al., in press). An earlier communication from this laboratory showed that substituted SV40 DNA molecules, though defective in plaque formation, can replicate in multiply infected cells (Lavi et al., 1972). We proposed that the high multiplicity serial passaging procedure provides conditions for the selection of substituted viral genomes possessing the capacity to replicate faster than standard SV40 DNA. The outcome of this selective process is that the serially passaged DNA becomes progressively enriched for a particular type of altered viral genome, as judged by reassociation kinetics (Frenkel et al., 1974) and by the pattern of restriction endonuclease cleavage (Brockman et al., 1973; Rozenblatt et al., 1972; Winocour et al., in press). A possible mechanism that could account for the advantageous replication of substituted SV40 DNA is amplification of signals for the initiation of DNA synthesis. The present series of experiments were undertaken to characterize the reiterated viral sequences in several serially passaged SV40 DNA populations and to determine if such sequences are derived from the region of the SV40 genome which contains the origin of replication.

MATERIALS AND METHODS

Cells and Viruses

The cultivation of the BS-C-1 line of African green monkey cells and the procedures for plaque purification and high multiplicity serial passage of SV40 have been published elsewhere (Lavi and Winocour, 1972; Lavi et al., 1972). Any independent set of serial passages initiated from plaque-purified virus is referred to as a serially passaged line and is designated according to the plaque-isolate, the line number, and the passage number. Thus, CVB/1/P3 indicates passage 3 of line 1 from plaque-isolate CVB (Figure 1).

Virus DNA

The SV40 DNA preparations are designated by the inoculum used to infect the cells; thus, CVB/1/P3 DNA is the closed-circular SV40 DNA produced in cells infected with CVB/1/P3 virus at high multiplicity. Virus DNA was extracted either from purified virions (Lavi and Winocour, 1972) or from infected cells using the Hirt procedure (Hirt, 1967). Closed-circular SV40 DNA was purified by equilibrium centrifugation in CsCl gradients supplemented with ethidium bromide, followed by sedimentation through 10-30% neutral

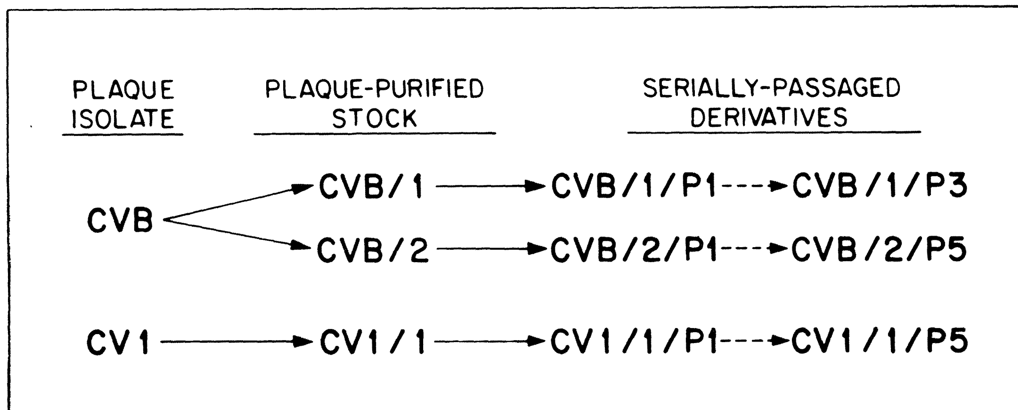


FIG. 1. History of the serially passaged lines studied. Full details on the plaque-isolates, the preparation of plaque-purified stocks grown at low multiplicity of infection, and the high-multiplicity serial passaging procedure, are presented elsewhere (Lavi and Winocour, 1972; Lavi *et al.*, 1972).

sucrose gradients or alkaline CsCl solution (Rozenblatt et al., 1972). To prepare radioactive SV40 DNA, the infected cells were labeled with ^{3}H-thymidine (18-26 Ci/mmole; 10 μc/ml medium) at 24-48 hours post-infection (when DNA was extracted from infected cells) or at 24 hours-6 days post-infection (when DNA was extracted from virions). DNA was sheared by sonic vibration as described elsewhere (Frenkel et al., 1974).

Digestion of SV40 DNA by *Hemophilus Influenzae* Restriction Enzyme

Plaque-purified SV40 DNA was digested to completion with *Hemophilus influenzae* (*Hin*) restriction enzyme (gift of Dr. D. Nathans) and the digestion products were fractionated on 5% polyacrylamide gels (Danna and Nathans, 1971; Rozenblatt et al., 1972). The DNA fragments were eluted from the gels (Rozenblatt et al., 1972) and were dialysed extensively against hybridization buffer.

DNA-DNA Hybridization

Hybridization was performed as previously described (Frenkel et al., 1974), in a buffer consisting of 0.05 *M* tris-HCl (pH 8.05) and varying NaCl concentrations (0.03 *M* to 0.4 *M*). Incubation was at 25°C below the melting temperature at the corresponding salt concentration. At the end of incubation, the hybridization mixture was digested with S1 nuclease from *Aspergillus oryzae* (Ando, 1966; Sutton, 1971). Detailed procedures for the nuclease assay, and for determining the extent of hybrid-formation resistant to S1, have been reported elsewhere (Frenkel et al., 1974). Under the conditions employed, 96-97% of heat-denatured SV40 DNA was digested and essentially 100% of non-denatured, sonicated SV40 DNA was resistant to the enzyme.

Estimation of the Fraction of Repetitive Sequences

DNA reassociation is ideally a second order reaction described by

$$\frac{D_t}{D_o} = \frac{1}{1+k\cdot D_o\cdot t} \tag{1}$$

where t is the time of hybridization, D_o and D_t are the molar concentrations of single-stranded DNA at the onset of hybridization and at time t, respectively, and k is the molar reassociation rate constant (Britten, 1969). Equation 1 has been used in the linear form (Wetmur and Davidson, 1968)

$$\frac{D_o}{D_t} = 1+k\cdot D_o\cdot t \qquad (2)$$

For plaque-purified SV40 DNA, of complexity 3.6×10^6 daltons (Tai et al., 1972)

$$D_o = \frac{C_o}{3.6 \times 10^6} \qquad (3)$$

where C_o is the DNA concentration in grams per liter and hence,

$$\frac{D_o}{D_t} = 1 + \frac{k}{3.6 \times 10^6} \cdot C_o \cdot t \qquad (4)$$

Thus the plot of D_o/D_t as a function of $C_o t$ should be linear with an intercept of 1 and a slope of $\frac{k}{3.6 \times 10^6}$. In the case of DNA consisting of several families of sequences differing in their molar concentrations, the curve will deviate from linearity. In this case, the kinetic analysis of hybridization may be ambiguous since similar reassociation curves can be generated by assigning varying combinations of complexities and molarities to the individual families of sequences. In the method described below for estimating the fraction of repetitive sequences, we have assumed that the observed non-linear reassociation curves of serially passaged SV40 DNA (see Results) arise from the presence of only two families of sequences which differ widely in their molar abundance (repetitive and unique).

Let α_1 and α_2 be the fractions of the repetitive and unique families of DNA sequences, respectively; and let D_{01} and D_{02} be the respective molar concentrations. The fraction of total DNA, which remains single-stranded at time t, equals the weighted sum of the single-stranded fractions of the two DNA species. Assuming that the two types of sequences hybridize with the same rate constant k, then:

$$\frac{D_t}{D_o} = \frac{\alpha_1}{1+k\cdot D_{01}\cdot t} + \frac{\alpha_2}{1+k\cdot D_{02}\cdot t} \qquad (5)$$

If the degree of sequence reiteration is high, then after a certain time of incubation, all the reiterated sequences will have reassociated whereas the hybridization of the more unique sequences will be incomplete. Hence, the contribution of the reiterated sequences to the single-stranded DNA fraction is negligible, and

$$\frac{D_t}{D_o} = \frac{\alpha_2}{1+k\cdot D_{02}\cdot t} \qquad (6)$$

However, $$D_{02} = \frac{C_o \cdot \alpha_2}{M_2} \qquad (7)$$

where C_o is the total DNA concentration in grams per liter and M_2 (grams per mole DNA) is the complexity (Britten, 1969) of the unique sequences. The complexity of the unique sequences was assumed to be that of plaque-purified DNA (3.6×10^6 daltons) since, as shown in Figures 2A and 2C, the reassociation pattern of serially passaged DNA is similar to that of plaque-purified DNA at higher C_ot values. Substituting in Equation 6, above,

$$\frac{D_o}{D_t} = \frac{1}{\alpha_2} + \frac{k}{3.6 \times 10^6} \cdot C_o \cdot t \qquad (8)$$

Therefore, at later stages of the hybridization reaction, the plot of D_o/D_t will become linear with a slope of $\frac{k}{3.6 \times 10^6}$. The value for $\frac{1}{\alpha_2}$ was obtained from the extrapolated intercept of the linear part of the reassociation plot at higher C_ot values. The fraction of repetitive (α_1) was obtained from $(1-\alpha_2)$.

RESULTS

Presence of Fast-Reassociating Sequences in Serially Passaged SV40 DNA

We have studied the reassociation kinetics of DNAs from several serially passaged SV40 populations. The C_ot curves (Britten and Kohne, 1968) for two such DNA preparations as well as that for plaque-purified SV40 DNA are shown in the left panels of Figure 2. The plots of D_o/D_t as a function of C_ot for the initial part of the annealing reactions are shown in the right panels of Figure 2. It will be noted that the plot for plaque-purified SV40 DNA is linear, indicating that the bulk of the DNA sequences reassociate at a single rate and therefore appear to be present in uniform molarity. In contrast, the reassociation of the serially passaged DNAs proceeds initially at a fast rate, then at a rate similar to that of plaque-purified DNA, and finally at a rate slightly slower than that of the plaque-purified DNA. Assuming that the differences in the reassociation rates are due to differences in the molar concentrations of sequences, and not to different reassociation rate constants, we conclude that some of the sequences in serially passaged DNA are present at higher molarities than in plaque-purified DNA, and are therefore present more than once per DNA molecule.

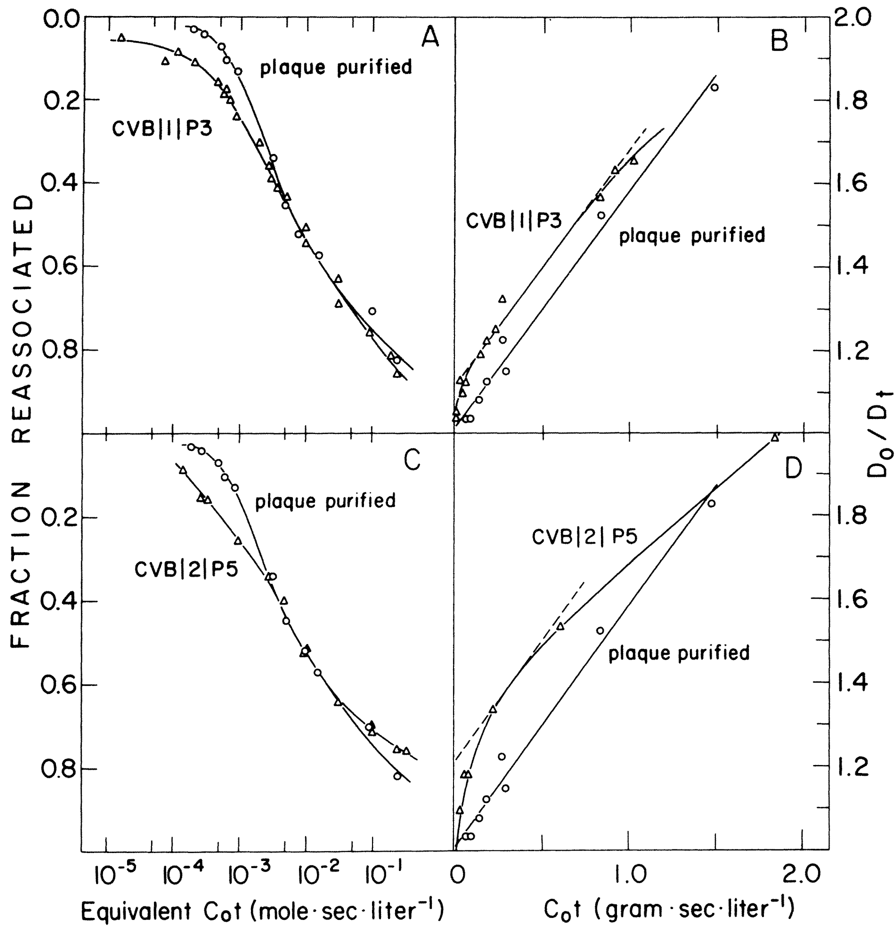


FIG. 2. Fast-reassociation sequences in serially passaged SV40 DNAs. Plaque-purified SV40 DNA (CVB) and the two serially passaged SV40 DNAs (CVB/1/P3) and CVB/2/P5) were self-annealed. In Panels A and C, the fraction reassociated is plotted as a function of C_0t. The data originally obtained at various salt concentrations (from 0.05 M to 0.4 M monovalent cation) were standardized to 0.18 M monovalent cation concentration (Frenkel et al., 1974). In Panels B and D, the initial phase of the reaction is plotted as D_0/D_t versus C_0t adjusted for 0.18 M monovalent cation. The plot for plaque-purified DNA is a linear regression line. The broken lines show the extrapolated intercepts (see text).

The Fraction of Fast-Annealing Sequences in Serially Passaged SV40 DNA

The fraction of repetitive DNA sequences in serially passaged SV40 DNA was estimated from the extrapolated intercepts in Figures 2B and 2D. As outlined in Methods, Equation 8 is derived for the case where the DNA contains two families of sequences which differ significantly in their molarities (repetitive and unique). On this basis, the reassociation of serially passaged SV40 DNA is expected to proceed initially at a fast rate and then at a slower linear rate with a slope identical to that of plaque-purified DNA (that is $\frac{k}{3.6 \times 10^6}$. Apparently the situation is more complex since at the higher C_ot values, the reassociation rates for serially passaged DNAs are slower than those of plaque-purified DNA (Figure 2), indicating that some sequences are present less than once per molecule. To minimize the errors resulting from multiphasic reassociation plots, we have drawn a line which is tangential to the reassociation curve of serially passaged DNA and which is parallel to the reassociation plot for plaque-purified DNA (broken lines in Panels B and D). It is this tangent which is extrapolated to t=0 to obtain an approximation of the size of the repetitive fraction. The data are summarized in Table 1, where it will be noted that the fraction of fast-reassociating sequences was found to be 12% for CVB/1/P3 DNA, 17% for CV1/1/P5 DNA and 18% for CVB/2/P5 DNA. In addition, Table 1 shows that no quantitative correlation was observed between the fraction of fast-annealing sequences and the fraction of host sequences.

Enrichment of the Fraction of Fast-Annealing Sequences

Since the fast-reassociating fraction did not exceed 20% of the total DNA (Table 1), we next attempted to obtain a DNA fraction which was enriched for the fast hybridizing sequences. Passage 1 DNA (identical to plaque-purified DNA in its reassociation pattern) and passage 5 DNA were self-annealed to a point where approximately 20% of the DNA sequences hybridized in each case. At that point, the hybridization mixture was digested with S1 enzyme. The nuclease resistant fraction (henceforth called "initially hybridized fraction") was phenol extracted, dialyzed extensively, heat denatured and reassociated again. The resulting kinetics of reassociation are shown in Figure 3. The reassociation plot for passage 1 "initially hybridized DNA" is linear (for at least 70% of the DNA) with an intercept of 1.02, indicating that the bulk of the sequences are present in uniform molarity. In contrast, passage 5 "initially hybridized DNA" does not reassociate homogeneously. The extrapolated intercept of the second (linear) part of the curve is 1.63, indicating that an estimated 39% of the sequences are fast-annealing (com-

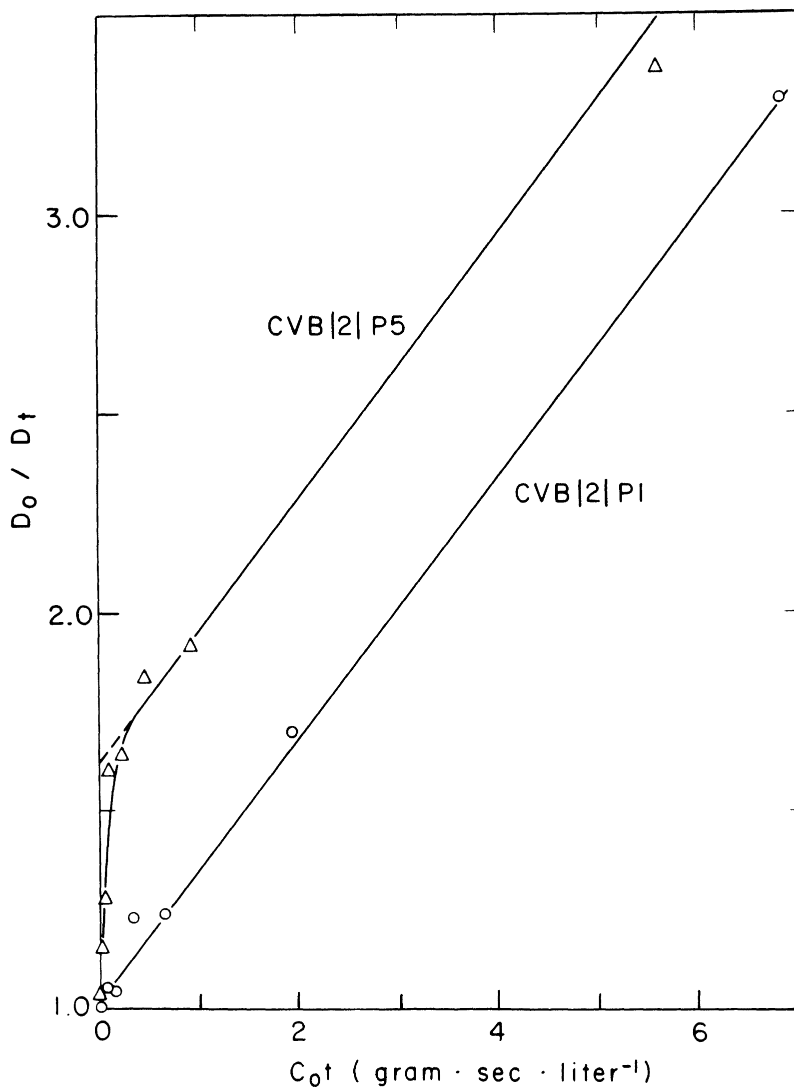


FIG. 3. Reassociation of the "initially hybridized fraction." DNAs from passage 1 (circles) and passage 5 (triangles) of the CVB/2 line were self-annealed until approximately 20% of the sequences reassociated. The hybridized fraction was isolated by S1 nuclease treatment, denatured and rehybridized. The C_0t values are adjusted to 0.18 M salt concentration.

TABLE 1. The Size of the Fast Reassociating Fractions in Serially Passaged SV40 DNA Preparations

SV40 DNA from Cells Infected with:		Fraction of Host Sequences[1]	Extrapolated Intercept[2]	Fast Reassociating Fraction[2]
CVB/1	Plaque purified	0	1.02	0.02
	Passage 3	0.44	1.13	0.12
CVB/2	Passage 1	NT[3]	1.00	0.00
	Passage 5	0.21	1.22	0.18
CV1/1	Plaque purified	0	1.03	0.03
	Passage 5	0.17	1.20	0.17

[1]Estimated by DNA-DNA annealing experiments in which the labeled serially passaged DNAs were incubated with a large excess of unlabeled plaque-purified SV40 DNA (data from Frenkel et al., 1974).

[2]See text for explanation of the extrapolated intercept and the calculation for the size of the fast reassociating fraction. The data are taken from Figure 2 for the plaque-purified, CVB/1/P3 and CVB/2/P5 DNA populations and from similar reassociation plots for CVB/2/P1 and CV1/1/P5 DNAs (not shown in Figure 2).

[3]Not tested.

pared to 18% in unfractionated CVB/2/P5 DNA). This substantial enrichment for the fast-reassociating sequences excludes the possibility that the multiphase reassociation pattern of the unfractionated passage 5 DNA is due to diminished reassociation rates resulting from breakdown or damage to the DNA; since, if this were the case, the fraction of fast-annealing sequences should have decreased rather than increased in the second round of reassociation.

Origin of the Fast-Annealing Sequences

To determine whether the repetitive sequences in serially passaged DNA are of host or viral origin, we hybridized the fraction enriched for repetitive sequences (the "initially hybridized DNA") in the presence of excess, sheared, unlabeled plaque-purified DNA or unfractionated serially passaged DNA. The data from these reactions are given in Table 2. It will be noted that addition of excess plaque-purified DNA accelerates the reassociation of the "initially hybridized fraction" to the same extent as the addition of the same

TABLE 2. Viral Origin of the Fast-Annealing Sequences[1]

Experiment	Unlabeled DNA	Cot Labeled DNA	Cot Unlabeled DNA	Percent Hybridized
1	None	4.2×10^{-5}	0	25.0
	Plaque-purified	4.2×10^{-5}	9.4×10^{-2}	72.1
	Serially passaged	4.2×10^{-5}	9.4×10^{-2}	71.5
2	None	6.0×10^{-4}	0	35.2
	Plaque-purified	6.0×10^{-4}	1.3	77.5
	Serially passaged	6.0×10^{-4}	1.3	79.7

[1]The "initially hybridized fraction" of ^{3}H-labeled, serially passaged SV40 DNA (CVB/2/P5) was isolated as described in Figure 3 and incubated in the presence of excess unlabeled plaque-purified DNA, or CVB/2/P5 DNA, or no DNA.

excess of serially passaged DNA. Thus, the bulk of the fast annealing sequences is present in plaque-purified DNA, and must therefore be of viral origin.

Comparison of the Fast-Annealing Sequences in Two Independently Derived, Serially Passaged DNA Populations

We next investigated the question if the fast-annealing sequences are the same in different serially passaged populations or whether in each case different viral sequences are reiterated. To answer this question, we hybridized the labeled "initially hybridized fraction," obtained from CVB/2/P5 DNA, in the presence of excess unfractionated unlabeled CVB/2/P5 DNA or CVB/1/P3 DNA. The concentrations of labeled DNA and the time of incubation were kept constant while the concentrations of unlabeled DNAs were varied. The data for these reactions are plotted in Figure 4. The reassociation of the CVB/2/P5 "initially hybridized fraction" in the presence of excess unfractionated CVB/2/P5 DNA is biphasic, as expected. Similarly, a biphasic reassociation pattern was obtained in the presence of increasing concentrations of CVB/1/P3 DNA. Therefore, sequences present in the "initially hybridized fraction" of CVB/2/P5 DNA are also present in CVB/1/P3 DNA in non-uniform molarity. We conclude from this result that a significant fraction of the reiterated sequences in CVB/2/P5 DNA are also reiterated in CVB/1/P3 DNA.

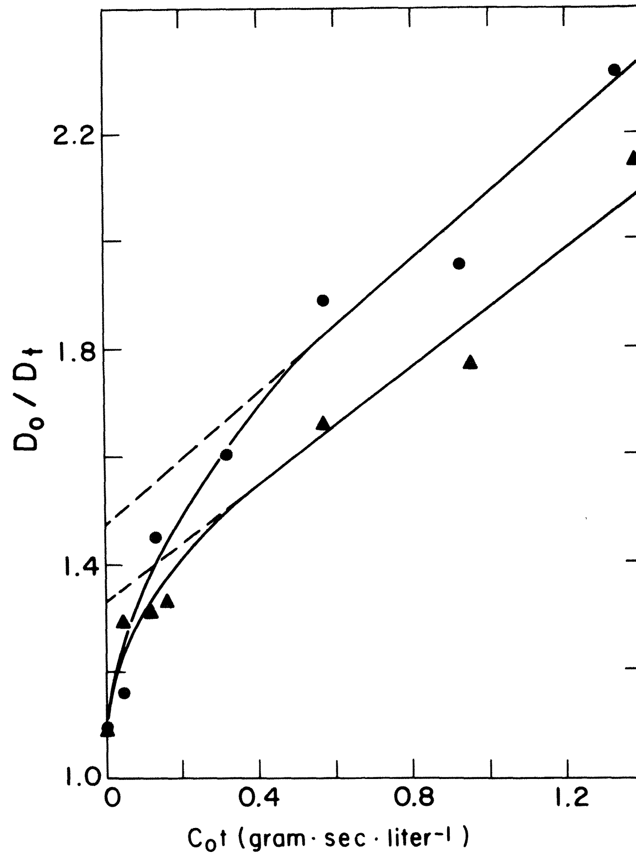


FIG. 4. Similarity of the fast-annealing sequences present in two independently derived populations of serially passaged SV40 DNAs. 0.008 μg/ml of the "initially hybridized fraction" derived from CVB/2/P5 DNA (circles) or unfractionated CVB/1/P3 DNA (triangles). The broken lines show the extrapolated intercepts.

Identification of the Fast-Annealing Sequences

The following series of experiments was designed to determine which part of the plaque-purified SV40 genome becomes reiterated during the serial passaging procedure. Labeled plaque-purified DNA was digested with the *Hin* restriction enzyme, and the cleavage products were fractionated by gel electrophoresis. The pattern shown in Figure 5 is similar to that reported by Danna and Nathans (1971), except that under the conditions used, *Hin*-fragments C and D were not resolved. Each of the labeled *Hin*-fragments was eluted from the gel and incubated at a constant concentration and for constant time in the presence of increasing concentrations of unlabeled sonicated plaque-purified viral DNA or unlabeled sonicated serially passaged DNAs (Figure 6). In all cases, the reassociation of labeled *Hin*-fragments in the absence of added unlabeled DNA did not exceed 4%. Under these conditions, the rate of reassociation of the labeled sequences is dependent only on the molarity of the corresponding sequences in the unlabeled DNA preparations.

From the data in Figures 6 and 7, it will be seen that the reassociation of *Hin*-fragment C + D sequences (in contrast to all the other *Hin*-fragments) is *initially* faster in the presence of the two serially passaged DNAs compared to plaque-purified DNA. As estimated by the extrapolated intercepts (see legend to Figure 6), 8% and 12% of *Hin*-fragment C + D sequences hybridize faster in the presence of CVB/2/P5 DNA and CV1/1/P5 DNA, respectively. Taking the sum total of C + D sequence complexity as 20% that of complete SV40 DNA (Danna, Sack, and Nathans, 1973), we estimate the size of the repetitive units in CVB/2/P5 DNA and CV1/1/P5 DNA to be 1.6% and 2.4%, respectively, the complexity of plaque-purified DNA. We conclude, therefore, that a sequence of either *Hin*-fragment C or *Hin*-fragment D, (6-9) x 10^4 daltons in molecular weight, becomes reiterated in the two serially passaged SV40 DNAs we have examined.

If *plaque-purified* SV40 DNA contains a unique set of sequences without duplications, we would ideally expect identical reassociation rates for all the labeled *Hin*-fragments in the presence of unlabeled excess plaque-purified DNA. The calculated slopes of the reassociation plots (Figure 6) for each of the *Hin*-fragments are tabulated in the first column of Table 3. Inspection of these values shows an unexpected degree of non-uniformity. For example, fragment J hybridizes more than twofold faster than fragment H. The variability in the observed rates could stem from three sources: (1) hybridization measurement errors, (2) different hybridization rate constants due to varying base composition of the different *Hin*-fragments, and (3) sequence duplication in plaque-purified SV40 DNA. These possibilities will be discussed later. The slopes of the reassociation plots for labeled *Hin*-fragments in the presence of the two serially passaged DNAs (Table 3, columns 2 and 3) reveal

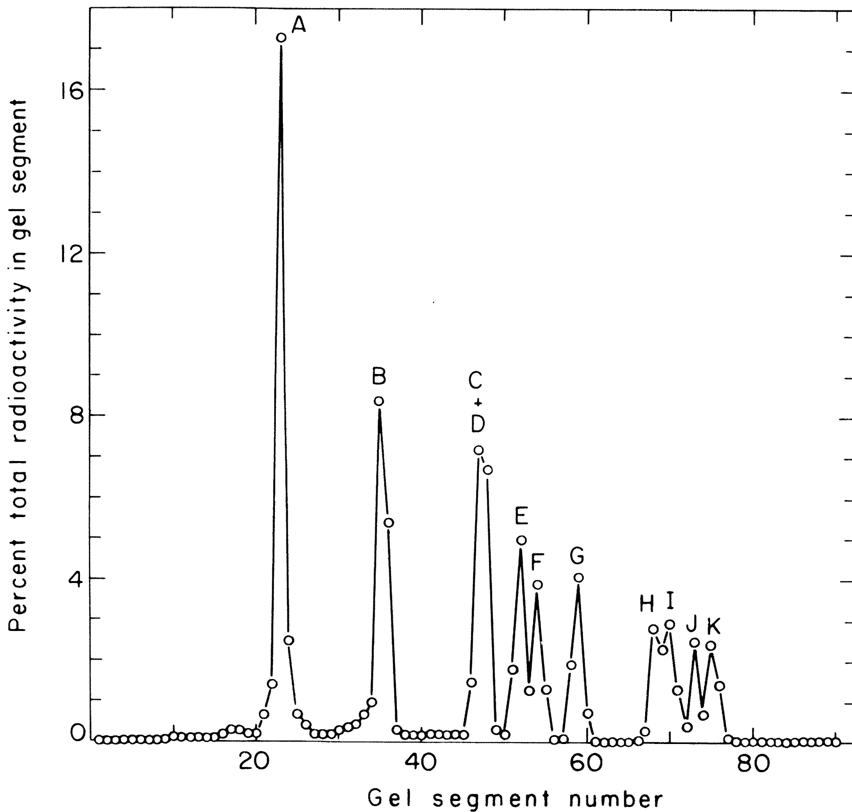
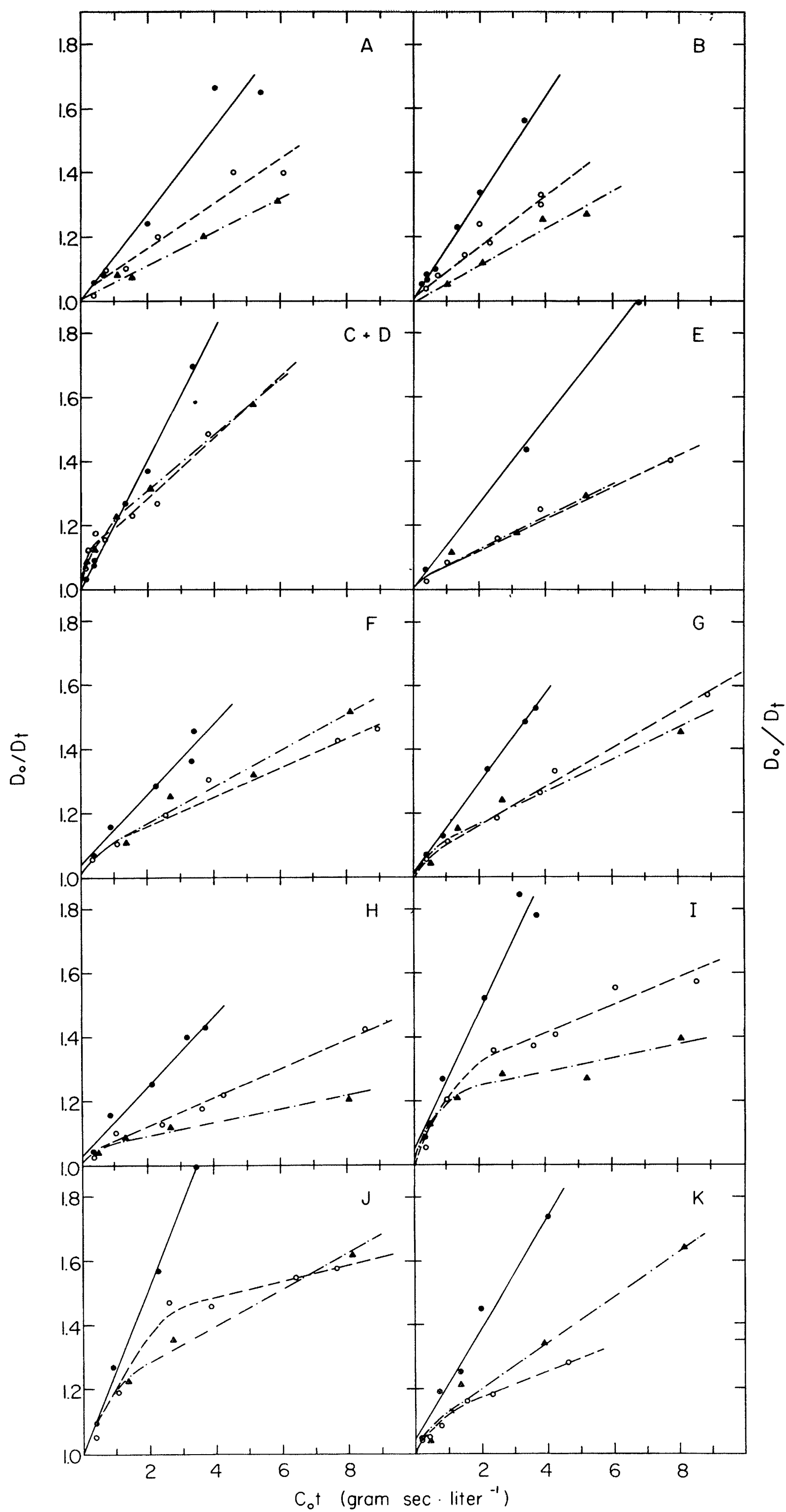


FIG. 5. Polyacrylamide gel electrophoresis of Hin-fragments from plaque-purified SV40 DNA. ^{3}H-labeled SV40 CVB DNA was digested with the Hin-restriction endonuclease and the resulting products fractionated on polyacrylamide gels as described in Methods. The different classes of fragments were eluted from the gel (Rozenblatt et al., 1972) and hybridized as described in the legend to Figure 6.

FIG. 6. Identification of the fast-reassociating viral sequences. 0.1 μg/ml of each of the ^{3}H-labeled Hin-fragments derived from plaque-purified SV40 DNA (see Figure 5) were reassociated for 0.3 hours in 0.032 M tris-HCl, 0.045 M NaCl (pH 8.05), in the presence of increasing concentrations of sonicated unlabeled plaque-purified DNA (●——●), CVB/2/P5 DNA (o---o), or CV1/1/P5 DNA (▲-·-▲). The D_o/D_t data are plotted against the C_ot values of the unlabeled DNAs. The plots were fitted to the data points by linear regression analysis. In the case of Hin-fragments C+D, I, J, and K (where the reassociation pattern is strongly biphasic) only the data points of the second phase of the hybridization reaction were used in the regression analysis.



A
B
C + D
E
F
G
H
I
J
K
1.0
1.2
1.4
1.6
1.8
Do/Dt
2
4
6
8
Cot (gram sec · liter ⁻¹)

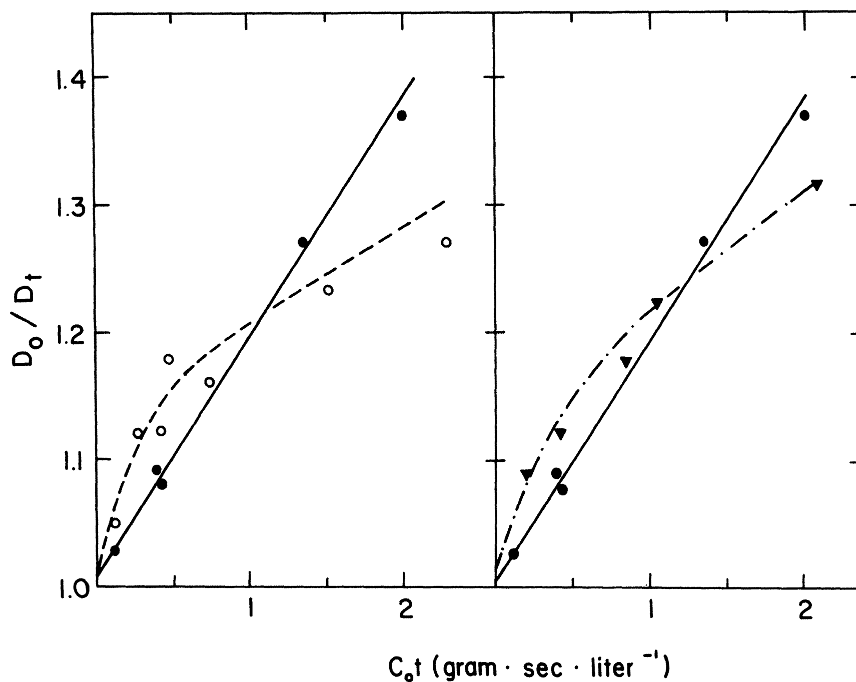


FIG. 7. Initial phase of the hybridization reaction between Hin- fragments C+D and plaque-purified or serially passaged DNA. The data are taken from Figure 6, C+D panel, and are plotted on an expanded scale. Plaque-purified DNA, ●——●; serially passaged CVB/2/P5 DNA, o---o; serially passaged CV1/1/P5 DNA, ▲-·-▲.

TABLE 3. Reassociation of Hin-Fragments in the Presence of Plaque-Purified or Serially Passaged DNA[1]

	Slope of Reassociation Plot in Presence of:		
Hin-fragment	Plaque-Purified DNA	Serially Passaged CV1/1/P5 DNA	Serially Passaged CVB/2/P5 DNA
A	.133	.051 (38)	.069 (52)
B	.159	.057 (36)	.078 (49)
C+D	.203	.086 (42)	.101 (50)
E	.132	.051 (39)	.051 (39)
F	.111	.056 (50)	.047 (42)
G	.140	.050 (36)	.060 (43)
H	.111	.021 (19)	.045 (40)
I	.220	.021 (9)	.041 (19)
J	.260	.056 (21)	.024 (9)
K	.174	.072 (41)	.040 (23)

[1]The values in the body of the table are the slopes of the reassociation plots shown in Figure 6, calculated by linear regression analysis. In the cases of fragments C+D, I, J, and K, where the reassociation pattern is strongly biphasic, only points in the second phase of the plot were used in the regression analysis. The figures in brackets denote the percentage ratio:

$$\frac{\text{slope in presence of serially passaged DNA}}{\text{slope in presence of plaque-purified DNA}}$$

the following features: (1) the sequences of all the Hin-fragments are present at a lower molarity in the two serially passaged DNAs, and (2) the sequences of Hin-fragments I, J, and H are present at markedly reduced molarities in CV1/1/P5 DNA (and, similarly, the sequences of Hin-fragments I and J are present at very much reduced molarities in CVB/2/P5 DNA) suggesting preferential deletion of these sequences in the serially passaged DNAs.

DISCUSSION

1. The Repetitive Sequences in Serially Passaged SV40 DNA

Previous communications have reported the presence of repetitive viral sequences in the supercoiled DNAs of serially passaged SV40 (Brockman et al., 1973; Martin et al., 1973; Winocour et al., in press), SV40-like human papova virus (Fareed, Byrne, and Martin, 1974) and polyoma virus (Falk and Wang, 1974). In the present study, we have characterized the reiterated sequences of serially passaged

SV40 DNA and determined their origin with respect to the Hin-cleavage map of the plaque-purified SV40 genome.

Of the ten classes of Hin-fragments derived from plaque-purified SV40 DNA, only sequences contained in the C + D class of fragments reassociate at a rate which is initially faster in the presence of serially passaged DNA than in the presence of plaque-purified DNA. Hence, some sequences of the C + D fragment are present at higher molarities in two independently derived passaged SV40 DNAs (CVB/2/P5 and CV1/1/P5) than in plaque-purified SV40 DNA. The third serially passaged line examined, CVB/1/P3, contains reiterated sequences similar to those in CVB/2/P5 DNA (Figure 4). We therefore conclude that in three independently derived lines of passaged SV40, essentially the same segment of the viral genome underwent amplification. It should be pointed out, however, that since the passaged viral populations studied were not cloned, the hybridization data relate only to the most abundant species of reiterated sequences. We have estimated the size of the repeat units in two serially passaged SV40 DNAs to be 1.6% and 2.4% the complexity of plaque-purified viral DNA. The fractions of total DNA sequences which are reiterated in these populations were estimated as 17% and 18% (Table 1). Thus, on the average, the repetitive unit (6-9 x 10^4 daltons) is repeated 8-11 times per molecule.

We have previously observed that serial passage of SV40 generates populations which become progressively enriched for a given type of defective viral genome (Frenkel et al., 1974), and we proposed that the high-multiplicity passaging procedure provides conditions for the selection of altered SV40 genomes which replicate faster than the helper virus DNA molecules (Frenkel et al., 1974; Lavi et al., 1972). The Hin-C fragment has been shown to contain the origin of viral DNA replication (Danna and Nathans, 1972; Fareed et al., 1972). The identification of the reiterated viral sequences as corresponding to some of those in the C + D Hin-fragments thus suggests a possible explanation for the selective replication advantage of passaged SV40 DNA. The reiteration of polymerase binding sites, for example, may greatly enhance the probability that a given viral DNA molecule, in a given time, will enter into replication. If such altered viral DNA molecules integrate into the host genome, then the addition of amplified DNA replication signals may also profoundly affect the control of cellular DNA synthesis.

2. Are Some Plaque-Purified Viral DNA Sequences Duplicated?

Assuming that plaque-purified SV40 DNA contains only a unique set of sequences, we would ideally expect identical reassociation rates in the hybridization reactions between each of the Hin-

fragments and excess plaque-purified DNA. Unexpectedly, we observed substantial variations in the reassociation rates (Table 3, column 1). These variations are unlikely to be due to measurement errors since each of the slope values listed in Table 3 is derived from multiple data points. Furthermore, the variations in hybridization rates cannot be correlated with differences in the GC-content of the Hin-fragments. Thus, fragments I and F have the same GC-content of 37% (Danna and Nathans, 1972); yet, in the presence of plaque-purified DNA, fragment I hybridizes twice as fast as fragment F. A possible explanation for the non-uniform reassociation rates of Hin-fragments in the presence of complete SV40 DNA is that certain sequences are duplicated in the plaque-purified viral genome. The data in the first column of Table 3 suggest that sequences in the I, J, and C + D Hin-fragments may indeed be duplicated. If such duplications occur in different parts of the genome (for example, if sequences in Hin-fragments I and J were repeated in Hin-fragment C or D),then, conceivably, such regions could provide preferred sites for intramolecular and intermolecular recombination events leading to genome rearrangements. It is perhaps not entirely fortuitous that Hin-I and Hin-J segments are preferentially lost in two serially passaged SV40 DNAs (Table 3, columns 2 and 3). Recently, it has been suggested that duplication of sequences at two separate loci in the genome of an SV40-like human virus may be involved in recombination events leading to sequence rearrangements (Khoury *et al.*, 1974).

ACKNOWLEDGEMENTS

We would like to thank Mr. B. Danovitz for excellent technical assistance and Dr. Sara Lavi for stimulating and helpful discussion. This work was supported by Public Health Service Research Grant CA-14530 from the National Cancer Institute.

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SOME PROPERTIES OF SUPERHELICAL OLIGOMERIC AND INTEGRATED SV40 DNA SEQUENCES

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This paper deals with some properties of SV40 DNA sequences which are covalently linked with host cell DNA. First oligomeric SV40 DNA with integrated host DNA sequences will be discussed, and we shall then describe linear duplex DNA consisting of SV40-host DNA sequences in productively infected cells.

We find that oligomeric superhelical SV40 DNA obtained during a regular type of low multiplicity infection of CV-1 cells with SV40 contains, in part, host DNA sequences covalently linked to the viral DNA. Figure 1 shows the purification and identification of the oligomeric SV40 DNA. First the dense band is isolated from the cesium chloride ethidium bromide gradients and sedimented through preformed cesium chloride alkaline gradients shown in the upper panel. The fast sedimenting section indicated by bars is isolated and then resedimented through an alkaline gradient, which gives high resolution and reveals the dimeric, trimeric and tetrameric molecules as well as some contaminating monomeric and some relaxed oligomeric DNA. These oligomers were immobilized, after denaturation, on filters and hybridized with either monomeric SV40 superhelical DNA or with host DNA in DNA-DNA hybridization reactions. The result is shown in Table 1. Almost 95% of monomeric SV40 DNA component I was capable of self-hybridizing. In contrast, only 80% of SV40 oligomers showed base sequence homology with monomeric SV40 superhelical DNA, while hybridization of oligomers to host DNA immobilized on filters reveals almost no base sequence homology. Thus, there is a large discrepancy in base sequence homology between oligomers and monomeric SV40 DNA which cannot be explained by the

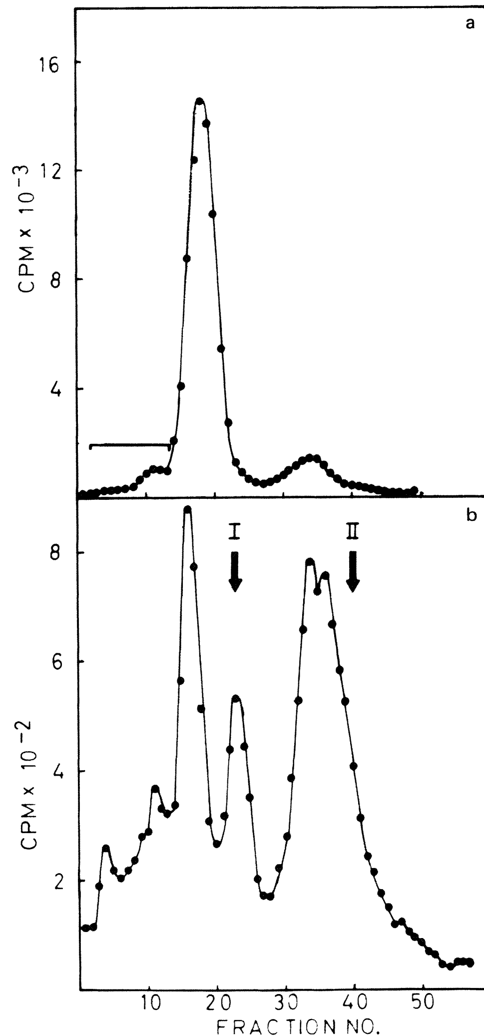

FIG. 1. The purification of oligomeric SV40 DNA.

a) Alkaline velocity sedimentation through a preformed CsCl-gradient (buffer: 0.02 M Tris; 0.002 M EDTA; pH 12.7; initial density 1.57 g/ml; SW 50.1, 38,000 RPM, 20°C, 16 h). The DNA sample obtained from the dense band of a CsCl-EtBr equilibrium centrifugation was denatured with 1/10 vol. of 1 N NaOH for 5 min at 20°C and layered on top of the preformed gradient and centrifuged. SW 50.1, 40,000 RPM, 20°C, 150 min.

b) Alkaline velocity sedimentation through preformed CsCl-gradient (for details, see legend to Figure 1a). The fractions indicated by bars (Figure 1a) were neutralized and dialysed against 0.1 x SSC. A desalted DNA sample was denatured as stated above and recentrifuged. SW 50.1, 40,000 UPM, 20°C, 2 h. The arrows indicate the positions of marker SV40 DNA component I and II.

TABLE 1. DNA-DNA hybridization of SV40 DNA I and of oligomeric SV40 DNA

DNA in solution	DNA on filter	% hybridized
SV40 I	SV40 I	94.4
SV40-oligom.	SV40 I	80.4
SV40-oligom.	cell	0.8

presence of host DNA sequences in oligomeric SV40 DNA molecules that are capable of hybridizing under these conditions. Since only reiterated or repetitive host DNA sequences can hybridize under the conditions employed in the DNA-DNA filter hybridization technique, this result shows that repetitive host DNA cannot be integrated in oligomeric DNA molecules. This conclusion is also supported by a study of the reassociation kinetics of oligomers with and without the addition of host DNA to the reaction mixture (Figure 2). It may be seen that addition of host DNA, in this case in approximately 25-fold excess, to the oligomeric DNA does not enhance its reassociation. The formation of DNA duplexes proceeds at the same rate, whether or not host DNA is added. These data and the data shown in the previous figure lead us to the conclusion that oligomeric SV40 DNA must contain approximately 15% of unique host DNA sequences which are incapable of hybridizing either with monomeric SV40 DNA or with repetitive host DNA.

The significance of this phenomenon is presently not understood. These molecules may, however, be useful in transducing unique host DNA sections into suitable recipient cells, where one could study if particular new functions are being expressed.

Covalent linkage between host DNA and viral DNA sequences is also observed in the case of integrated SV40 DNA during the course of a productive cycle of infection. Integration of SV40 DNA sequences into the host DNA during productive infection is a regularly occurring event which is independent of the multiplicities of infection. It has been observed by several groups which have used different methods for the detection of such molecules in the case of SV40 and Polyoma (Hirai and Defendi, 1972; Ralph and Colter, 1972; Babiuk and Hudson, 1972; Waldeck, Kammer, and Sauer, 1973). The most striking feature is that substantial amounts of viral DNA sequences occur in the form of covalently linked viral-host DNA sequences. Babiuk and Hudson (1972) have reported recently that as much as 50% of the total macromolecular DNA isolated from cells infected with Polyoma consist of integrated Polyoma DNA sequences.

SV40 DNA sequences are particularly enriched in small DNA

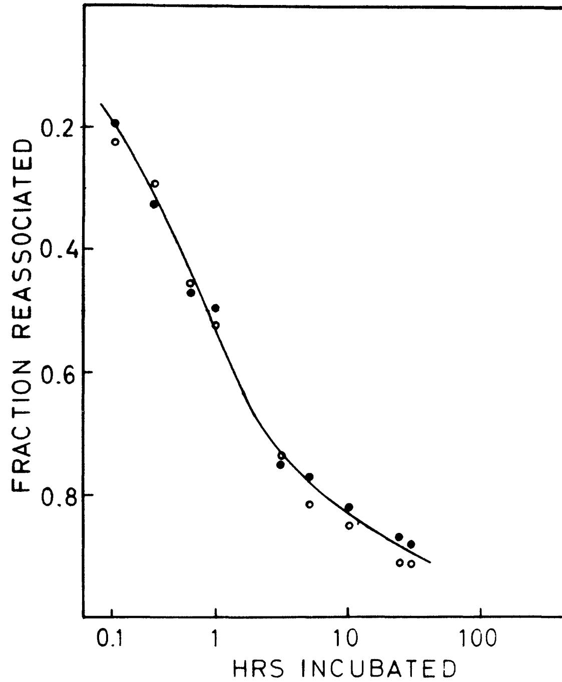

FIG. 2. Kinetics of reassociation of oligomeric SV40 DNA in the presence (o) or absence (•) of highly reiterated, unlabeled CV-1 DNA. The purification of the DNA and the conditions of reassociation are described elsewhere (Waldeck et al., 1973). Oligomeric DNA was derived from Figure 1a indicated by bars. CV-1 DNA was incubated at a C_0t of 100. Then the DNA mixture was fractionated by elution from hydroxyapatite. After washing with 0.12 M PO_4 buffer, the double-stranded DNA was eluted with 0.4 M PO_4 buffer and the fraction was dialysed against 0.1 x SSC. The fragmented and denatured DNA was reannealed in 0.4 M PO_4 buffer (pH 6.8) at 60°C, and the amount of single- and double-stranded DNA at each point was determined by elution from hydroxyapatite. Concentration of DNA used in the reaction: oligomeric DNA, 0.4 µg/ml; highly reiterated CV-1 DNA, 10 µg/ml. Each point represents a total of 200 cpm. At zero time about 3-5% of the input radioactivity eluted as double-stranded DNA.

duplex molecules which are contained in the Hirt supernatant after selective extraction of the DNA at the end of the productive cycle (Waldeck et al., 1973). Integration also takes place in the macromolecular host DNA but to a much lesser extent. The strategy of the experiments for isolation is as follows: Cells were infected at low multiplicities and labeled throughout the period of infection for two days and then the DNA in the Hirt supernatant after phenol extraction was centrifuged into a dye-buoyant density gradient. The peak at the light density which contains the relaxed and linear duplex DNA molecules was isolated, the dye was removed and the DNA was then subjected to a selective hybridization using either immobilized SV40 or host DNA on filter for selection. SV40 DNA was immobilized on the filters and hybridized with the DNA from the light band which comprises a variety of molecules, either of pure viral or pure host origin, or viral-host sequences covalently linked to each other. The DNA which contains viral base sequences is selectively bound to the viral DNA on the filters. After hybridization, the filters were washed and the non-specifically bound DNA was effectively removed, as shown by various controls. We have established that less than 0.1% of heterologous DNA remains non-specifically bound to the filters after washing. The filters were then eluted and the eluted DNA was subjected to ultrasonic treatment. Then it was rehybridized to either purified viral or to host DNA in order to demonstrate the covalent linkage of heterologous DNA sequences to viral DNA sequences. These experiments permit isolation of large amounts of viral-host DNA sequences that are covalently bound. Usually between 0.5 and 3% of the input DNA can be eluted from the filters. Figure 3 shows the size class distribution of these particular viral-host DNA sequences. The DNA contained in the light band of the CsCl-EtBr gradient when subjected to alkaline velocity sedimentation shows a rather heterogeneous sedimentation profile. The peak being about 15 S is, therefore, somewhat smaller than SV40 DNA component II. This particular DNA was hybridized as shown in the previous slide to SV40 DNA, the filters were washed, and the specifically bound DNA was eluted. The sedimentation profile of the eluate is shown in the same graph. The specifically isolated DNA molecules which contain viral DNA sequences seem to reflect the proportion of the size classes which were assayed in the DNA hybridization experiment. However, large DNA molecules which are larger than approximately 22 S are not present in the eluate. This could be either because there are no such large viral DNA sequences or because such molecules are lost during the washing procedure since they might be detached from the filters. A test revealed that the latter possibility is true in case of both host and viral DNA sequences as shown by sonication of the large, fast sedimenting DNA molecules and hybridization of the small fragments. Since the fragmented DNA was able to hybridize, it is clear that large DNA molecules cannot be recovered in the eluate because they are lost during washing.

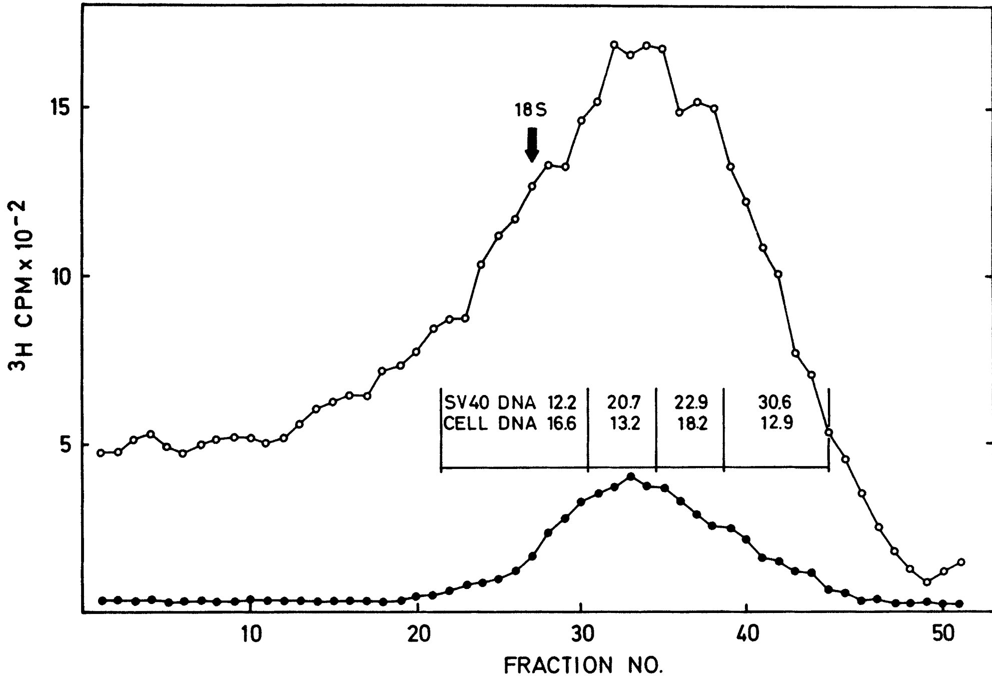

FIG. 3. Alkaline velocity sedimentation of DNA selectively isolated from infected CV-1 cells before (o—o) and after hybridization to and elution from SV40 DNA (●—●). The supernatant fraction from selectively isolated DNA was centrifuged into equilibrium in a CsCl-EtBr gradient. The light band was isolated and part of the material was sedimented for 240 min. at 44,000 rpm through a preformed CsCl alkaline gradient (initial density 1.49 g/cm^3, pH 12.6, 18 hr. 36,000 rpm, Spinco rotor SV 50.1). Another part of the DNA was hybridized to SV40 DNA on filter and eluted from the hybrid complex. The size of the eluted DNA (●—●) was determined after sedimentation through a preformed alkaline CsCl-gradient. The eluted DNA was divided into 4 pools and hybridized to CV-1 and SV40 DNA on filters. The arrow indicates the position of a ^{14}C SV40 DNA component II marker.

The distribution of heterologous DNA sequences, in this particular case host DNA, was determined by fractionating eluted DNA into 4 size classes and rehybridization after ultrasonic treatment of the indicated 4 pools to either viral or host DNA. Of particular interest is the distribution of the heterologous host DNA which was obtained together with the eluted viral DNA sequences. It may be seen that regardless of the size of the eluate the host DNA sequences are evenly distributed over the entire range covered by the specifically isolated viral host DNA molecules. We can conclude, therefore, that there are rather large as well as small DNA molecules which contain viral and host DNA covalently linked to each other.

It is unknown if SV40 DNA integrates as an intact open molecule or if it integrates in the form of subgenomic fragments. In case SV40 DNA was integrated in these molecules as an intact unit it should be possible, by fragmentation of these molecules by ultrasonic treatment and subsequent hybridization to SV40 DNA followed by elution, to eliminate host DNA sequences. In contrast, if viral DNA sequences were integrated in the form of small fragments with host DNA sequences adjacent on either side, fragmentation of these molecules followed by hybridization to SV40 DNA should not enable us to remove efficiently the heterologous host DNA sequences from the eluates. Therefore, we have sheared the DNA by ultrasonic treatment to various extents and we have determined the size of the ultrasonically treated DNA prior to hybridization. After hybridization and elution the actual size of the specifically hybridized DNA was determined again in alkaline velocity sedimentation. Also the homology distribution of viral or host DNA sequences in these molecules was determined in DNA-DNA hybridization experiments. The data are summarized in Figure 4. The upper panel contains DNA which was not sheared and which was obtained from the Hirt supernatant. Again the eluates regardless of whether they were obtained from hybridization to SV40 or from hybridization to host DNA reflect approximately the size classes of DNA which was assayed in the hybridization mixture, except that very large molecules are not contained in the eluates. Furthermore, regardless of whether SV40 DNA or host DNA was immobilized on the filters the eluates contain approximately the same size classes of DNA molecules. In essence, the resulting homology distribution shows that it is not possible to eliminate heterologous DNA sequences by shearing the DNA to small fragments and subjecting these fragments to selective hybridization. No loss of heterologous DNA sequences is encountered when the DNA is sheared to pieces of approximately 7.4 S, and, even when the DNA is sheared further, again the homology distribution remains precisely the same. We have now obtained pieces of 6.5 S fragments. These fragments represent about 6 to 800 bases in length, that is, approximately the size of about 1 or 2 cistrons. Again, we were unable to remove efficiently heterologous DNA sequences from the eluted DNA molecules after selective isolation from either SV40 or host

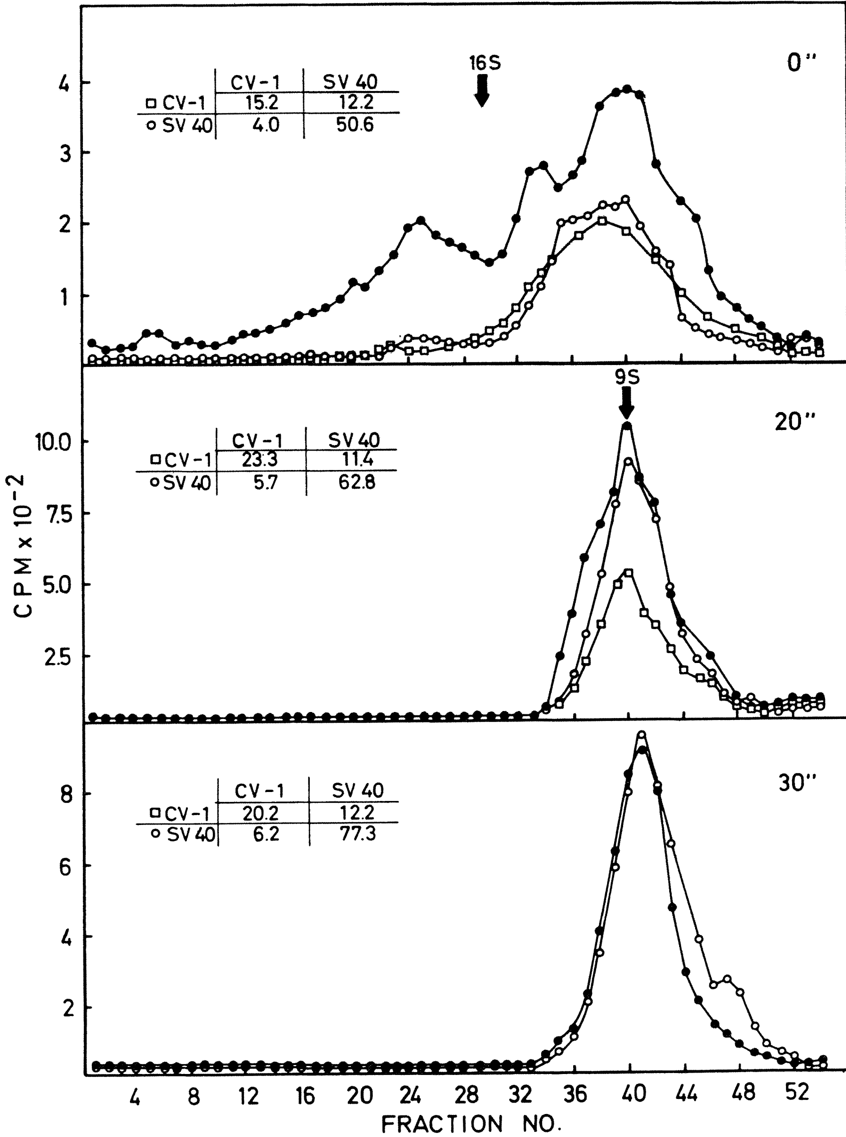

FIG. 4. Ultrasonic treatment (20 sec and 30 sec with Braunsonic 300 S) and alkaline velocity sedimentation (for conditions, see legend to Figure 3) of material recovered from the light band of the Hirt supernatant before (●—●) and after hybridization to and elution from SV40 (o—o) and CV-1 DNA (□—□). The upper panel illustrates the sedimentation profile of the unsheared light band before (●—●) and after hybridization to and elution from SV40 (o—o) and CV-1 DNA (□—□). The numbers in the tables describe the homology between the unsonicated and sonicated DNA molecules and CV-1 and SV40 DNA. The arrow indicates the position of 16 s marker DNA.

DNA. These data suggest to us that at least some of the SV40 DNA sequences must be integrated in these particular molecules in the form of subgenomic fragments with host DNA adjacent on either side. One could imagine that under certain conditions--for example, under high multiplicities of infection--such sequences could be excised, circularized and replicated, thus generating superhelices with integrated host DNA sequences (Lavi and Winocour, 1972). Although this is, for the time being, speculation, our data allow an important conclusion on the properties of the site of integration in productively infected cells: It is clear that within the range of 800 bases the integration site in the mammalian DNA in these particular molecules must consist of reiterated host DNA sequences which are either directly adjacent to the viral DNA sequences or which are separated only by a few unique base sequences from the integrated viral sections. This must be concluded, because only reiterated host DNA can be detected in the DNA-DNA filter hybridization technique which is being used here. Unique host sections would not reveal themselves under the conditions employed in the filter technique.

Further experiments are in progress now to determine the time course of development of such molecules during productive infection and to clarify the biological significance of these structures.

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SIMIAN VIRUS 40 IN SYNCHRONIZED MONKEY CELLS: INTEGRATION OF VIRAL DNA INTO THE CELL GENOME AND TIMING OF REPLICATION

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SUMMARY

Synchronized CV_1 cells which were in the G_1 period at the time of infection, were able to promote the onset of SV40 DNA replication during the S period immediately following infection. In cells which had passed the end of G_1 at the time of infection, viral DNA replication did not start until the S period of the next mitotic cycle. This showed that a cell controlled event, situated at the G_1-S interval, conditioned the permissiveness of the host cell to the replication of the viral genome. In addition, initiation of SV40 DNA synthesis required the presence of at least one functional viral gene product, as was shown through the use of the tsA30 mutant of SV40.

Integration of SV40 DNA into the host cell chromosomal DNA was progressive with time after infection and seemed independent of the timing of the host cell cycle.

INTRODUCTION

The early, prereplicative phase of the growth cycle of Simian Virus 40 (SV40) is characterized by the synthesis of early viral mRNA (Oda and Dulbecco, 1968; Aloni, Winocour, and Sachs, 1968; Sauer and Kidwai, 1968) and the appearance of virus-specific T, U, and transplantation antigens (see reviews in John Tooze, [Editor], <u>The Molecular Biology of Tumour Viruses</u>, Cold Spring Harbor Labora-

tory, 1973; and Girard and Manteuil, Biochimie, in press). The amount of several of the cellular enzymes participating in DNA metabolism is increased, and cellular DNA synthesis is induced in most resting cells (Gershon, Sachs, and Winocour, 1966; Hatanaka and Dulbecco, 1966 and 1967; Kit, 1968; Ritzi and Levine, 1970; Levine, 1971), together with that of histones (Winocour and Robbins, 1970) and of nuclear acidic proteins (Rovera, Baerga, and Defendi, 1972). In addition, the cell surface membrane eventually undergoes a series of structural changes (Ben Bassat, Inbar, and Sachs, 1970). SV40 DNA molecules apparently become integrated into the DNA of the host cell (Hirai and Defendi, 1972; Waldeck, Kammer, and Sauer, 1973; Sauer et al., this symposium).

The expression of the early viral genes is only transient in most non-permissive cells. The cells revert to the normal state soon after infection, and show no further sign of infection, although they apparently bear integrated SV40 DNA sequences in their genomes (Smith, Gelb, and Martin, 1972). Early SV40 genes remain permanently expressed in only a small percentage of the cell population, which eventually gives rise to stably transformed cells. In no case is SV40 DNA replicated in a detectable fashion.

In permissive cells, however, the expression of the early viral genes is followed by the replication of viral DNA, which, in turn, triggers the expression of the late viral genes (Rapp et al., 1965; Carp, Sauer, and Sokol, 1966; Sauer, 1971). This process apparently necessitates the intervention of still unknown cellular factor(s), since the permissivity of a cell depends mostly on the species from which the cell was derived. In addition, it requires the product of at least one early viral gene, since thermosensitive SV40 mutants of complementation group A are unable to replicate their DNA at elevated temperatures (Tegtmeyer, 1972). A similar finding was reported earlier for polyoma virus mutants (Eckhart, 1969).

The reason why monkey cells are permissive to the replication of SV40, whereas mouse or hamster cells are not, is still obscure. As a preliminary attempt at studying this question, we have examined what relationship might exist between the early events of the SV40 growth cycle in monkey cells, the initiation of viral DNA replication, and the timing of the host cell cycle periods. For this purpose, subcloned CV_1 cells were synchronized, then infected with SV40 at different times of their mitotic cycle, and the time course of both the replication of viral DNA and of some of the early events of the virus growth cycle was determined.

RESULTS AND DISCUSSION

I - The Onset of SV40 DNA Synthesis Seems to Be Temporally Related to That of a Host Cell S Phase

Using this synchronized CV_1 cell system, we have shown that the timing of SV40 DNA replication is controlled by that of the host cell S phase (Pages et al., 1973). Thus, whether the cells were infected at the beginning or the end of an S period, or during the following G_2 period, no viral DNA synthesis could be detected before the cells reached the S period of their following mitotic cycle. In cells infected during a G_1 period, viral DNA synthesis was however initiated within the same mitotic cycle, as soon as the cells entered an S phase. Initiation of viral DNA replication was therefore apparently dependent on initiation of host cell DNA synthesis. Similar findings have been reported for polyoma virus (Kaplan and Ben Porat, 1968; Thorne, 1973).

Once viral DNA replication had begun, it was no longer dependent on the timing of the host cell cycle. In cells synchronized by a double thymidine excess, SV40 DNA synthesis lasted from the beginning of an S period to the end of the following one, and did not cease nor decrease during the time interval which separated both periods. It therefore appears that only the initiation of the first round, or rounds, or viral DNA synthesis was under the control of the host.

SV40 DNA synthesis was the most delayed in cells which were at the beginning of an S period at the time of infection, and the least delayed in those which were in G_1. It was suppressed when cells, infected in either S or G_2, were prevented from entering their next mitotic cycle through arrest in metaphase. These, and other considerations (Pages et al., 1973) lead to the conclusion that the initiation of viral DNA replication can occur only if, and after, the infected cell proceeds from a G_1 to an S period. The timing of initiation of SV40 DNA synthesis with respect to the host cell S phase suggests that it is conditioned by the occurrence of a critical cellular event, which apparently takes place in the infected cell at, or near, the G_1-S interval.

It is not clear however to what extent this event is related to the onset of the host cell S phase itself. In other words, it can be questioned whether cellular DNA synthesis actually has to begin before viral DNA replication can start. The existence of a BSC_1 cell line in the resting cell cultures of which cellular DNA synthesis is not induced, or induced to a very limited extent only, after infection by SV40, although these resting cell cultures fully support the growth of the virus (Gershon et al., 1966; Ritzi and Levine, 1970), suggests that cell DNA synthesis *per se* is not

mandatory for the initiation of viral DNA replication. Moreover, host conditional mutants of polyoma virus which can grow only in transformed permissive cells, and not in non-transformed cells, seem normally to induce host cell DNA synthesis in resting non-transformed cells (Benjamin, 1970, and this symposium). This shows that the mutants require a cellular function which is spontaneously, or permanently, expressed in transformed cells, but not in resting normal cells, and which is different from cell DNA synthesis. It also implies that this function is induced in resting cells in the case of infection by wild type virus.

It is tempting to assume that this cellular function is the same as that which is expressed in the synchronously growing cells at the G_1-S interval. According to this hypothesis, the critical event of the host cell cycle which promoted the replication of SV40 DNA in the synchronized CV_1 cell system would be related to the onset of the host cell S phase in a temporal fashion only, and would bear no direct relationship with actual cellular DNA synthesis. The nature of this event is still obviously a matter for speculation at the present time. It might be related to the exposure of the wheat germ agglutinin sites on the cell surface membrane, since the host-range mutants of polyoma virus fail to induce such exposure in resting cells (Benjamin and Burger, 1970).

In short, it seems well established that both SV40 and polyoma virus require the expression of a cellular function for the initiation of the replication of their DNA in permissive cells. This function seems to be expressed by the cell only at the time, or near the time, of a G_1-S interval. Since growing cells naturally come to express that function through the progression of their successive mitotic cycles, they offer the most favorable environment for the growth of these viruses. Resting cells, on the other hand, being arrested at some early stage of their G_1 period, should be unable to support the replication of viral DNA as long as they have not been induced by the virus to complete their G_1 period and reach the G_1-S interval. In this respect, induction of the host cell from the resting to the growing state should be a mandatory characteristic of SV40 and polyoma virus. Indeed, the thermosensitive ts3 mutant of polyoma virus, which cannot induce host cell DNA synthesis and exposure of the wheat germ agglutinin sites in resting cell cultures maintained at elevated temperature, is blocked at that temperature in the early phase of its growth cycle, and its DNA is not replicated (Dulbecco and Eckhart, 1970; Eckhart, Dulbecco, and Burger, 1971). The viral function involved is required only for the initiation of the first round or rounds of viral DNA synthesis, since when permissive cells infected with this ts mutant are shifted from permissive to non-permissive temperatures after viral DNA replication has begun, this is not followed by arrest of viral DNA synthesis. Interestingly, the same viral function is implied in the

maintenance of the transformed state in non-permissive cells (Eckhart et al., 1971; Dulbecco, this symposium). The SV101 mutant of SV40 (Robb and Martin, 1972) seems to share many of the properties of the ts3 mutant of polyoma.

The existence of such SV40 and polyoma virus mutants shows that, in resting cells at least, initiation of viral DNA synthesis requires the prior synthesis of an early virus gene product, which, triggering the progression of the cell through its mitotic cycle, brings it to the stage at which it expresses a cellular function which in turn allows viral DNA replication to start.

II - The Initiation of SV40 DNA Synthesis Requires the Presence of a Functional Viral Initiator

As was shown by Tegtmeyer (1972), thermosensitive SV40 mutants such as tsA30 are unable to replicate their DNA in cells maintained at high temperature (41°C), but grow normally at low temperature (33°C). A shift of tsA30 infected cell cultures from 33° to 41°C at any time of the replicative phase of the virus growth cycle is followed by a rapid arrest of SV40 DNA synthesis (Tegtmeyer, 1972, and Figure 1). The viral function involved is thus continuously required for viral DNA replication, and not only for the establishment of the replicative phase, as was the case for the SV101 class of mutants.

Both the elongation and termination of the replicating tsA30 DNA molecules are normal at high temperature. This can be shown by briefly pulse labeling tsA30-infected cells with labeled thymidine at 33°C, then chasing the label at either 33° or 41°C, and following the fate of the labeled DNA molecules at both temperatures (Figure 2). In this experiment, supercoiled SV40 DNA component I molecules were distinguished from nicked SV40 DNA component II and replicative intermediates (R.I.) molecules through analysis by alkaline sucrose gradients centrifugation. The results, expressed in Figure 2 as the percentage of label in both categories of molecules, show that the appearance of radioactivity into DNA component I was very fast at first, then slower and slower with time. Three hours after the chase, 5% of the label still remained in R.I. and DNA II. However, no difference could be detected between the kinetics of the chase at 33°C (circles) and that at 41°C (triangles). Since viral DNA synthesis was rapidly halted at 41°C, this shows that the tsA30 defect bears on the initiation of each round of DNA replication, as previously demonstrated by Tegtmeyer (1972).

This, and the fact that tsA30 can be complemented by a mutant of complementation group B (Tegtmeyer, 1972), also show that the gene product which is thermolabile in the case of tsA30, is a

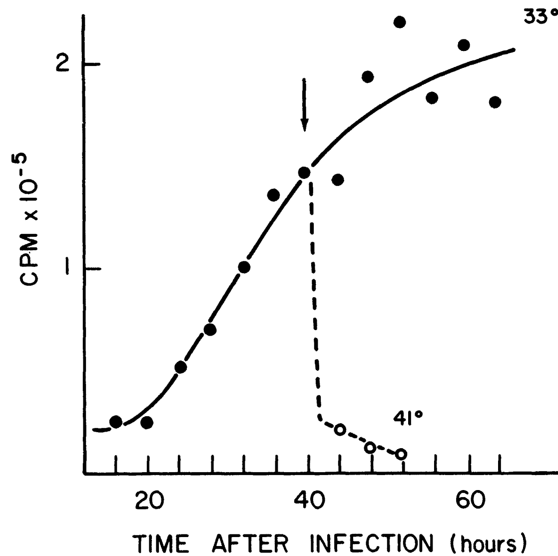

FIG. 1. Arrest of SV40 DNA synthesis in tsA30-infected cells upon transfer from 33°C to 41°C. SV40 DNA synthesis was followed by a series of pulse labelings with 10 μCi/ml ^3H thymidine and selective extraction of viral DNA (Hirt, 1967) in randomly growing Patas monkey kidney cells infected with the tsA30 mutant of SV40 at a multiplicity of 1 PFU/cell, and either maintained at 33°C throughout the virus cycle (•) or transferred to 41°C at the time indicated by the arrow (o).

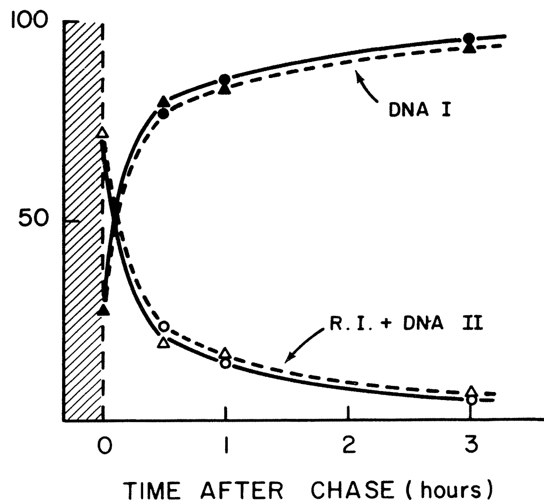

FIG. 2. Comparison of the elongation and termination of nascent tsA30 DNA chains at 33°C and 41°C. tsA30-infected Patas monkey kidney cells were labeled after 39 hours at 33°C with 200 μCi/ml ^3H thymidine for 20 minutes (hatched area). Part of the cell monolayers was then washed twice with, then overlaid with growth medium prewarmed to 33°C and containing 10^{-5} M unlabeled thymidine, whereas another part was similarly washed and overlaid with medium prewarmed to 41°C. Samples of the cultures incubated at 33°C (o——o) and of those incubated at 41°C (Δ——Δ) were withdrawn at the indicated times, viral DNA was selectively extracted (Hirt, 1967) then analyzed by centrifugation in the SW 41 rotor of a Spinco for 5 hours at 41,000 rpm, 4°C, through 5-20% sucrose gradients in .3 M NaOH, .7 M NaCl, .001 M EDTA, pH 13. The results were expressed as percent of the total radioactivity recovered on each gradient. Open symbols, radioactivity in R.I. and DNA component II; closed symbols, radioactivity in DNA component I.

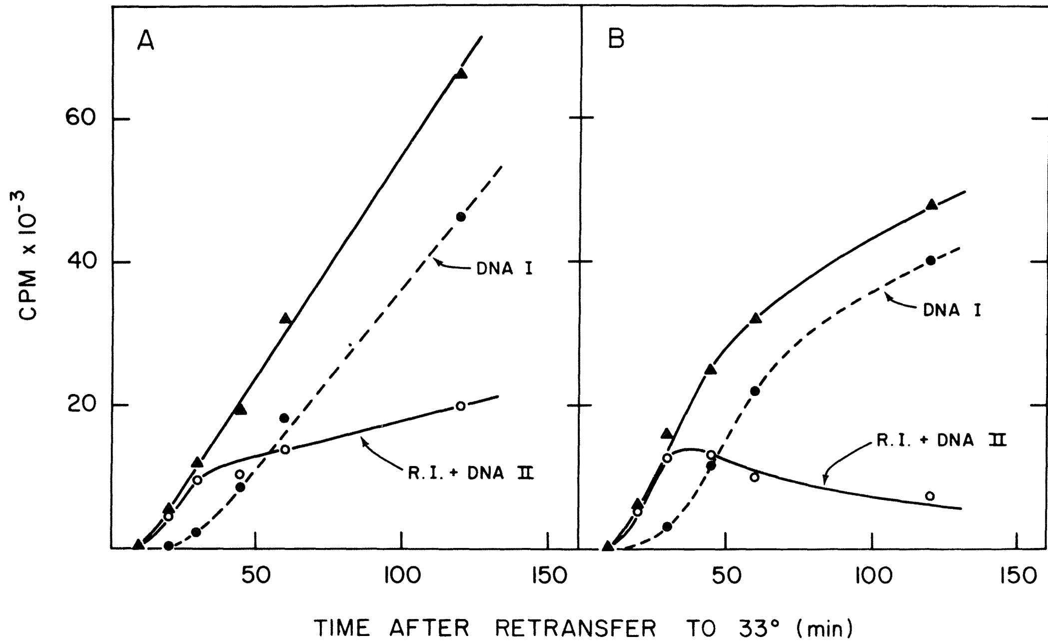

FIG. 3. Reinitiation of tsA30 DNA synthesis in the absence of protein synthesis. tsA30-infected CV_1 cells, which had been maintained for 40 hours at 33°C, then 5 hours at 41°C, were transferred back to 33°C either in the presence (panel B) or absence (panel A) of 150 µg/ml cycloheximide. Reinitiation of viral DNA synthesis was followed through labeling the cells with 100 µCi/ml ^{3}H thymidine added at the time of reversal of the temperature block. Radioactivity accumulating into total viral DNA (▲——▲) was analyzed by alkaline sucrose gradient centrifugation as described for Figure 2. Symbols: ●——●, radioactivity in DNA component I; o——o, radioactivity in R.I. and DNA component II.

diffusible factor specifically required for initiation of replication on the viral chromosome.

It is not clear however whether a continuous synthesis of this viral initiator is required for the continuous replication of viral DNA, or whether the same molecules of initiator could be repeatedly used for each successive round of replication throughout the replicative phase of the virus cycle. In order to study this question, we have investigated the time course of reinitiation of SV40 DNA synthesis at 33°C in tsA30-infected cell cultures which had first been maintained at 33°C, then were shifted to 41°C for various lengths of time, and finally were transferred back to permissive temperature.

When tsA30-infected cells were transferred to 41°C before viral DNA synthesis had begun, initiation of viral DNA synthesis upon back transfer of the cells to 33°C was found to depend upon the phasing of the host cell cycle. In synchronized cells, initiation was apparently immediate in cells which were in the vicinity of a G_1-S interval at the time when the temperature block was reversed. It was, on the contrary, delayed in cells which were still in S at that time (unpublished results). In randomly growing cultures, on the other hand, no or very little lag could be observed between the time when the temperature block was reversed, and that when viral DNA synthesis was first initiated. This suggested that the tsA30 thermosensitive block was immediately reversible, but that initiation of viral DNA replication did, or did not, take place immediately after returning the tsA30-infected cells to permissive temperature, depending upon the timing of the transfer from 41°C to 33°C with respect to the critical event of the host cell cycle.

In order better to demonstrate that the defect in the tsA30 initiator was reversible, randomly growing CV_1 cells infected with tsA30 were first maintained at 33°C until viral DNA synthesis was well underway, then transferred to 41°C for a few hours. At this point, the cells were labeled with ^{3}H thymidine, and part of the monolayers was shifted back to 33°C in the presence of cycloheximide in order to inhibit further protein synthesis, whereas another part was shifted without any inhibitor. Viral DNA was selectively extracted and analyzed for radioactivity in both SV40 DNA component I and DNA component II and R.I. (Figure 3). In the control untreated cultures (panel A), reinitiation was somewhat slow and asynchronous, as judged from the fact that the accumulation of radioactivity into replicating DNA molecules did not plateau for the whole duration of the experiment, and that it took approximately 60 minutes before the amount of label in DNA component I molecules became equal to that in R.I. and SV40 DNA component II (see also Manteuil and Girard, Virology, in press). In the cultures shifted back to 33°C in the presence of cycloheximide (panel B), the kinetics of labeling

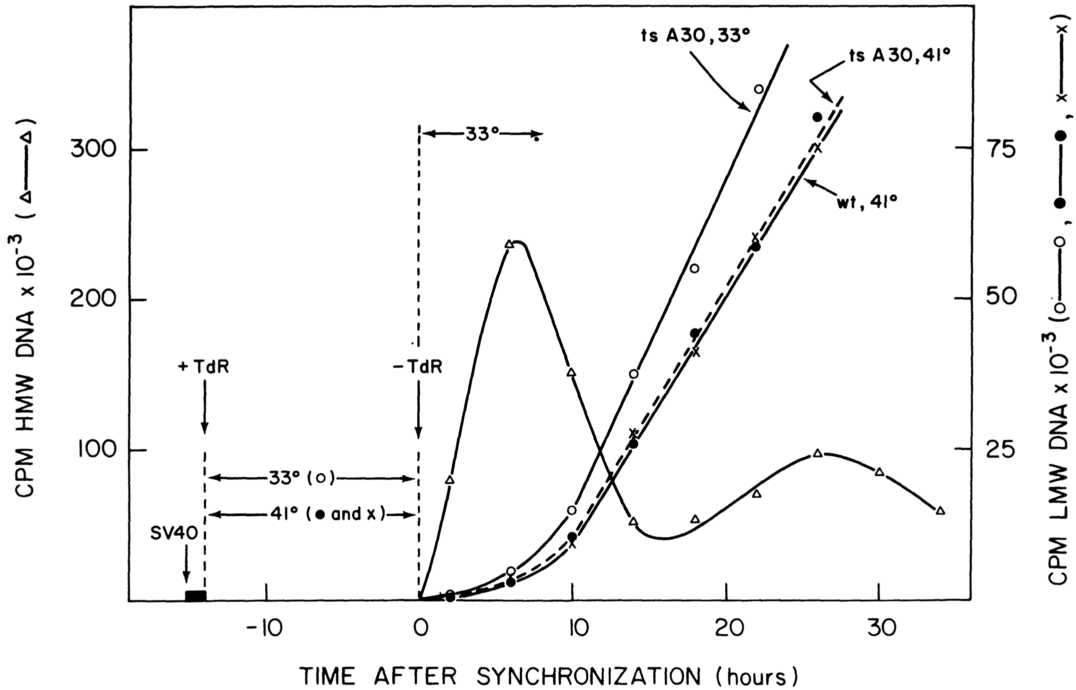

FIG. 4. Time course of the replication of tsA30 DNA in synchronized CV_1 cells. CV_1 cells synchronized overnight with excess thymidine (Pages et al., 1973) were allowed to proceed through a first S phase, then a G_2 period, at which time they were infected with either tsA30 or wild type virus, both at a multiplicity of 20 PFU/cell, before being again blocked by excess thymidine. Part of the tsA30-infected cell cultures was then kept at 33°C (o——o) whereas another part was transferred to 41°C (●——●). The wild type virus-infected cells were also incubated at 41°C (X——X). Fourteen hours later, all thymidine blocks were released, and all cell cultures were then incubated at 33°C. Cellular and viral DNA syntheses were followed by a series of pulse labelings with 20 μCi/ml ^3H thymidine, followed by selective extraction of viral DNA (low M.W. DNA, right hand scale of the figure). Radioactivity in cellular DNA (Δ——Δ) was taken as that recovered in the extraction pellet (high M.W. DNA, left hand scale in the figure). Note that radioactivity in viral DNA has been represented in a cumulative fashion, and not as rates of synthesis.

of the various SV40 DNA species was identical to that in the control cultures for at least the first 30-50 minutes following the transfer back to 33°C, showing that reinitiation occurred normally in these infected cells in spite of the absence of new protein synthesis. However, after 50-60 minutes, radioactivity in R.I. progressively decreased, and synthesis of SV40 DNA_I progressively turned off.

This experiment demonstrates that the thermolability of the tsA30 initiator protein is readily reversible, but that, in permissive conditions, synthesis of new initiator molecules has to take place continuously in the infected cell in order to sustain the full rate of viral DNA replication.

Finally, initiation of tsA30 DNA synthesis in cells shifted from non-permissive to permissive temperature was studied in the synchronized CV_1 cell system. The cells were synchronized through a first excess thymidine, infected with either tsA30 or wild type virus when reaching G_2, then further synchronized through a second excess thymidine. The second synchronization step took place at 33°C for part of the cultures, and at 41°C for another part. Excess thymidine was then removed, and all cells were allowed to proceed through S at 33°C. Figure 4 shows that in the tsA30-infected cell culture maintained at all times at 33°C, viral DNA synthesis was initiated at some time during the beginning of the first S period to follow release of the thymidine block (open circles), as previously demonstrated (Pages et al., 1973). In the tsA30-infected cell cultures which had been maintained at 41°C during the entire early phase of the virus growth cycle, and which had gone through a G_1-S interval at that temperature, viral DNA synthesis was also initiated soon after the thymidine block was released (closed circles), and the time course of accumulation of progeny viral DNA molecules was identical to that found in cell cultures infected with wild type virus and similarly treated (crosses). The fact that there was slightly less viral DNA synthesized in the two cultures blocked at 41°C as compared to that blocked at 33°C might reflect the fact that some cells suffered from their passage at 41°C.

These results lead to the conclusion that all the early events, cellular as well as viral, which lead to the onset of viral DNA replication in tsA30-infected cells, can normally take place at 41°C, and that, provided the cells are transferred back to permissive temperature at the right time with respect to the occurrence of the cell controlled critical event, situated at, or near, the G_1-S interval, initiation of viral DNA synthesis occurs without delay at the permissive temperature.

The tsa and ts25 mutants of polyoma virus share many of the properties of the tsA30 mutant of SV40 (Fried, 1965; Eckhart, 1969; Vogt, 1970). Interestingly, all these mutants are thermosensitive

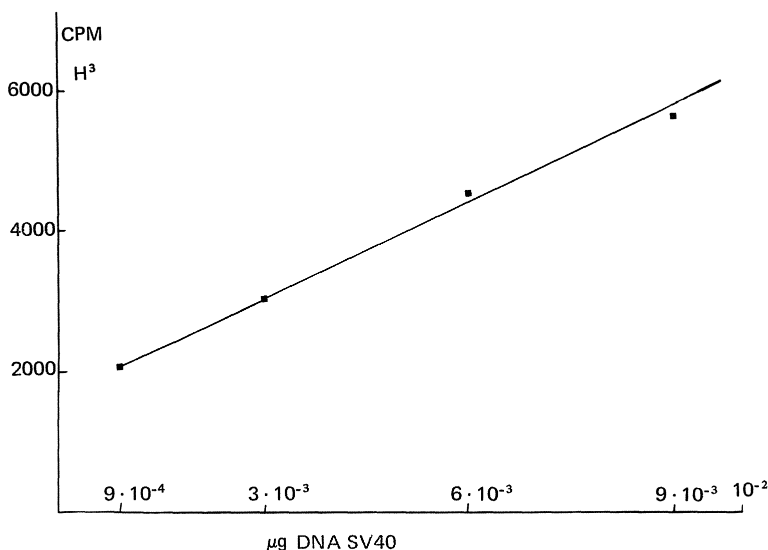

FIG. 5. Calibration curve for the hybridization of SV40 cRNA with SV40 DNA. Labeled SV40 cRNA was synthesized with E. coli RNA polymerase as described by Sambrook, Sharp, and Weller (1973), using the four ^{3}H-labeled ribonucleoside-triphosphates. Specific radioactivity was estimated as 10^7 cpm/µg or more, but could not be actually measured because of the too limited amount of RNA synthesized. All SV40 DNA preparations were made from cells infected at multiplicities of 10^{-3} to 10^{-6} PFU/cell with virus passaged repeatedly at similarly low multiplicities. For the preparation of the filters, SV40 DNA component I was purified twice through banding in CsCl in the presence of ethidium bromide, then converted to the extent of 70% into DNA component II through treatment with 5 x 10^{-4} µg/ml RNase free pancreatic DNase for 70 minutes at 37°C in 0.035 M Tris HCl pH 8, 0.03 M $MgCl_2$, 0.005 M EDTA, 0.05 X SSC. Resulting component II DNA molecules were purified through centrifugation in the SW25.1 rotor of the Spinco for 19 hours at 25,000 rpm, 4°C, through 15-30% sucrose gradients in 0.1 M NaCl, 0.01 M EDTA buffer adjusted to pH 11.8 with NaOH. Denatured unit length DNA thus obtained was diluted as required into 5 X SSC and slowly filtered through Sartorius M50 filters. In order to minimize the formation of perfect DNA-RNA hybrids which could have been released from the filters during hybridization (Hass et al., 1972), cRNA was fragmented prior to use through treatment for 1.5 minutes with 0.1 M NaOH, then immediately neutralized with 0.5 M Tris-HCl pH 7.4. A fixed amount (7 x 10^5 cpm) of this RNA was hybridized for 4 days at 37°C in the presence of formamide and SDS (Conaughy, Laird, and McCarthy, 1969) with the indicated amounts of SV40 DNA fixed on nitrocellulose filters. The filters were then soaked repeatedly into 2 X SSC, incubated in the presence of 10 µg/ml pancreatic RNase and 5 units/ml T_1 RNase for 30 minutes at 37°C in 2 X SSC, rinsed again, and analyzed for radioactivity.

for the establishment of transformation in non-permissive cells, but not for its maintenance. It is not known whether this is due to the fact that viral DNA has to undergo a limited replication in the cell before it can eventually become stably integrated and give rise to a permanently transformed cell line, or whether the viral initiator has a dual function, one for initiation of replication in the permissive cell, and the other for the ordered recombination of host and viral chromosomes in non-permissive cells.

III - The Integration of SV40 DNA into the Host Cell Chromosome Is Independent of the Onset of a Host Cell S Phase

It has been recently shown that SV40 DNA integrates into the chromosomal DNA of permissive cells during the lytic cycle (Hirai and Defendi, 1972; Waldeck et al., 1973; Sauer, this symposium). We have investigated the time course of such integration using the synchronized CV_1 cell system.

In order to detect integration, labeled viral cRNA was prepared in vitro through the use of E. coli RNA polymerase and SV40 DNA component I. Most of the product thus synthesized was asymmetric, as judged from the fact that it was only 10-15% autoannealable. Its size was that expected from a full SV40 transcript, since it sedimented at approximately 28 S in sucrose gradient in 0.1 M NaCl (unpublished results). The cRNA was fragmented prior to hybridization to prevent the formation of perfect RNA-DNA hybrids. Hybridization of 7×10^5 cpm of this cRNA with an increasing amount of SV40 component II DNA fixed on nitrocellulose filters, gave a calibration curve (Figure 5) which could be used to estimate approximately the number of genome equivalents for each amount of cRNA retained on a filter.

High M.W. cellular DNA from infected cells was obtained by lysing the cells on top of alkaline sucrose gradients, then centrifuging and isolating the DNA molecules sedimenting at 200 S or more. After fixation on nitrocellulose filters, this DNA was allowed to hybridize with a fixed amount of fragmented cRNA as described in the legend to Figure 5 and Table 1. Reconstruction experiments using a known amount of purified SV40 DNA component I mixed with a lysate of non-infected cells before isolation of the high M.W. cellular DNA showed that contamination of cell DNA by viral DNA was virtually nonexistent in the conditions of the experiment (Table 1). Note, however, that there was a relatively high degree of hybridization of SV40 cRNA with uninfected CV_1 cell DNA (.3 to .5% of the input cpm), in spite of the fact that the SV40 DNA used for the preparation of cRNA had been purified from cells infected at a multiplicity of 10^{-6} pfu/cell, as recommended by Lavi and Winocour (1972) in order to minimize the formation of defective SV40 particles.

Similarly, reconstruction experiments in which uninfected CV_1 cells were mixed with increasing amounts of SV40 virions before isolation of the high M.W. cellular DNA failed to show contamination of cell DNA by viral DNA (Table 2).

In a first series of experiments, randomly growing CV_1 cells were infected with SV40, and both the time course of viral DNA replication and that of the appearance of viral DNA sequences in the high M.W. DNA of the cell were determined (Figure 6). Viral DNA synthesis (closed squares) began at approximately 10-12 hours after infection, as previously reported (Manteuil et al., 1973). The amount of radioactive cRNA hybridized with the high M.W. DNA molecules extracted from the infected cells at the times indicated was

TABLE 1. Absence of contamination of high M.W. cellular DNA by added SV40 DNA

Amount of viral DNA added (µg)	cpm hybridized per 100 µg cellular DNA
--	3500
0.1	2450
0.5	3500
1.0	3500
5.0	2500
10.0	5500

4×10^6 uninfected CV_1 cells were washed twice with ice cold sterile PBS and the monolayers were scraped of their supports. The cells were centrifuged, resuspended in 0.2 ml of 0.01 M Tris-HCl pH 7.4, 0.01 M NaCl, 0.0015 M $MgCl_2$, then layered on top of a 10-30% sucrose gradient in 0.3 M NaOH, 0.5 M NaCl, 0.01 M EDTA. The cells were lysed by gently mixing them on top of the gradient with 2 ml 0.5 M NaOH, 0.1 M EDTA. The gradients were then left to stand at 4°C for 16-24 hours, after which they were centrifuged for 2.5 hours at 25,000 rpm, 4°C, in the SW27 rotor of the Spinco. Fractions were collected from the bottom of the tubes, monitored for optical density at 260 nm, and the material sedimenting at 200 S or more (high M.W. DNA) was pooled, diluted in the required amount of 5 X SSC, and used to prepare nitrocellulose filters containing 20-40 µg DNA.

In this reconstruction experiment, the indicated amounts of purified SV40 DNA component I were each added to a cell suspension (4×10^6 cells) on top of the sucrose gradients prior to lysis with NaOH. The DNA filters were hybridized with 7×10^5 cpm cRNA as described in the legend to Figure 5. RNase-resistant radioactivity remaining on the filters has been expressed as cpm hybridized per 100 µg cellular DNA per filter.

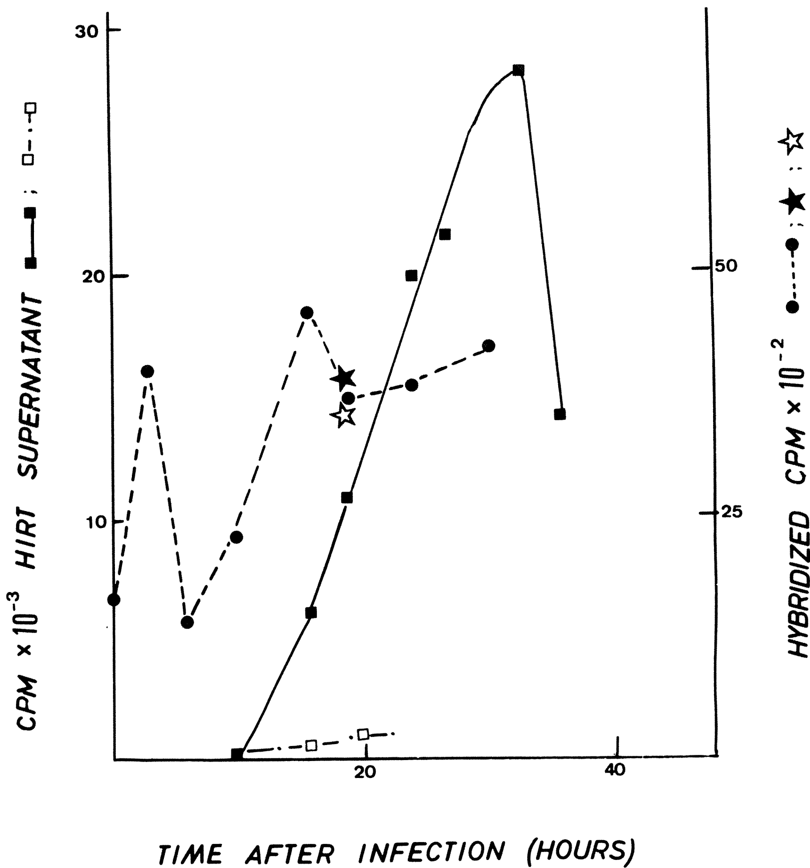

FIG. 6. Time course of integration of SV40 DNA in randomly growing cells. Growing CV_1 cells were infected at a multiplicity of 20 PFU/cell and the time course of SV40 DNA synthesis was determined on part of the monolayers by a series of pulse labelings with labeled thymidine and selective extraction of the DNA according to Hirt (■—■). At the indicated times, 4 x 10^6 infected cells were lysed on top of alkaline sucrose gradients, high M.W. cellular DNA was prepared, and its content in SV40 DNA sequences (●——●) was measured through hybridization with labeled cRNA (see legends to Table 1 and to the preceding figure). Part of the infected cell culture was treated with 40 μg/ml cytosine arabinoside at the time of infection, and both the extent of viral DNA synthesis (□—□) and the amount of SV40 DNA integration at 19 hours after infection (☆) were determined. Part of the cell monolayers was also treated with 7 mM unlabeled thymidine immediately after infection, and the extent of SV40 DNA integration was measured 19 hours later (★). Hybridization has been expressed as cpm cRNA hybridized per 100 μg high M.W. cellular DNA.

increased more than twofold over background in the immediate hours following infection, then dropped back to background levels at 6 hours after infection (closed circles). This early and transient rise in the annealing capacity of cellular DNA with SV40 cRNA immediately after infection has been reproducibly observed in all the experiments we have done so far, as well with non-synchronized cells (see for instance Figure 7). We have no explanation for its occurrence, since control experiments where SV40 was mixed extemporaneously with uninfected cells failed to reveal the presence of SV40 DNA in the high M.W. cellular DNA recovered from the alkaline sucrose gradients (see Table 2). Moreover, the fact that after a few hours, the level of hybridization returned to background values in all these experiments shows that the increase in hybridization observed immediately after infection could not be due to trivial contamination of the host cell DNA by parental SV40 DNA molecules. This increase therefore either implies a massive and temporary integration of SV40 DNA into the host cell chromosomes immediately after infection, or suggests that the freshly decapsidated parental SV40 DNA molecules could be trapped in an alkali resistant fashion in the DNA of the host cell. However, no data are available at the present time to support either hypothesis.

Following the return to background level, there was a progressive increase in the hybridization capacity of cellular DNA with viral cRNA, until a plateau was reached at approximately 16 hours after infection (Figure 6, closed circles). From the calibration curve shown in Figure 5, this plateau can be estimated to correspond to about 6×10^8 molecules of SV40 DNA per 100 μg cell DNA, i.e., 100-150 molecules per tetraploid cell genome equivalent.

TABLE 2. Absence of contamination of high M.W. cellular DNA by added SV40

Amount of virus added (PFU)	cpm hybridized per 100 μg cellular DNA
--	3300
4×10^6	3400
4×10^7	3000
1.6×10^8	3000

The indicated amounts of SV40 were mixed with 4×10^6 uninfected CV_1 cells on top of alkaline sucrose gradients as described in the legend to Table 1. High M.W. cellular DNA was then purified, and used to prepare nitrocellulose filters containing 20 μg cell DNA. These filters were hybridized with cRNA as described in the legend to Figure 5. Radioactivity remaining on the filters has been expressed as cpm hybridized per 100 μg cell DNA.

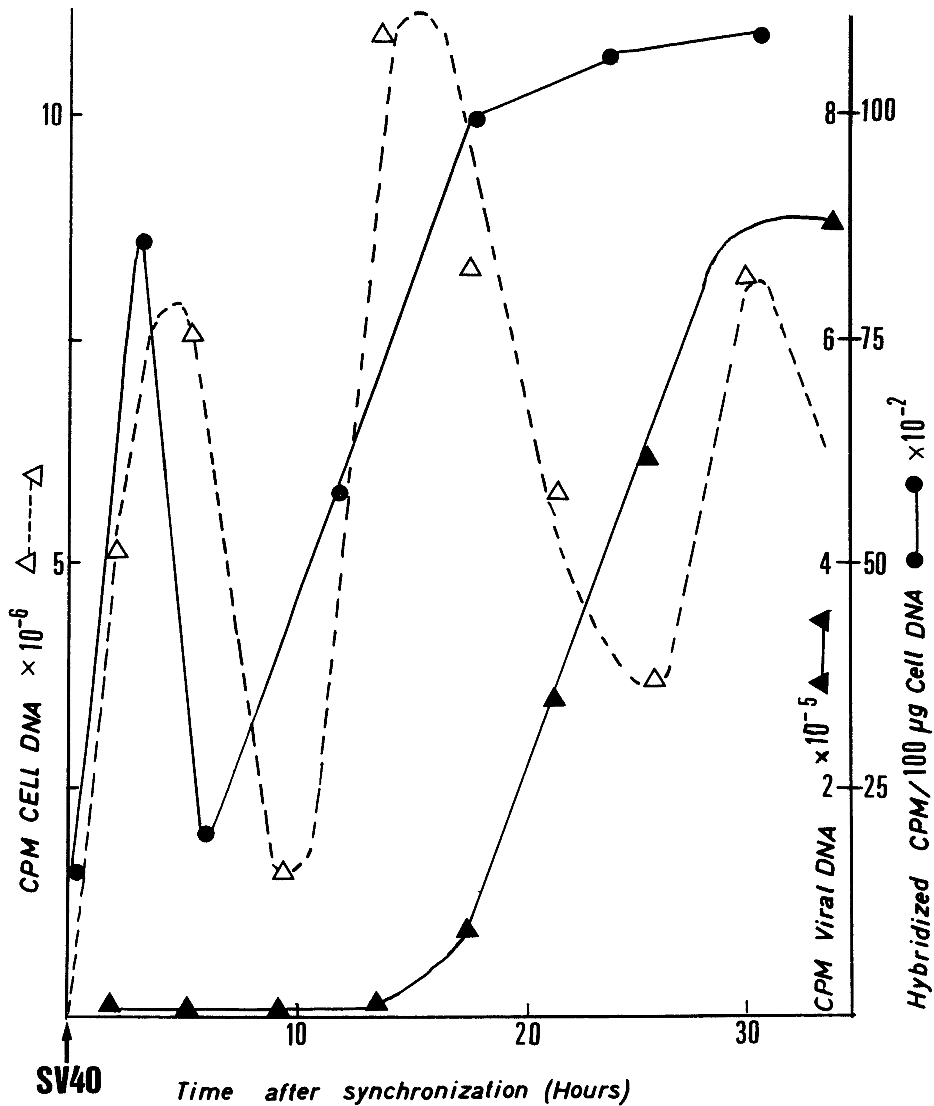

FIG. 7. Time course of integration of SV40 DNA in synchronized CV_1 cells. Synchronized CV_1 cells were infected with SV40 at a multiplicity of 20 PFU/cell at the beginning of an S period. Cellular (△——△) and viral (▲——▲) DNA syntheses were followed by a succession of pulse labelings with 4 μCi/ml ^3H thymidine and selective extraction of the DNA according to Hirt. The time course of integration was followed as described in the legend to the preceding figure (●——●).

It should be pointed out that this figure can only be tentative in view of possible discrepancies in the establishment of a correct calibration curve (Hass, Vogt, and Dulbecco, 1972). An identical figure was also obtained in cells treated at the time of infection with either cytosine arabinoside (closed star in Figure 6) or excess thymidine (open star), in spite of the complete prevention of SV40 DNA synthesis under these conditions (open squares in Figure 6). This is in agreement with the results of Hirai and Defendi (1972), and shows that the integration of SV40 DNA sequences into the host cell genome is independent of viral DNA replication.

In the next series of experiments, CV_1 cells were synchronized by the double excess thymidine procedure, and infected with SV40 at the time when the second thymidine block was released. Here again, both the time course of viral DNA synthesis and that of viral DNA integration into the host cell high M.W. DNA were determined (Figure 7). After the initial transient rise which was observed immediately after infection, the amount of SV40 DNA sequences detectable in the host cell DNA returned to background levels at 5 hours after infection, after which it progressively increased to reach a plateau at approximately 20 hours (closed circles). Viral DNA synthesis was not however initiated before approximately the 15th hour after infection (closed triangles). At that time, there were already approximately 400-500 viral genome equivalents integrated per tetraploid cell genome equivalent.

Altogether, these results first show that the integration of SV40 DNA into the host cell chromosomes is an early event, which precedes viral DNA synthesis, and can normally occur even if viral DNA replication is prevented. Second, they show that integration is not an all or none process, but rather takes place progressively with time, beginning a few hours after infection and reaching a plateau 12 to 15 hours later, and this as well in synchronized as in randomly growing cell cultures. Third, integration does not seem to depend upon any particular stage of the cell cycle, although the time when most of the integration took place in the synchronized cell system corresponded to that of an S phase (Figure 7). Here again, however, the temporal relationship with an S phase could be coincidental, as is suggested by the fact that integration could normally occur in cells in which all DNA synthesis had been suppressed.

It is not known whether integration of SV40 DNA into the DNA of the permissive cell might be an actual prerequisite to viral DNA replication, nor, if so, why. The fact that the number of integrated SV40 DNA copies remained fairly constant during at least 15 hours at the time when the rate of viral DNA synthesis was rapidly increasing in the infected cell suggests that the integrated copies were not excised and that they did not play any direct role in the repli-

cation process, at least for most of them and during most of the replicative phase. The high number of viral equivalents integrated per infected cell however casts some doubt on the actual significance of the integration observed here in the lytic cycle. Thus, the possibility cannot be overlooked that in spite of the precautions taken for the growth of our virus stocks, these were still contaminated by defective virus particles which played no direct role in the growth of the virus, but the preferential recombination of which with the host cell genome (Lavi and Winocour, 1972; Lavi et al., 1973; Martin et al., 1973; Rozenblatt et al., 1973) was readily detected. Obviously, the answers to these crucial questions will have to await further experiments, and possibly, the use of a more refined technological approach.

ACKNOWLEDGEMENTS

We thank Peter Tegtmeyer for the tsA30 mutant of SV40, James Robb for the ts101 mutant, Ed Brody for the gift of E. coli RNA polymerase, and Nardi Horodniceanu for the kind gift of Patas monkey kidney primary explants cells. We also should like to acknowledge the efficient technical assistance of Louise Marty, Françoise Suarez and Danielle Bénichou. This work benefited from the help of the Délégation Générale à la Recherche Scientifique et Technique and the Direction des Recherches et Moyens d'Essai.

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TRANSCRIPTION OF SV40 IN LYTICALLY INFECTED AND TRANSFORMED CELLS

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As long as we take reasonable care, we can grow stocks of Simian Virus that are remarkably homogeneous. Each infectious particle contains a single molecule of DNA weighing 3.4 x 10^6 daltons--enough to code for perhaps 5-8 polypeptides of average size. The manner in which this virus-coded information is expressed is highly cell-dependent. For example, after infection with SV40, monkey cells undergo a productive or lytic response, during which there is an ordered appearance of virus-specific functions, with some virus products present at all times and others detectable only in the late stages of the viral growth cycle. The infection progresses through a well-defined series of episodes, which culminate in cell death and the concomitant liberation of a new crop of virus particles. The events are multiplicity-independent in that they occur in the same order, albeit at different rates, in cells infected either with one infectious particle or with a hundred. By contrast, the outcome of exposing mouse cells to SV40 is very different. Firstly, if the multiplicity of infection is low, most of the cells show little or no effect; secondly, whatever the multiplicity of infection, very few cells of the population die as a consequence of their exposure to the virus; and thirdly, no new virus is ever produced but many of the cells that receive a high multiplicity of SV40 are stimulated to divide a few times and there is expression of certain virus-specific antigens. After a few generations, these usually disappear and most of the cells in the population revert to the somnolence typical of the untransformed phenotype. However, it is from this population of abortive transformants that the rare stable transformants emerge. These cells

retain indefinitely the properties that were transiently displayed by their abortively transformed siblings: They contain virus-specific DNA in an integrated form, and there is genetic evidence available to indicate that continued expression of certain virus-coded functions may be necessary not only to initiate, but also to maintain the transformed state (see review, Sambrook, 1972).

So, when different types of cells are presented with the same set of virus-specific information, they deal with it in very different ways, and it is tempting to believe that if we were able to find out the molecular basis for this regulation, we would learn a good deal about the ways in which cells conduct their affairs. Clearly, modulation of SV40 gene expression can be achieved at any one of a number of levels; our major concern in this paper will be to discuss the control of viral transcription in lytically infected cells and in stable lines of SV40 transformants.

A. THE LYTIC CYCLE

All the events which occur during the transcription of SV40 DNA in monkey cells are carried out against a backdrop of cellular RNA synthesis. Apparently, the virus has no mechanism to dampen the host's transcription machinery, and no artificial way has been found to inhibit cellular RNA synthesis selectively. Therefore, the only way we can monitor the production of viral RNA sequences is by nucleic acid hybridization, using either the binding of pulse-labeled RNA to SV40 DNA immobilized on cellulose nitrate filters, or the annealing in solution of unlabeled RNA to radioactive separated strands of viral DNA. By either technique, SV40-specific RNA can be detected as soon as 3-6 hours after infection--a time when the virus has been stripped of its outer shell of protein and has penetrated the nucleus of the cell. As infection proceeds, there is a slow but steady accumulation of this RNA; but at no time before 12-15 hours after infection do SV40 sequences account for more than 0.001% of the total RNA of the cell (Sambrook, Sharp, and Keller, 1972). However, at the time that replication of SV40 DNA starts, the cell begins to feel the full weight of viral RNA synthesis: More and more of the cell's transcription machinery becomes devoted to turning out SV40 RNA sequences until at late times after infection, 3-6% of newly synthesized RNA is virus-specific (Tonegawa _et al._, 1970). Despite the rising level of SV40 RNA sequences, the cell nevertheless manages to continue making its own RNA (Oda and Dulbecco, 1968a). Whether this RNA plays any role in SV40 lytic infection is unclear; most people seem to think of the ongoing cellular transcription as little more than a kind of ballast which the host carries along with its main cargo of SV40 sequences.

The beginning of SV40 DNA replication is contemporaneous not only with an increase in the rate of viral RNA synthesis but also with a change in the pattern of transcription. Competition hybridization experiments published as early as 1968 (Oda and Dulbecco, 1968b; Aloni, Winocour, and Sachs, 1968; Sauer and Kidwai, 1968) very clearly established that only about one-third of the viral sequences present in cells at late times during lytic infection can be detected before SV40 DNA synthesis has begun. From these results, it was at once obvious that at least some control of virus gene expression is exerted at the level of RNA. Clearly, the next steps were to identify which strand of the viral DNA was used as template for early and late species of viral RNA, to map the early and late regions of the SV40 genome, to determine the orientation of RNA around the circular viral DNA, and to learn which of the RNA polymerases in infected cells was responsible for transcription of SV40 sequences. Modest as these goals were, results proved to be elusive and progress was dilatory. In the past year or two, however, the pace has quickened somewhat and now the answers to these questions are almost complete.

1. Strand Separation

Each molecule of SV40 DNA consists of two polynucleotide strands, each of which is closed upon itself and wrapped around its partner. The strands are topologically joined and cannot be dissociated by conditions such as high pH or temperature which destroy hydrogen bonds and cause loss of base-pairing (see review, Vinograd and Lebowitz, 1966). So before attempting to separate one strand of SV40 DNA from the other, a single-strand nick or double-strand scission must be introduced into the DNA, in order that it may escape from its topological constraints. Even when this has been done, it turns out that the complementary strands of SV40 DNA cannot be separated by any of the methods such as equilibrium centrifugation at alkaline pH or in the presence of copolymers which work so well for other types of DNA. The problem remained unsolved until 1970, when Westphal showed that the closed-circular form of SV40 DNA is transcribed _in vitro_ by _E. coli_ RNA polymerase in an asymmetric manner, and that the resulting RNA product (cRNA) could be used as a convenient tool to separate the strands of the viral DNA. The first step in the method which is now used routinely (Sambrook _et al._, 1972; Khoury, Byrne, and Martin, 1972) is to convert ^{32}P-labeled, circular SV40 DNA to a linear form with the restricting endonuclease EcoRI. The DNA is then denatured and incubated for a brief period with a twenty- to fiftyfold excess of asymmetric RNA. One of the strands of DNA anneals to cRNA very rapidly in virtually quantitative yield, and the resulting DNA-RNA hybrid can be separated from the remaining single-stranded DNA by chromatography on hydroxylapatite. The strand of SV40 DNA that is

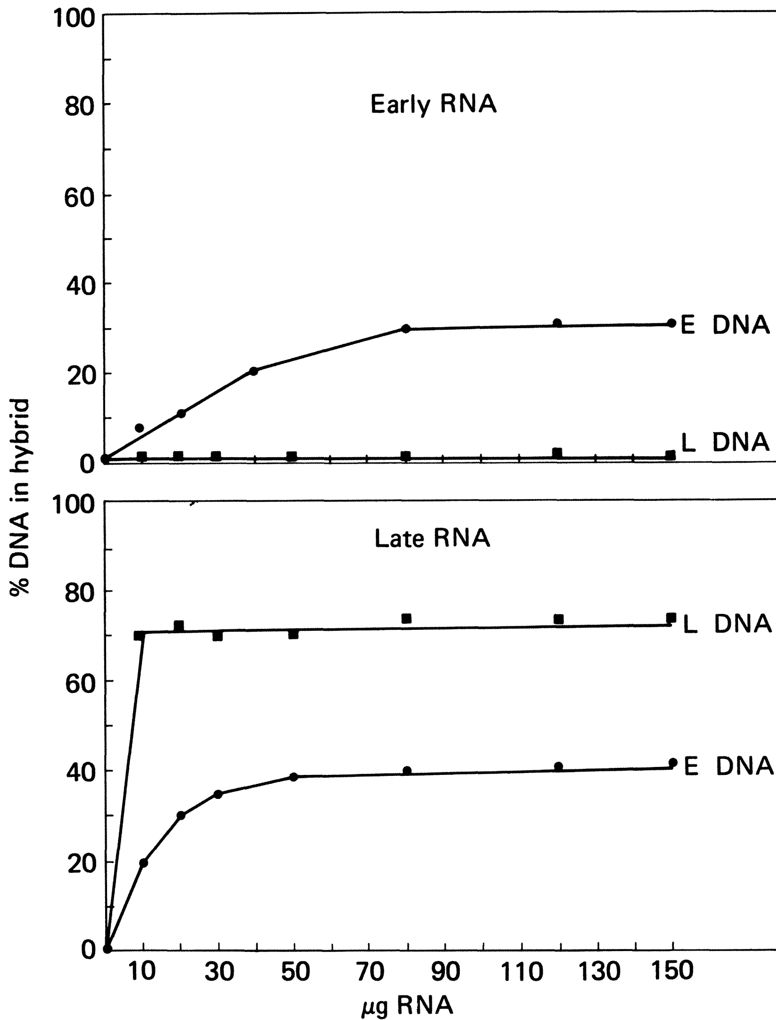


FIG. 1. Hybridization of E-DNA (●-●-●) and L-DNA (■-■-■) to RNA isolated from cells early and late during lytic infection. Monolayers of CV-1 cells were infected with SV40 at a multiplicity of 60-80 plaque-forming units per cell. After 1 hour at 37°C, Dulbecco's medium containing 2% fetal bovine serum was added. 5-fluorodeoxyuridine (5 x 10^{-5} M) was added to those cultures from which early RNA was to be extracted. Early (14 hour) and late (32 hour) RNA were extracted by Scherrer's (1969) protocol.

The hybridization mixtures contained 2.10^{-3} μg of ^{32}P-labeled E or L-DNA (sp. act. 8 x 10^{5} cpm/μg) and different amounts of RNA in a total volume of 0.125 ml of 1.0 M NaCl, 0.14 M phosphate buffer pH 6.8. After incubation at 68°C for 36 hours, the samples were assayed by chromatography on hydroxylapatite.

complementary to cRNA is called the E or (-) strand; the other is called the L or (+) strand. After the initial separation, the two strands are freed from trace amounts of cross-contamination by a second cycle of hybridization, this time in the absence of cRNA. They are then sheared and used in hybridization experiments. Because the ^{32}P-labeled DNA is degraded to small size (approximately 300 nucleotides) the fraction of the radioactivity which enters hybrid after incubation with saturating concentrations of RNA is a direct measure of the proportion of the DNA sequences that is represented in RNA in the sample under test.

When the separated strands of SV40 DNA are incubated with RNA extracted from monkey cells at early times after infection with SV40, 30-35% of the sequences of the E or (-) strand of viral DNA enter hybrid. At late times after infection transcripts corresponding to about 35-40% of the sequences of the E strand and 70% of the L strand can be detected (see Figure 1) (Sambrook et al., 1972; Khoury et al., 1972). These results confirm the relative proportions of the early and late regions of the viral genome that had been established by the earlier competition hybridization experiments, and they corroborate other experiments which had indicated that at late times after infection, 100% of the sequences of SV40 (i.e., the equivalent of the strand of the DNA) are transcribed into stable species of RNA (Martin and Axelrod, 1969). In addition, they imply that there is at least one strand switch operative during transcription of late SV40 RNA, they show that all of the early functions of SV40 are coded by the E or (-) strand of the DNA, and they suggest that the great majority, and perhaps all of the late viral functions are coded by the L or (+) strand.

2. Which RNA Polymerase?

There are two principal forms of DNA-dependent RNA polymerase in mammalian cells (Roeder and Rutter, 1970), which differ in their chromatographic properties, cation requirements, intranuclear location, salt optima and sensitivity to α-amanitin. Enzyme II is active at high salt concentrations but is totally inhibited by α-amanitin; enzyme I is unaffected by the toxin but is inactive in high salt. To test the characteristics of SV40-specific transcription we extracted nuclei from monkey cells at late times after infection and used them to synthesize RNA in vitro in the presence or absence of α-amanitin at different salt concentrations (Jackson and Sugden, 1972). The RNA was purified and tested for its ability to hybridize to SV40 DNA. Whereas the RNA synthesized by nuclei from SV40-infected monkey cells in the absence of α-amanitin at high salt concentrations contained sequences complementary to SV40 DNA, that synthesized in the presence of the toxin showed no detectable hybridization to the viral DNA (see Figure 2). The simplest

FIG. 2. Hybridization to SV40 DNA of RNA synthesized by nuclei from SV40 infected MA-134 cells in the presence and absence of α-amanitin. Thirty hours after infection with 20 plaque forming units per cell of SV40, MA-134 cells were removed from the petri dishes by treatment with trypsin. After washing in ice-cold phosphate-buffered saline, the cells were washed once with ice-cold swelling buffer (0.01 M potassium phosphate pH 7.9, 0.002 M $MgCl_2$) and were resuspended in the same buffer at a concentration of 1.5 x 10^6 cells/ml. After about 1 minute at 0°C, Triton-X was added to a final concentration of 0.5% and dithiothreitol to 0.001 M and the cells were lysed by gentle pipetting for 3 minutes at 0°C. The nuclei were sedimented for 3 minutes at 800 x g and resuspended in swelling buffer at 10^7 - 4 x 10^7 nuclei/ml. 0.05 ml samples of nuclear suspension were used per assay.

RNA was synthesized by incubating nuclei for 30 minutes at 37°C in a total volume of 0.15 ml in 4% glycerol, 0.001 M dithiothreitol, 0.01 M $MgCl_2$, 0.01 M Tris-HCl pH 7.9, 0.05 M adenosine triphosphate, 0.001 M cytidine triphosphate and guanosine triphosphate, 0.1 μCi of ^3H-UTP (26 Ci/mmole) in the presence and absence of 0.9 μg of α-amanitin/ml. in different concentrations of NH_4Cl.

RNA was extracted by the method of Scherrer (1969) and hybridized to SV40 immobilized on filters as described by Martin (1969).

Each point represents an average of the percent of input counts bound for two different RNA concentrations. Backgrounds have not been subtracted.

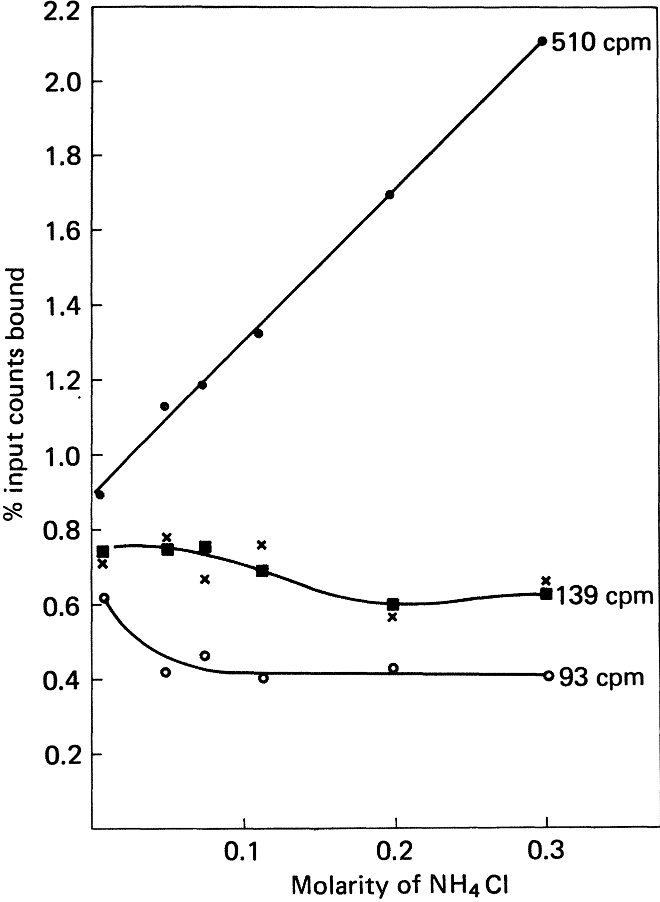
(Data from Jackson and Sugden (1972))

●—● percent input counts synthesized without α-amanitin bound to SV40 DNA filters

o—o percent input counts synthesized without α-amanitin bound to blank filters

X—X percent input counts synthesized with α-amanitin bound to SV40 DNA filters

■—■ percent input counts synthesized with α-amanitin bound to blank filters


2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
% input counts bound
510 cpm
139 cpm
93 cpm
0.1
0.2
0.3
Molarity of NH_4Cl

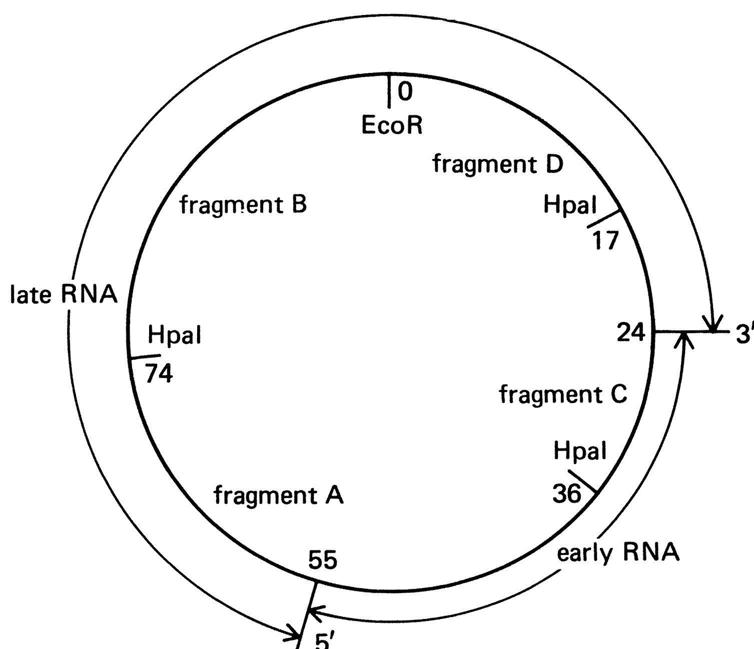


FIG. 3. A map of the SV40 genome. The cleavage site of E. coli EcoRI endonuclease is at zero and map distances are given as percentage lengths of SV40 in a clockwise direction. The positions of the Hpa I cleavage sites are taken from Danna et al. (1973) and Sharp et al. (1973). The assignment of the early and late regions of SV40 DNA, which is described in the text, assumes that early RNA is transcribed from a contiguous portion of the viral genome.

interpretation of these experiments is that in monkey cells late in SV40 infection, it is host polymerase II that transcribes the viral DNA; because α-amanitin totally inhibits the synthesis of SV40-specific RNA, we conclude that in all probability the same enzyme is responsible for the synthesis of both early and late sequences of viral RNA. However, we cannot rule out the possibility of a viral-coded polymerase or a viral modified host polymerase.

3. Mapping of Early and Late Regions of the Viral Genome and Orientation of RNA Synthesis

Central to the progress we have made in attacking these problems has been the availability of a suite of restricting endonucleases--enzymes which introduce double-strand cuts into DNA at particular nucleotide sequences. Danna and Nathans (1971) were the first to realize the value of these enzymes for dissecting the

SV40 genome, and the eleven fragments that they obtained after cleavage of SV40 DNA by endonuclease R·Hin (H. influenzae) have been precious objects in studies of replication (Nathans and Danna, 1972) and transcription of the viral genome (Khoury et al., 1973b). In our laboratory, we have used four specific fragments of SV40 DNA generated by sequential cleavage of closed, circular viral DNA by endonucleases EcoRI (E. coli) and Hpa I (H. parainfluenzae) (Sharp, Sugden, and Sambrook, 1973) to map the location of the early and late regions of the viral genome (Sambrook et al., 1974). The four specific fragments correspond to 38%, 26%, 19%, and 17% of the total viral genome and their location is known (Sharp et al., 1973; see Figure 3).

^{32}P-labeled SV40 component I DNA was digested sequentially with endonucleases EcoRI and Hpa I. After separation through 0.7% agarose, 2.2% polyacrylamide gels (Figure 4), the strands of each of the DNAs were separated using asymmetric complementary RNA as described above. The separated strands of each fragment were sheared and hybridized to unlabeled RNA extracted from the polysomes of monkey cells at different times after infection. The results are shown in Figure 5 (lefthand column). "Early" RNA hybridized only to part of the E strand of fragments A and C and

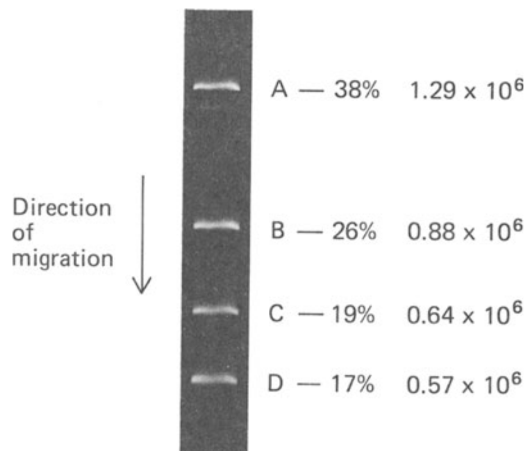


FIG. 4. Photograph of an 0.7% agarose, 2.2% polyacrylamide gel after separation of the four specific fragments of SV40 DNA. 2 μg of component I SV40 DNA was digested sequentially with EcoRI and Hpa I as described by Sharp et al. (1973). The fragments were separated by electrophoresis through an 0.7% agarose, 2.2% polyacrylamide gel for 12 hours at 4 v/cm as described by Pettersson et al. (1973). The gel was stained for 30 minutes in a solution of 0.4 μg/ml ethidium bromide and examined directly by short-wavelength ultraviolet illumination.

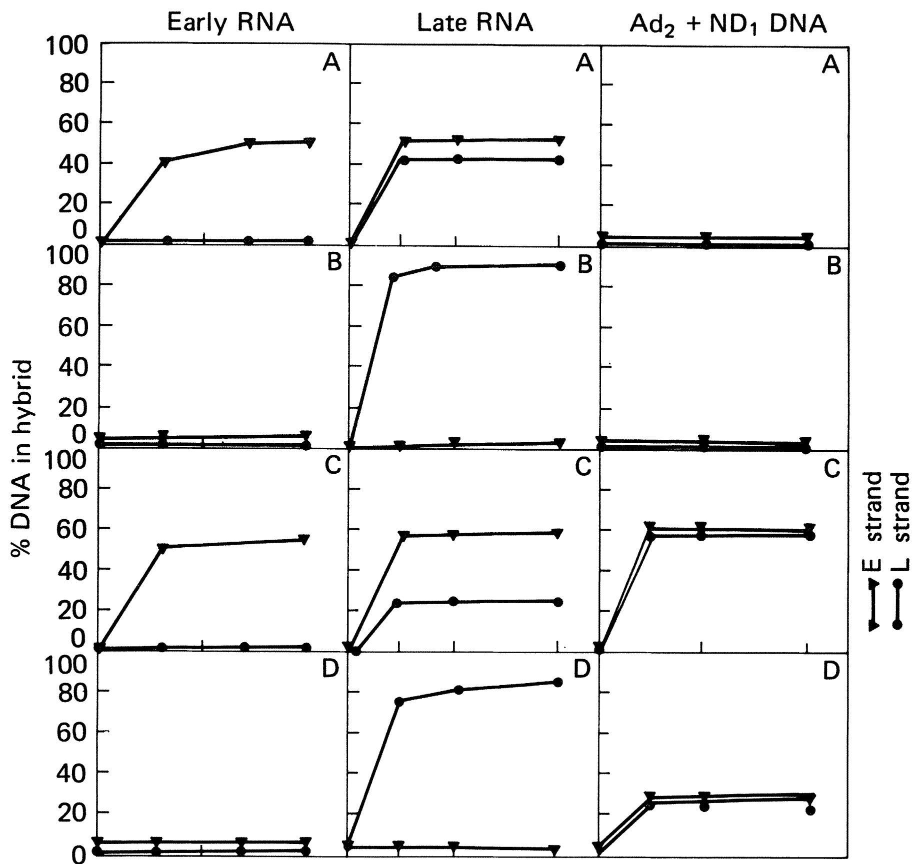


FIG. 5. Hybridization of early and late polysomal RNA to the separated strands of the EcoRI - Hpa I fragments of SV40 DNA. The separated E and L strands of ^{32}P-labeled SV40 fragments were boiled for 30 minutes in 0.3 N NaOH, neutralized and hybridized for 36 hours at 68°C to increasing amounts of early RNA (left column) polysomal "late" RNA (center column) or Ad2+ND1 DNA (right column). Each hybridization mixture contained about 250 cpm of ^{32}P-labeled DNA in total volume of 0.1 ml of 0.14 M sodium phosphate pH 6.8, 0.4% SDS, 1 M NaCl. The proportion of denatured and hybrid ^{32}P-labeled DNA was determined by chromatography on hydroxylapatite. (From Sambrook et al., 1974).

not to DNA derived from any other portion of the viral genome. At saturating levels of RNA, about 50% of the E strand sequences of fragment A and 60% of the E strand sequences of fragment C entered hybrid. The sum of these percentages is equivalent to 31% of the sequences of the entire E strand of SV40 DNA--a figure which agrees well with the previously published estimates given for the length of the early region of the viral genome. If early RNA is transcribed from a contiguous region of the viral genome, we calculate that one

end of the early region is located at position 55 and the other at position 24 on the SV40 map (see Figure 3).

To check the precision of these estimates we hybridized sheared DNA of the adenovirus 2 SV40 hybrid, Ad2$^+$ND1 (Levin et al., 1971) which contains the SV40 sequences located between map positions 12 and 28 (Morrow and Berg, 1972). Because the border between fragments C and D is located at map position 17, 58% of the sequences of fragment C and 29% of those of fragment D are present in Ad2$^+$ND1 DNA. We found that at saturation, 62% of the sequences of the E and L strands of fragment C and 32% of the E and L strands of fragment D formed duplexes with Ad2$^+$ND1 DNA (see Figure 5, righthand column). These values are very close to those predicted and for this reason, we believe that our estimates for the percentages of fragments A and C that are transcribed into RNA are not seriously in error.

When RNA extracted from polysomes late in lytic infection was hybridized to the separated strands of each fragment, the results shown in Figure 5 (center panel) and Table 1 were obtained. At saturating levels of RNA, 45% of the L strand and 52% of the E strand of fragment A, 90% of the L strand and none of the E strand of fragment B, 26% of the L strand and 60% of the E strand of fragment C and 92% of the L strand and none of the E strand of fragment D behaved as hybrid. The sum of these percentages is equivalent to 64% of the sequences of the L strand and 31% of those of the E strand--figures which are in good agreement with results obtained with unfractionated DNA.

From these data we conclude that 1) all the early functions of SV40 are located within fragments A and C; 2) all the sequences of fragments B and D and part of the sequences of A and C code for late functions; 3) if all the early genes of SV40 are contiguous, then the strand switches between early and late RNAs occur at positions 55 and 24.

The final question asks whether the synthesis of early RNA occurs in a clockwise or counterclockwise direction on SV40 DNA. In the experiment shown in Figure 6 we took advantage of the fact that endonuclease EcoRI has been shown to cleave SV40 DNA at a single site (Morrow and Berg, 1972; Mulder and Delius, 1972) by making single-strand scissions that are four-bases apart (Mertz and Davis, 1972). Because the resulting linear molecules contain a 3' hydroxyl group and a protruding 5' phosphoryl tail, they serve as excellent primer templates for DNA synthesis (Hedgpeth, Goodman, and Boyer, 1972) and we were able to incorporate ^{3}H-dTMP at the ends of the molecules using RNA-dependent DNA polymerase isolated from avian myeloblastosis virus. The DNA was then cleaved with endonuclease R·Hpa I and the four DNA fragments were separated by electrophoresis, denatured and hybridized to asymmetric SV40

TABLE 1. Hybridization of the separated strands of SV40 DNA fragments to late polysomal or late total cell RNA

		% of ^{32}P-labeled DNA entering hybrid at saturation	
Fragment	Strand	Late polysomal	Total cell RNA
A	E	52	50
A	L	45	68
B	E	0	24
B	L	90	92
C	E	60	60
C	L	26	75
D	E	0	25
D	L	92	90
SV40	E	34	44
SV40	L	70	77

(From Sambrook et al., 1974.)

cRNA. Because the ^{3}H-dTMP was incorporated only into the two fragments (B and D) which flank the EcoRI site (see Figures 3 and 6), and because of the nature of the cleavage produced by this enzyme, the ^{3}H-dTMP must be present in one of the two DNA strands of fragment B and in the opposite strand of fragment D. Thus, the ^{3}H counts incorporated into only one of the two fragments should hybridize to asymmetric cRNA. The results given in Table 2 show that whereas less than 6% of the ^{3}H counts of fragment D were complementary to cRNA, greater than 90% of the ^{3}H counts in fragment B annealed to cRNA. Because RNA-dependent DNA polymerase catalyzes the incorporation of nucleotides into DNA in a 5' - 3' direction, and because fragment B lies to the left of the EcoRI site, then the direction of DNA synthesis at the end of fragment B must be clockwise, as drawn on the conventional SV40 map (see Figure 3). Asymmetric cRNA, therefore, must be made in a counterclockwise direction; because cRNA and early RNA are both complementary to the E strand of SV40 DNA, then early RNA also must be synthesized in a counterclockwise orientation. A similar conclusion has been reported by Khoury et al. (1973b). Since late RNA hybridizes to the L strand of SV40 DNA, then it must be made in a clockwise direction. These results mean that the 5' ends of both early and late RNA are located in fragment A, and the 3' ends in fragment C.

From all this, it is clear that we know a good deal about the nuts and bolts of transcription of SV40 during the lytic cycle. Unfortunately, our understanding of the way viral RNA synthesis is

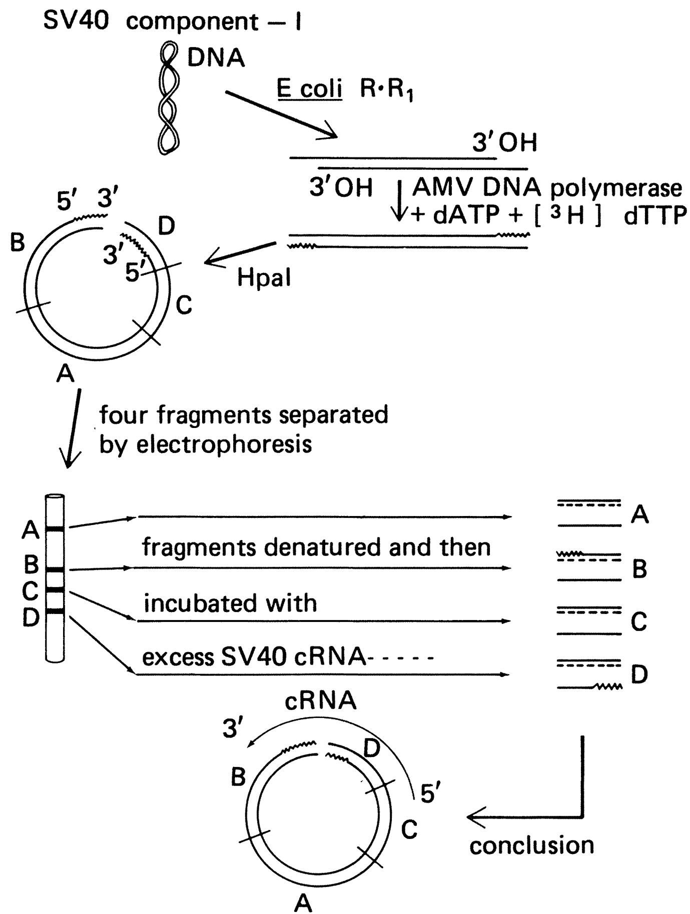


FIG. 6. Determination of the direction of synthesis of SV40 cRNA. For details see text (from Sambrook _et al._, 1974).

TABLE 2. Hybridization to cRNA of fragments of SV40 DNA specifically labeled at the EcoRI cleavage site

	-cRNA				+cRNA			
	^{32}P		^{3}H		^{32}P		^{3}H	
DNA fragment	cpm*	% double-stranded	cpm*	% double-stranded	cpm*	% double-stranded	cpm*	% double-stranded
A	272/3972	6.8	----	----	1694/3810	44.4	----	----
B	184/2805	6.5	17/261	6.5	1467/2957	49.6	260/285	91.2
C	139/2014	6.9	----	----	931/1835	50.7	----	----
D	143/2183	6.5	12/215	5.6	1083/2175	50.8	7/221	3.2

^{32}P-labeled SV40 EcoRI linear RNA was labeled with ^{3}H-dTMP at the Eco cleavage site and the four specific fragments were isolated as described in the legend to Figure 4. Approximately 0.02 μg of the DNA of each fragment was denatured by boiling and allowed to reanneal in the absence or in the presence of 2 μg of cRNA. The reaction was carried out for 15 min at 68°C in 1 ml of 0.14 M sodium phosphate pH 6.8, 0.4% SDS and the hybrids were assayed by chromatography on hydroxylapatite. (From Sambrook et al., 1974).

controlled, and the role of such regulation in the overall management of SV40 gene expression is very poor. It seems likely, however, that at early times after infection, viral RNA synthesis is used as the major determinator of viral function, because we can detect transcripts complementary to only a part of the sequences of one strand of the viral DNA. It seems fair to think that the reason that late viral functions are not expressed is because "late" viral RNA is not present. There are three main hypotheses to explain how this regulation is achieved: Firstly, it may be that the state of the viral DNA is such that synthesis of late viral RNA is impossible. Perhaps the viral DNA, after it has shed its outer coats, retains some internal proteins which block RNA polymerase; or maybe the determining factor is whether the viral DNA is integrated or not. Secondly, it may be that the RNA polymerase that transcribes the early region of SV40 cannot synthesize RNA from the "late" genes. SV40 particles do not contain DNA-dependent RNA polymerase, so that early RNA must be transcribed from the incoming viral DNA by a pre-existing cellular RNA polymerase. It is conceivable that this

polymerase can recognize only the early promoter(s) on the viral genome. Thirdly, it is possible that "late" RNA is made at all stages of infection but is rapidly broken down at early times. As far as we know, no experiments have been done specifically to test this idea; indeed, in a certain sense, it cannot be tested. One could always claim that no matter how short the pulse-label, no matter how sensitive the hybridization technique, the viral RNA is broken down too rapidly to be detected. However, we can put an upper limit on the number of such unstable late viral RNA molecules using data from the hybridization experiments that have been performed using the separated strands of SV40 DNA. Assuming that each cell contains 10^{-5} μg of RNA, it turns out that at early times after infection, there are less than 4 molecules of late RNA present per cell.

The mechanism of the shift from "early" to "late" RNA synthesis which occurs at about the time that viral DNA replication begins is unknown but there are three possible explanations: Firstly, there could be an alteration of the physical state of the viral genome, either as a consequence of removal of internal proteins, or excision from an integrated state, or during DNA synthesis, so that new regions of the DNA become available for transcription. Secondly, there could be a synthesis of a new virus-coded RNA polymerase which recognizes the late SV40 promoter(s). Alternatively, there could be a virus-induced modification of a preexisting host polymerase. Thirdly, it may be that at late times after infection, the entire sequence of both strands of SV40 DNA begins to be copied into RNA, and control is carried out by post-transcriptional processing. This suggestion merits serious consideration in the light of the experiments of Aloni (1972, 1973) who has shown that late after infection with SV40, cells contain short-lived species of RNA which self-anneal and seem to be transcribed from the entire sequence of SV40 DNA. In addition, we have reported (Sambrook _et al._, 1974) that when ^{32}P-labeled E and L strand DNA of each of the four specific SV40 fragments was hybridized with the _total_ RNA extracted from cells late in lytic infection, results were obtained which were very different to those observed with polysomal RNA. For each of the four fragments, the sum of the transcripts of the E and L strands amounted to much more than the equivalent of the DNA strand (see Table 1 and Figure 7). From the results shown, it is clear, (1) that _total_ cell RNA contains sequences complementary to a minimum of 77% of the L strand and 44% of the E strand of SV40 DNA; and (2) that symmetric transcription of at least 20% of the sequences of fragment A, 14% of fragment B, 15% of fragment C and 17% of fragment D are present in cells at late times after infection. The origin and fate of these symmetric sequences is not clear; if control is exerted at the post-transcriptional level, they could indeed by the primary transcription products and the precursors of stable, asymmetric poly-

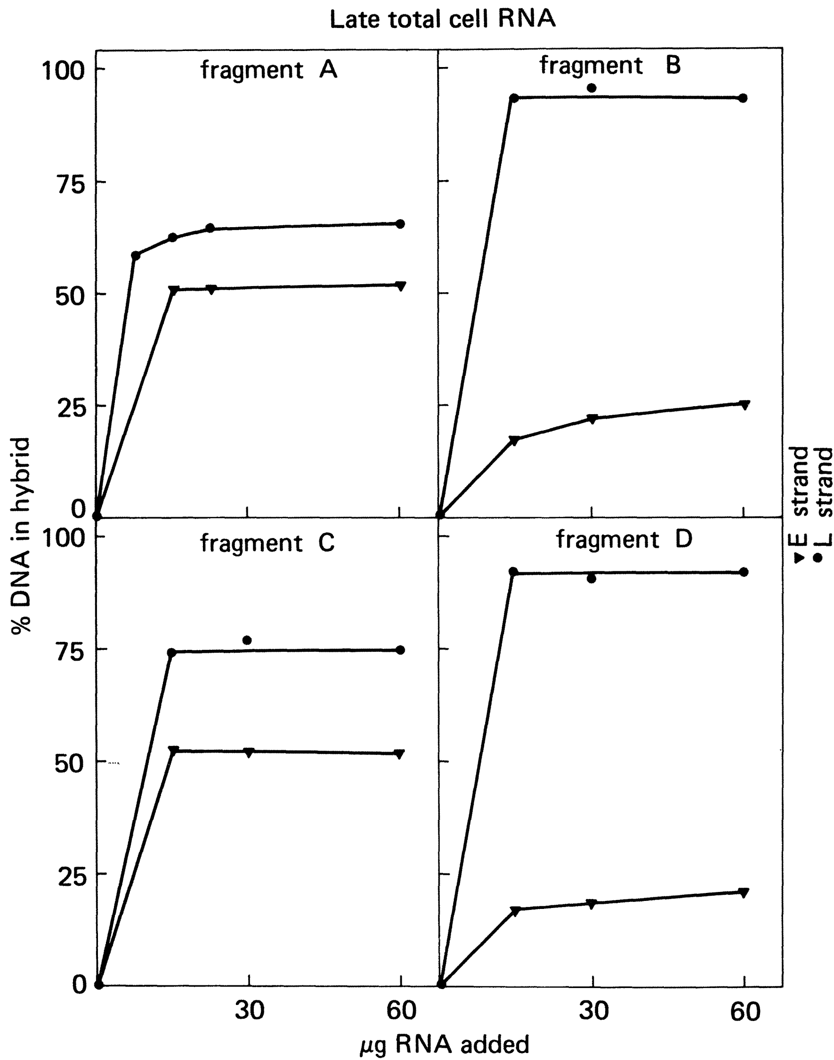


FIG. 7. Hybridization of total cell RNA extracted at late times after infection to the separated strands of the EcoRI - Hpa I fragments of SV40 DNA. Total cell RNA was extracted (Scherrer, 1969) 48 hours after infection of BSC-1 cells with 50 plaque forming units of SV40 per cell, and hybridized to the separated strands of the four fragments of SV40 DNA as described in the legend to Figure 5.

somal RNA as Aloni has suggested (1972, 1973). However, it is also conceivable that they may be aberrant transcription products originating not from the free replicating pool of SV40 DNA but from the viral sequences which become integrated into the host genome during lytic infection (Hirai and Defendi, 1972). In this case, they may play no functional role in SV40 gene expression and may be no more than flotsam drifting around in a degenerating cell. It seems to us that the best hope of resolving this problem lies in the isolation of discrete species of SV40 RNA, both transient and stable, which can be mapped against specific fragments of SV40 DNA.

Before leaving transcription of SV40 during the lytic cycle, we should point out two aspects that need further analysis. Firstly, we do not know whether the early region is one contiguous piece of the SV40 genome. There is no evidence so far published to suggest that the early genes are broken into two or more clumps, but the data obtained by Khoury et al. (1973b) and by ourselves (Sambrook et al., 1974) do not rule out such a possibility. Secondly, there is a discrepancy between the size of the early region of SV40 calculated from hybridization data, and the size of early SV40 messenger RNA obtained by velocity centrifugation through density gradients. All the published hybridization data indicate that the early region accounts for about 30% of the total SV40 genome--that is equivalent to a weight of single-stranded RNA of 6×10^5 daltons. Yet the velocity centrifugation experiments performed by several groups show that early SV40 messenger RNA has an apparent molecular weight of 9×10^5 (Tonegawa et al., 1970; Weinberg, Warnaar, and Winocour, 1972). Assuming that both pieces of data are correct, the only way to explain the discrepancy is that there are host RNA sequences attached to the viral transcript. Quite clearly, this hypothesis which has many implications for the way in which early SV40 RNA synthesis is controlled needs to be tested extensively.

B. TRANSFORMED CELLS

Mouse cells stably transformed by SV40 contain viral DNA sequences in an integrated state (Sambrook et al., 1968). The quantity of viral DNA differs from cell line to cell line. Some contain as little as 1-2 copies of viral DNA per diploid quantity of cell DNA (Gelb, Kohne, and Martin, 1971; Ozanne, Sharp, and Sambrook, 1973), others as much as 9-10 copies (Ozanne et al., 1973). Virus-specific RNA is invariably present (Benjamin, 1966; Khoury et al., 1973a; Ozanne et al., 1973) and from saturation hybridization data it appears that about 0.001-0.002% of the total cellular RNA is complementary to SV40 DNA (Sambrook et al., 1973). We have used the separated strands of SV40 DNA to measure the percentage of the viral genome that is transcribed into RNA in different cell lines, and we have mapped the position of these sequences using the four specific fragments of SV40 described previously.

TABLE 3. Percentage of the SV40 genome transcribed in different transformed cell lines

Cell line	Experiment	Percentage of viral genome transcribed	
		E strand	L strand
SV3T3 clone 9	1	73	15
	2	68	14
	3	67	17
SV101	1	77	3
	2	68	5
	3	72	3
SVT2	1	58	0
	2	52	0
	3	49	0
SVB30	1	62	11
	2	65	10
SVPy11	1	54	0
	2	58	0
	3	65	0
SVuv30	1	24	22
F1SV10	1	32	3
	2	36	3
3T3		0	0

For origin of cell lines and methods, see Ozanne et al., 1973.

1) The Extent of Transcription of SV40 DNA in Different Lines of Transformed Cells

Separated strands of ^{32}P-labeled SV40 DNA prepared as described elsewhere (Sambrook et al., 1972) were sheared and hybridized exhaustively to increasing amounts of RNA.

The data obtained with RNA extracted from four SV40 transformants are given in full in Figure 8 and the results of experiments on other cell lines are summarized in Table 3. The striking conclusion is that nearly all the cell lines contain RNA complementary to more sequences of E-strand DNA than do lytically-infected cells.

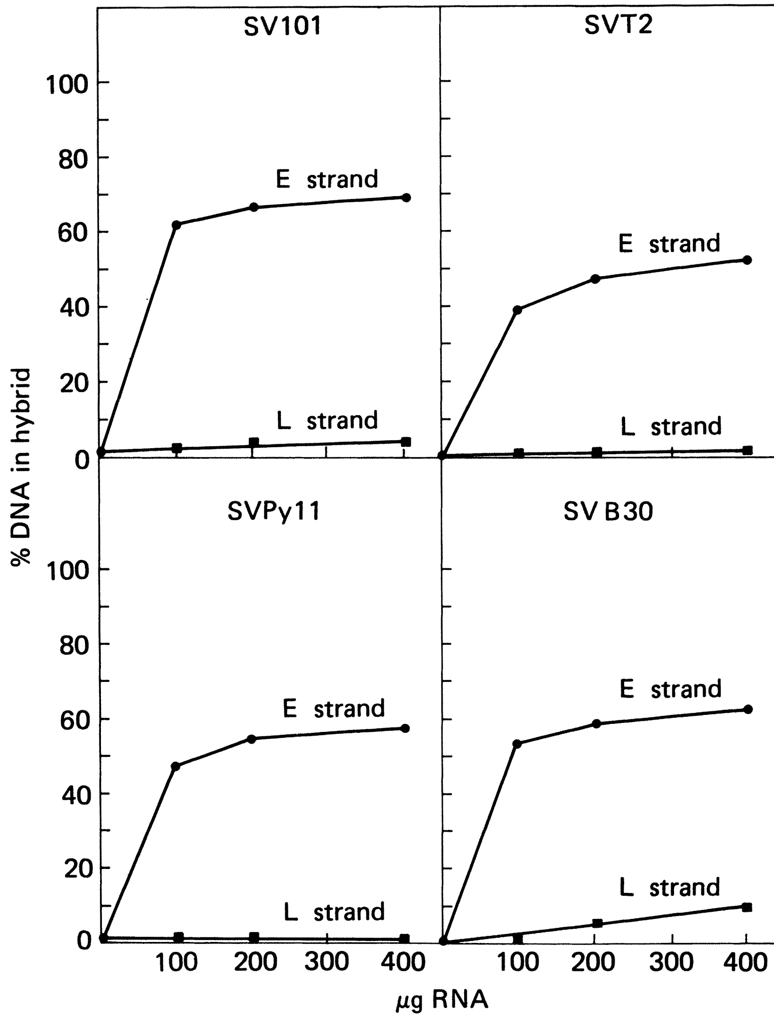


FIG. 8. Hybridization of the separated strands of total SV40 DNA to RNA isolated from transformed cells. Hybridization mixtures contained 2 x 10^{-3} μg of sheared ^{32}P-labeled E or L-strand DNA (specific activity 9 x 10^5 cpm/μg) prepared as described earlier (Sambrook et al., 1972) and different amounts of RNA in a total volume of 0.125 ml of 1 M NaCl, 0.0002 M EDTA, 0.001 M Tris pH 7.5. After incubation for 36 hours at 68°C, the percentage of DNA reacting with RNA was assayed by chromatography on hydroxylapatite. (From Ozanne et al., 1973).

At no time during productive infection are more than 35-40% of the sequences of SV40 E-strand DNA detectable in stable species of RNA; consequently, at least a part of the viral RNA present in transformed cells must consist of anti-late sequences.

Some, but not all, lines of SV40-transformed mouse cells contain low levels of RNA that hybridize to L-strand DNA. Because we have never been able to add enough RNA into the hybridization mixtures to saturate the ^{32}P-labeled DNA probe, we do not know what fraction of the L strand sequences is transcribed in these cell lines. But we are sure that this RNA plays no role in maintaining the transformed state, since it is not present in all SV40-transformed cell lines.

2) Mapping of SV40 RNA Sequences in Transformed Cells

The separated strands of each of the four ^{32}P-labeled fragments of SV40 DNA were prepared as described elsewhere (Sambrook et al., 1974), sheared to a size of 300 nucleotides and hybridized to RNA extracted from three lines of mouse cells independently transformed by SV40. The results are shown in Figure 9. From the shape of the saturation curves, it is clear that transformed cells contain at least two types of virus-specific RNA. There are RNA sequences that are present in high abundance, and which hybridize to the part of the E-strand DNA of the contiguous fragments A and C. One line of cells (SV101) contains an additional abundant species of RNA that is complementary to part of the E-strand of fragment B. In addition, all three of the cell lines contain less abundant species of virus-specific RNA. These hybridize to parts of the E-strand sequences of fragments A and C and also in the case of SV101 to the L-strand DNA of all four fragments. Because these RNAs are present in very low concentrations, we were never able to reach saturation and we cannot calculate the exact percentage of the sequences of any fragment that are transformed by RNA.

The simplest patterns of viral transcription are shown by SVT2 and SVPy11 cells. The RNAs present in high concentrations are those which hybridize to the E-strand fragments A and C. For two reasons it seems highly likely that these RNA sequences are identical to those found in permissive cells at early times after infection. Firstly, competition hybridization experiments have established that there is a high degree of sequence homology between early RNA and transformed cell RNA (Oda and Dulbecco, 1968a; Aloni et al., 1968; Sauer and Kidwai, 1968) and, secondly, the percentage of the sequences of fragments A and C that anneal to early and to transformed cell RNA is very similar (Sambrook et al., 1974). For these reasons, it is probable that the viral function responsible for maintenance of transformation is an early gene product--an idea

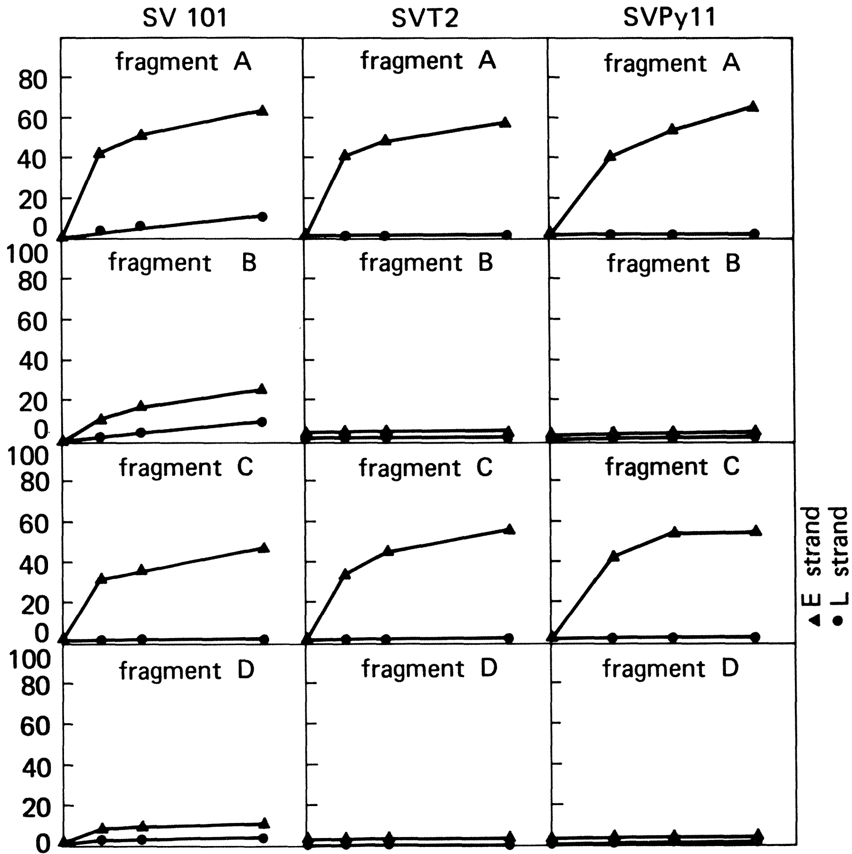


FIG. 9. Hybridization of RNA from three lines of transformed cells to the separated strands of the EcoRI - Hpa I fragments of SV40 DNA. For experimental details, see legend to Figure 5.

which has already been suggested by others on the basis of genetic experiments (see review, Eckhart, 1972).

SV101 cells contain a more complicated set of viral RNAs than do the two other cell lines. The highly abundant RNA species are complementary not only to part of the E-strand DNAs of fragments A and C, but also to part of the E-strand of fragment B. In addition, there are low abundance RNAs complementary to part of the E-strand DNAs of fragments A and C and to the L-strand DNAs of all four fragments. We feel that the explanation of this plethora of RNA species lies in the large quantity of viral DNA sequences present in SV101 cells. This cell line contains about 9 copies of SV40 DNA per diploid quantity of cell DNA, and it does not seem too outrageous to propose that many of the viral sequences found in SV101 cells are transcribed from copies of the viral genome which are not required to maintain the cells in their transformed state.

The model we have proposed (Sambrook _et al._, 1972; Ozanne _et al._, 1973) to account for the patterns of viral RNA synthesis in transformed cells is based on three premises:

(i) that SV40 DNA integrates into host DNA with a break somewhere in its late genes, thereby preserving intact those viral functions which are required to maintain cells in the transformed state;
(ii) that integration does not necessarily occur at the same site within the viral genome in different cell lines; and,
(iii) that integration places viral DNA under the control of host promoter(s).

Given these assumptions, we can generate a simple model which is consistent with all the data so far accumulated. After initiating transcription at a host promoter, the RNA polymerase enters the integrated viral DNA on the E-strand somewhere in the late region of the genome. It continues along SV40 sequences synthesizing first "antilate" and then "early" RNA, before termination occurs at the 3' end of the early genes. Assuming that, in different cell lines, SV40 is integrated with breaks at different positions in the viral genome, then the percentage of the viral genome that is transcribed will vary, depending on the distance between the integration site and the 5' end of the early genes (position 55). Because the transcription of the integrated genome is under the control of a host promoter, RNA molecules should be synthesized that are considerably larger than a single-strand of SV40 DNA and that contain covalently linked host and viral sequences. Such molecules are in fact present in the nuclei of at least one line of SV40-transformed cells (Lindberg and Darnell, 1970; Wall and Darnell, 1971). To account for the observation that the viral sequences

present in the cytoplasm of these cells are not attached to host sequences (Wall and Darnell, 1971), it has been proposed that the primary transcription product undergoes processing during which polyadenylic acid residues are attached to the 3' hydroxyl end and the host sequences are removed from the 5' end. In order to explain the unequal representation in RNA of different regions of the SV40 genome, we suggest that processing of the primary transcript includes not only the host but also the "antilate" viral sequences which lie between the promoter and the beginning of the true early RNA.

Clearly, this model is tentative and several of its major predictions could turn out to be wrong. For instance, we have no evidence that the 3' end of the early genes really is a terminator for RNA synthesis--it could equally well be a processing point. And we cannot be certain that the only way to transcribe viral DNA is by synthesis of hybrid host-virus RNA molecules--we could explain the presence of viral RNA sequences in varying abundances if there is an early viral promoter which is active at least some of the time. Finally, it may turn out that integration in different cell lines always occurs at the same position in the SV40 genome and that the inconstancy of the proportion of the viral DNA that can be detected in RNA is the result of variations in the efficiency of processing from cell line to cell line. All of these points can be tested, and in one sense it hardly matters which of them turns out to be correct. The omphalos of this work is that cells transformed by viruses such as SV40 contain defined DNA sequences in a defined physical state; if we can understand the way that the expression of these sequences is controlled, we can reasonably hope to discover not only how viruses transform cells, but also how cells conduct their normal business.

Parts of this article have been used in a paper entitled "Transcription of the Smaller DNA Tumor Viruses" that was given at the Twenty-Fifth Symposium of the Society for General Microbiology and published by Cambridge University Press 1975.

ACKNOWLEDGEMENTS

This research was supported by grants from the National Cancer Institute and the American Cancer Society.

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THE STRUCTURAL PROTEINS OF SV40 AND POLYOMA VIRUS

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INTRODUCTION

Both SV40 and polyoma virus have as their genome a single DNA molecule containing approximately 5500 base pairs (Tai *et al.*, 1972). Each virus can therefore code for only a small number of polypeptides and the sum of their molecular weights cannot exceed 200,000 daltons.

Only approximately one half of the genetic information of the viruses is expressed early after infection, i.e. prior to the synthesis of viral DNA. Structural viral proteins are not synthesized before late after infection and are coded by the other half of the genome (Khoury, Burne, and Martin, 1972; Lindstrom and Dulbecco, 1972; Sambrook, Sharp, and Keller, 1972).

In the present work, we have analyzed the structural proteins of polyoma and SV40. Evidence is presented that the smaller viral polypeptides are similar for both viruses and that they consist of histones derived from the host cell.

MATERIAL AND METHODS

Virus Growth and Purification

Wild type polyoma virus (large plaque) was grown at high multiplicity on primary mouse kidney cells. SV40 of strain 777 was passaged and grown at high multiplicity on established African

green monkey cells (MA 134, BSC-1). Four days after infection, cells were frozen and thawed and the lysate sonicated. Cell debris were removed by low speed centrifugation and then the virus was pelleted by spinning at 60,000 g for 3 hours. The pellet was homogenized in a CsCl solution of a density of 1.32 g/cm^3 and centrifuged for 20 hours at 35,000 rpm in a SW 50.1 Spinco rotor. Bands of virus and empty capsids were visible, and they were removed from the side of the tube with a syringe. After dialysis the samples were purified by sedimentation in a 5-20% sucrose gradient at room temperature for 20 min. at 40,000 rpm in the same rotor. The virus band moved about halfway down.

Radioactive Labeling

^{35}S-methionine (specific activity >100 Ci/mM, obtained by hydrolysis of ^{35}S labeled E. coli) was added 24 h after infection at concentrations of 10-50 μC/ml in met-free medium. Five hours later, normal medium was added. ^{3}H-arginine and ^{14}C-arginine was purchased from Amersham and added at the indicated times in arg-free medium.

SDS-polyacrylamide gel electrophoresis and estimations of molecular weight were carried out according to Weber and Osborn (1969). Bovine serum albumin, aldolase, L-chymotrypsinogen and cytochrome C (from Boehringer Mannheim) were used as standards. High resolution gels were made with the discontinuous buffer system of Laemmli (1970). Virus was disrupted by boiling in sample buffer containing 5% of sodium dodecyl sulfate and 5% beta-mercaptoethanol.

Peptide Analysis

The gel regions containing the proteins were cut out and incubated in 0.1% sodium dodecyl sulfate, 0.1 M ammonium carbonate pH 8 for 24 hours in the presence of bovine serum albumin as carrier. The polypeptides were precipitated with trichloracetic-acid (20% final concentration) at 4°C overnight. The pellet was washed with acetone and with ether to remove traces of sodium dodecyl sulfate. The proteins were dissolved and then oxydized with performic acid for 1 hour at 4°C, lyophilized twice and dissolved in 0.05 M ammonium carbonate pH 8.5. Digestion with TPCK trypsine (Worthington) was performed for 3 hours at 37°C at a substrate to enzyme ratio of 40. The sample was then lyophilized and dissolved in 20 μl electrophoresis buffer (pyridine:acetic acid:water 5:50:945, pH 3.5) and applied to a cellulose thin layer plastic sheet (Mackerey and Nagel, Dueren, W. Germany). Electrophoresis was performed for 90 min at 13'000 volt under Varsol. The methionine containing peptides were revealed by autoradiography.

RESULTS

Disrupted polyoma virus shows six polypeptides on polyacrylamide gels (Figure 1). The three smallest polypeptides are absent in empty capsids. The major capsid protein migrates with a mobility corresponding to a molecular weight of 47,000 daltons. Two minor polypeptides of the capsid have molecular weights of 35,000 and 23,000 daltons respectively. The small polypeptides which seem to be contained in the core of the virus have molecular weights in the range of 10,000 to 15,000 daltons.

If the viral proteins are analyzed by high resolution gels, some of the bands split up into doublets (Figure 2), and at present we do not know the reason for this.

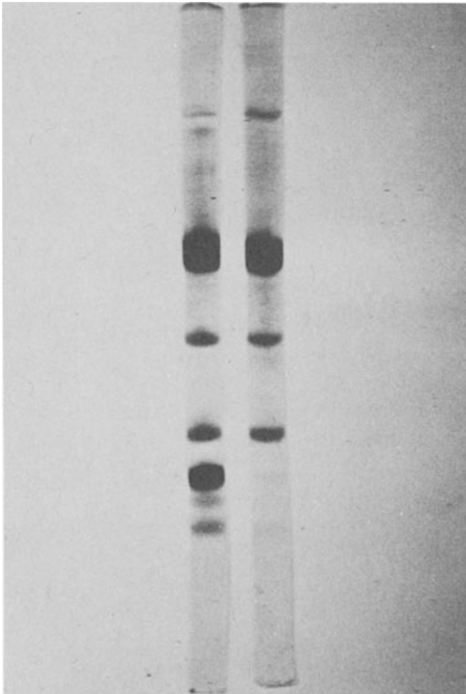

FIG. 1

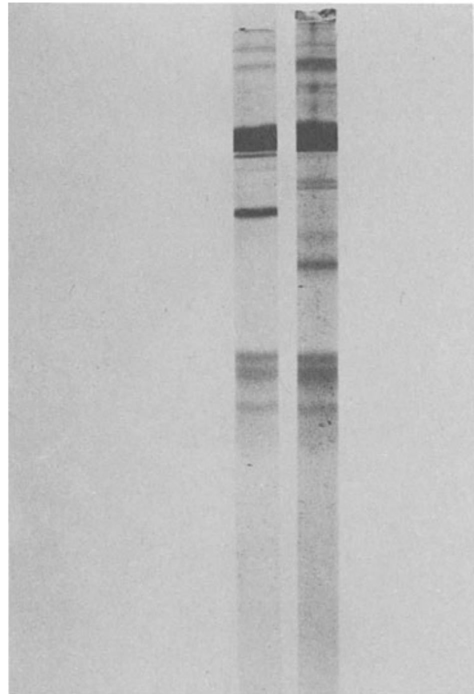

FIG. 2

FIG. 1. SDS-polyacrylamide gels of disrupted polyoma virus (left) and polyoma empty capsids (right). 12% polyacrylamide according to Weber and Osborn. Approx. 150 μg of protein per sample, stained with Coomassie Brilliant Blue.

FIG. 2. SDS-polyacrylamide gels of disrupted SV40 (left) and polyoma virus (right). 15% polyacrylamide according to Laemmli.

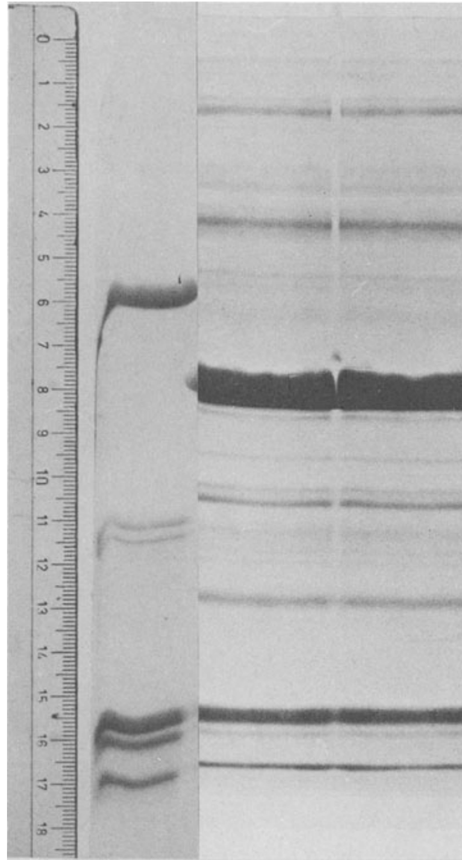

FIG. 3. SDS-polyacrylamide-slab gels of purified calf thymus histones (left, top band is a bovine serum albumine marker) and polyoma virus (right).

A comparative gel analysis of the proteins of SV40 and polyoma is given in Figure 2. The major capsid protein of SV40 is slightly smaller than the one of polyoma, and it is always preceded by a satellite band, the function of which is not known yet. An additional capsid protein migrates between the minor capsid proteins of polyoma and has a molecular weight of 30,000 daltons. The non-capsid proteins of the two viruses display the same electrophoresis pattern.

Disruption of the SV40 particles in alkaline buffer (pH 10.5) leads to a complex of DNA and non-capsid proteins (Huang, Estes, and Pagano, 1972).

The non-capsid proteins of polyoma virus have been analyzed by Frearson and Crawford (1972), and they came to the conclusion that these proteins are histones of the host cell. To check this finding, purified histones from calf thymus were compared by gel electrophoresis to polyoma proteins. As seen in Figure 3, the polyoma non-capsid proteins migrate like histones.

To characterize these proteins one step further, the methionine containing peptides were analyzed. ^{35}S-met labeled virus was prepared as described in material and methods. Growing uninfected cells were labeled in the same way, the nuclei prepared by use of Nonidet P-40 and the acid soluble proteins extracted in 0.1 N HCl. They were electrophoresed on gels, and the bands corresponding to

Basic Proteins — Fingerprint
Mouse Cell—Polyoma Comparison

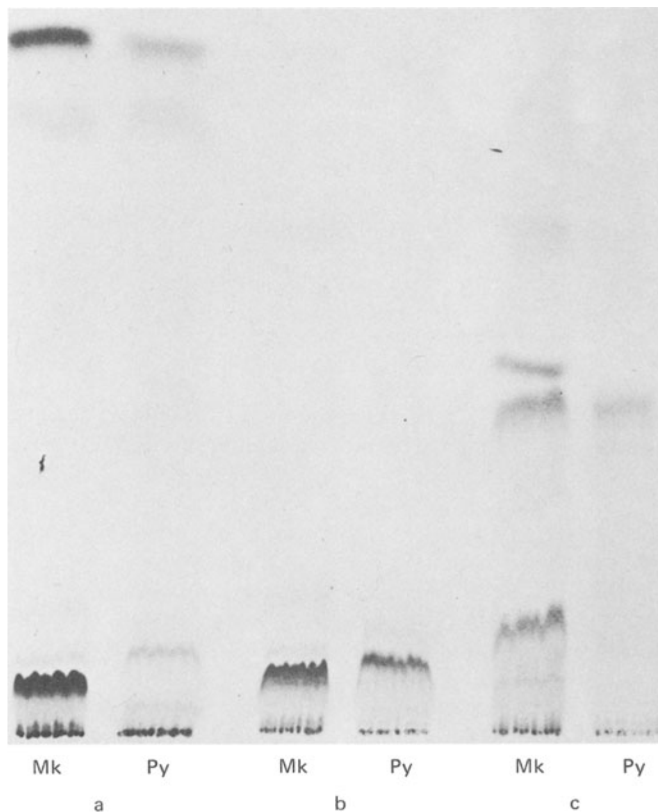

FIG. 4. Autoradiograph of ^{35}S labeled tryptic peptides separated by electrophoresis on thin layer. Comparison of mouse kidney (MK) and polyoma (Py) histones. a) slowest moving polypeptide on gels, c) fastest moving polypeptide.

Basic Proteins — met^{35}S Fingerprint
Polyoma–SV40 Comparison

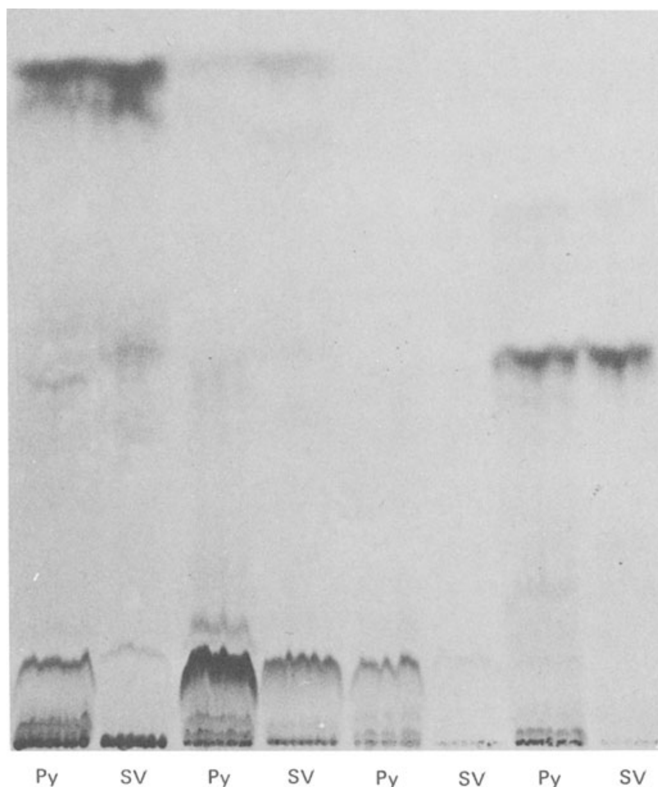

FIG. 5. As Figure 4, but comparison between polyoma (Py) and SV40 (SV). Slowest polypeptide to the left, fastest to the right.

the small viral proteins were eluted and subjected to peptide analysis as described in material and methods.

Figure 4 gives comparative peptide maps of mouse kidney histones and the corresponding proteins of polyoma virions. In Figure 5, the small proteins of SV40 and polyoma are compared, while Figure 6 gives the comparison of monkey and mouse histones. From these data, we conclude that the technique employed does not allow us to find a difference between the corresponding histones of mouse cells, monkey cells, SV40 and polyoma virus.

A further experiment was done to find out if histones synthesized previous to viral infection are incorporated into virions: Mouse kidney cells were labeled with ^{14}C-arg during 24 hours. They were then washed and infected with polyoma virus. 24 hours after

infection, ^{3}H-arg was added to the medium, and 2 days later the virus was harvested and purified. The proteins were separated on a gel and their radioactivity counted. It is obvious from Figure 7 that there was a high turnover of ^{14}C-arg, since all the viral proteins are labeled. The ratio of ^{14}C to ^{3}H is twofold higher for the histones than for the other viral proteins, indicating that histones synthesized before the infection are incorporated into the viral particles.

Basic Proteins — Fingerprint
Mouse–Monkey Comparison

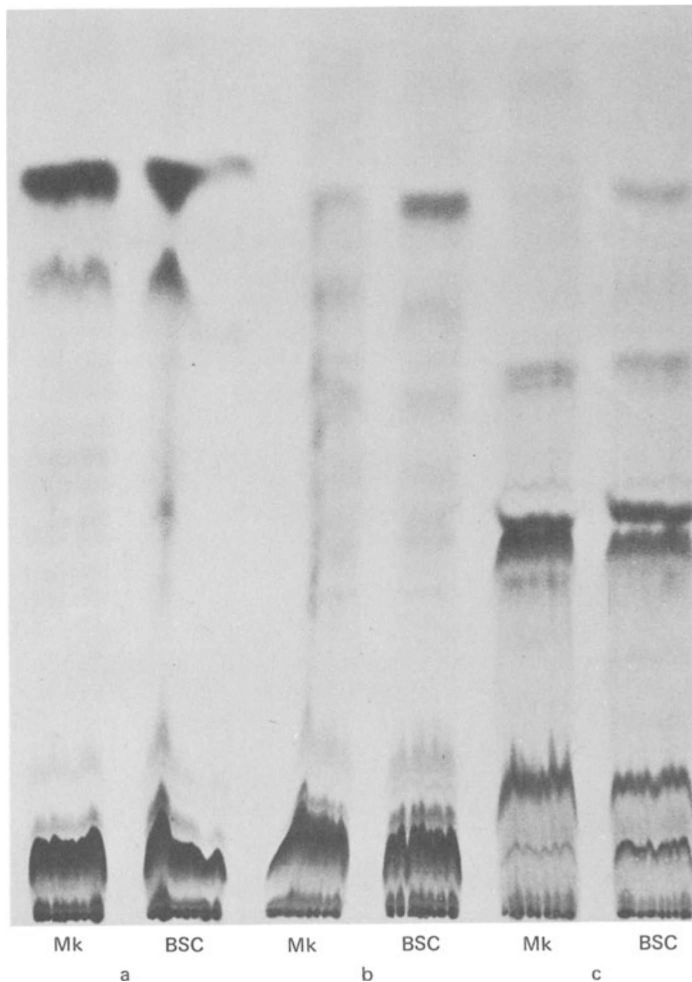

FIG. 6. As Figure 4, but comparison between mouse kidney (MK) and African green monkey (BSC) histones. a) slowest moving polypeptide, c) fastest polypeptide.

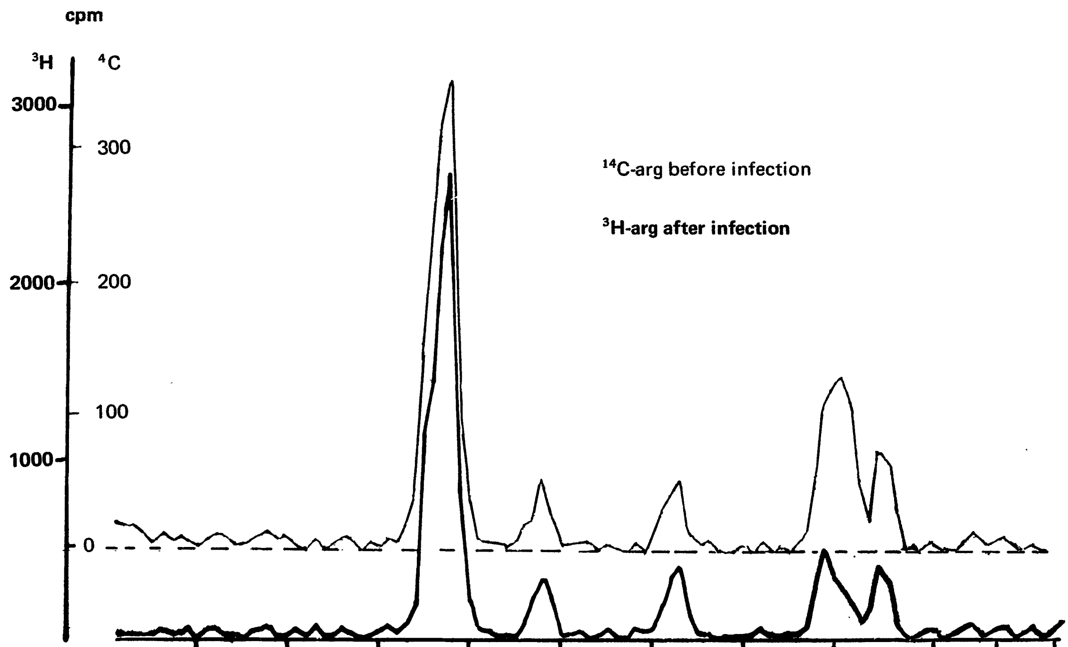

FIG. 7. SDS-polyacrylamide gel (10%) of polyoma virus grown in the presence of ^{3}H-arg on cells labeled prior to infection with ^{14}C-arg. The gel was fractionated, the proteins eluted in the presence of sodium dodecyl sulfate and the radioactivity counted in Aquasol (New England Nuclear Corporation).

DISCUSSION

On denaturing gels the protein patterns of polyoma virus and of SV40 are similar. (For a recent review, see Crawford, 1973). The majority of the protein of both viruses consists of a major capsid polypeptide with a molecular weight of approximately 45,000 daltons. The function of the minor capsid proteins is not clear yet. In high resolution gels of polyoma proteins, the minor polypeptides of the capsid split up into doublets. Roblin, Härle, and Dulbecco (1971) found a polypeptide larger than the major capsid protein, which was not consistently found in this work.

It is not clear whether the minor capsid protein of SV40 called VP 2 by Estes, Huang, and Pagano (1971) corresponds to the satellite band that is visible next to the major protein on Figure 2 or whether it is a different protein.

Associated to the viral DNA, there are basic proteins which, according to gel electrophoresis and methionine peptide analysis, behave like histones of the host cells.

A more detailed peptide analysis is under way to find out if the viral core proteins and the host histones are identical or if they contain midifications (G. Fey and B. Hirt, in preparation).

The fact that host histones are bound to the small DNA of SV40 and polyoma viruses provides a convenient model to study histone DNA interactions in mammalian cells.

ACKNOWLEDGEMENT

This work was supported by the Swiss National Science Foundation.

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THE CELL FREE TRANSLATION OF PURIFIED SIMIAN VIRUS 40 MESSENGER RNA

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INTRODUCTION

In order to understand the expression of the Simian Virus 40 (SV40) genome, it is important to be able to ascertain which new proteins appearing after infection are virus specified. This is especially true considering the potent transforming properties of the virus where the number of changes induced in the transformation process are far too numerous to be accounted for directly by the SV40 genome. The cell free synthesis of viral polypeptides directed by purified viral genetic material is one clear method of establishing which proteins are virus specific. Since SV40 does not inhibit host RNA or protein synthesis after infection and viral proteins do not comprise more than 10% of the total proteins synthesized even late in infection (Walter, Roblin, and Dulbecco, 1972; Anderson and Gesteland, 1972), the translation of total messenger RNA from SV40 infected cells will not establish which products are virus specific. In order to obtain purified SV40 mRNA, we have hybridized poly(A)-containing RNA from SV40 infected monkey cells (BS-C-1 line) to SV40 DNA according to a technique described by Weinberg, Warnaar, and Winocour (1972). This selected SV40 RNA, when added to a cell-free translation system derived from extracts of wheat germ, directs the synthesis of the major SV40 capsid protein VP-1 (Prives *et al.*, 1974). Using this approach, it should eventually be possible to establish the identity of other virus specific polypeptides in permissive and transformed cells.

RESULTS AND DISCUSSION

Distribution of SV40 RNA Sequences in Infected BS-C-1 Cytoplasmic RNA

An estimate of the abundance of SV40 RNA relative to host cytoplasmic mRNA late in lytic infection was sought in order to estimate the possibility of selection of viral messenger by hybridization in quantities sufficient for cell-free translation. The proportion of virus specific proteins has been shown to be approximately ten per cent of the host cell's cell newly synthesized protein population (Walter et al., 1972; Anderson and Gesteland, 1972). A comparable value obtained for the proportion of SV40 RNA would indicate the feasibility of selection of SV40 mRNA on a preparative scale. To make this assessment, BS-C-1 monkey cells were labeled for 7 days with uridine-5-H^3 at daily intervals in order to bring about as uniform and complete labeling of the various cellular RNA components as possible. This takes into consideration the estimated 24-hour half-life of the long-lived class of mammalian messenger RNA reported by Singer and Penman (1973). The cells were infected under similar labeling conditions, and infection proceeded for 48 hours prior to extraction of cellular cytoplasmic RNA. Table 1 summarizes the results of this experiment. The cytoplasmic RNA was subjected to oligo(dT) cellulose chromatography (Aviv and Leder, 1972) and the proportions of SV40 specific RNA in the cytoplasmic total, poly A containing [oligo(dT) cellulose retained] and poly A-free [oligo(dT) cellulose non-retained] RNA classes were estimated by low temperature formamide hybridization to SV40 DNA immobilized on nitrocellulose filters (Weinberg et al., 1972). A small (0.395%) proportion of total cytoplasmic RNA consists of SV40 specific sequences, late in infection. When this RNA is subjected to oligo(dT) cellulose chromatography, there is an approximate hundredfold enrichment of the proportion of SV40 RNA in poly A containing RNA over that in poly A free RNA. 9.8% of poly A containing RNA is SV40 specific which is consistent with the reported proportion of virus specific proteins late in infection.

It is also noteworthy that there is a considerable proportion of poly A free SV40 RNA. The poly A free and poly A containing RNA fractions in Table 1 are derived from the same sample of total cytoplasmic RNA, and it can be estimated that 25% of the cytoplasmic SV40 RNA is free of poly A sequences. Whether this represents naturally occurring poly A free SV40 RNA, or occurs as a result of one or more of the various RNA extraction steps is not clear. That a substantial proportion of cellular messenger RNA consists of SV40 RNA sequences encouraged further translation studies.

TABLE 1. Distribution of SV40 specific RNA in cytoplasmic RNA

RNA added	Input	Hybridized counts per minute	Hybridized minus blank	% hybridized
Total	6.8×10^5	2728	2556	0.375
Poly A free	9.0×10^6	5777	5307	0.086
Poly A rich	1.4×10^5	14,206	13,958	9.80

Proportion of SV40 sequences in cytoplasmic Poly A free and Poly A rich RNA. 5 cultures of BS-C-1 cells (4×10^6 cells/culture) were labeled at 24 hour intervals with uridine-5-^{3}H (10 µCi/ml of culture medium; 29 Ci/mMole) for 7 days. The cultures were infected with SV40 (strain 777) at 100 plaque forming units per cell 48 hours after infection. Cultures were washed three times with phosphate buffered saline and lysed with 1.0 ml NP 40 buffer (1.0 NaCl, .01 M Tris ClpH 7.8, .005 M $NaCl_2$, 0.5% Non Iodet p. 40, Shell Co.). Nuclei and cytoplasm were separated by centrifugation at 2000 x g for 5 minutes. The supernatant containing the cytoplasmic portion of the cells was made 0.1 M with respect to EDTA and 1% with respect to SDS and RNA was extracted by phenol-chloroform-iso-amylalcohol procedure. Cytoplasmic RNA was subject to oligo(dT). Cellulose chromatography (Aviv and Leder, 1972) and SV40 specific RNA sequences were determined by low temperature formamide hybridization of the various RNA fractions to SV40 DNA immobilized on nitrocellular filters by a procedure described previously (Weinberg *et al.*, 1972).

Polypeptides Directed by RNA from SV40 Infected and Uninfected Cells

Total cellular RNA extracted from SV40 infected and non-infected BS-C-1 cells was subjected to oligo dT cellulose chromatography. Samples of crude poly A free and poly A rich RNA were added to a cell free system prepared from wheat germ. This translation system has several advantages in that it is easy to prepare, has virtually no endogenous protein synthesis, and responds efficiently and faithfully to a wide variety of messenger RNA species, including globin and TMV (Roberts and Paterson, 1973). It was found that crude and poly A free RNA fractions from SV40 infected or uninfected BS-C-1 cells were inactive in stimulating the incorporation of ^{35}S methionine into acid insoluble polypeptides in wheat germ extracts. However, poly A containing RNA was very efficient in this respect and reached comparable levels of stimulation to those observed with the other mRNA species mentioned above. Messenger RNA from infected and uninfected monkey cells stimulated protein synthesis to the same extent in the wheat germ system. The products directed

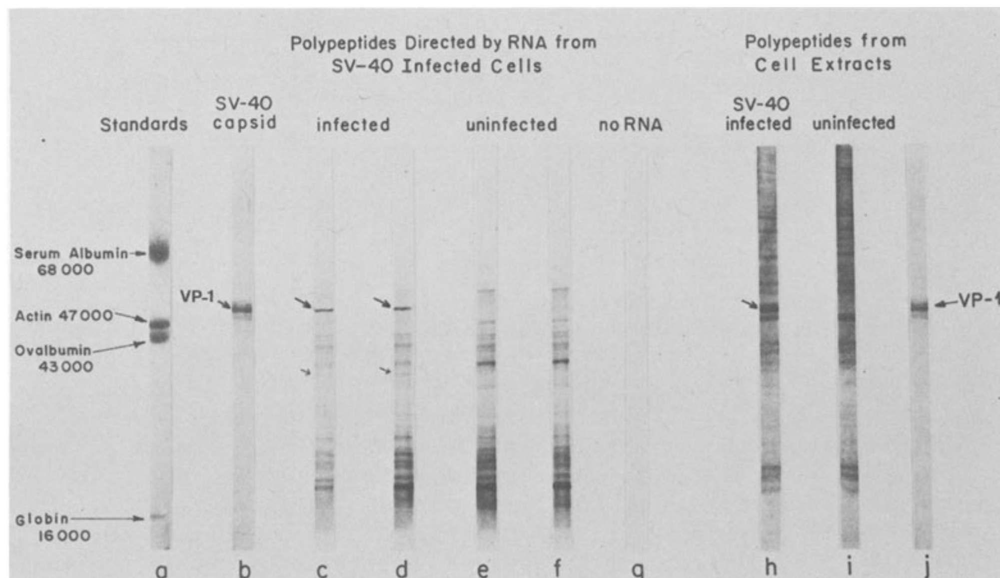

FIG. 1. Polypeptides directed by mRNA from SV40 infected cells.

^{35}S methionine labeled polypeptides were synthesized in extracts of wheat germ prepared and assayed as described by Roberts and Paterson (1973) in response to mRNA (2.5 µg RNA/100 µl reaction mixture) from SV40 infected or uninfected BS-C-1 cells prepared by oligo(dT) cellulose chromatography of total RNA. Cell free products were analyzed by SDS polyacrylamide slab gel electrophoresis followed by autoradiography.

a) Standard proteins stained with Coomasie Brilliant Blue (CBB).

b, j) Proteins from purified SV40 virions stained with CBB.

c, a) Autoradiogram of (^{35}S) methionine labeled polypeptides directed by poly A containing RNA from SV40 infected cells.

e, f) Autoradiogram of ^{35}S-methionine labeled polypeptides directed by poly A containing RNA from uninfected cells.

g) Autoradiogram of polypeptides synthesized in the absence of added RNA.

To analyze polypeptides of cell extracts, cells were collected from cultures 48 hours after infection (or mock infection), suspended in 1.0 ml buffer containing 0.05 M Tris HCl pH 6.8, 1% SDS 1% 2-mercaptoethanol and 10% glycerol, sonicated for 10 minutes and subjected to polyacrylamide gel electrophoresis.

h) CBB stained polypeptides of BS-C-1 cells 48 hours after SV40 infection.

i) CBB stained polypeptides of uninfected BS-C-1 cells.

by these mRNAs were analyzed by SDS polyacrylamide slab gel electrophoresis and found to consist of fairly similar series of polypeptides ranging in size from approximately 15,000 to greater than 60,000 daltons molecular weight as estimated by comparison with several standard polypeptides (Figure 1). Most products of mRNA from infected and uninfected cells corresponded in electrophoretic mobility although not necessarily in the quantity produced as estimated from the variable intensity of the autoradiographed bands (Figure 1). A marked exception was the appearance of a major novel polypeptide directed by RNA from SV40 infected cells which had the same electrophoretic mobility as the SV40 VP-1 marker. This polypeptide was not present in extracts directed by mRNA from uninfected cells. A second novel polypeptide, whose molecular weight is estimated as approximately 35,000 daltons was also directed by mRNA from infected cells. It does not have a similar mobility to SV40 VP-3 whose molecular weight has been estimated as 30,000 daltons (Crawford, 1973).

Polypeptides extracted from SV40 infected and uninfected BS-C-1 cells 48 hours after infection were similar except for a major novel polypeptide appearing in infected cells which had a similar electrophoretic mobility to the SV40 VP-1 marker (Figure 1). The proportion of this novel protein was estimated to be approximately 5-10% of the total polypeptides and is thus consistent with the finding that 9-10% of poly A containing RNA in infected cells is SV40 specific.

Preparation of Purified SV40 Messenger RNA

Assessment of the coding potential of the SV40 genome depends upon the isolation of a purified source of SV40 RNA. An approach that we thought promising was the selective hybridization of SV40 RNA to SV40 DNA. SV40 RNA thus extracted from infected BS-C-1 cells late in lytic infection has been shown to consist primarily of 2 classes of cytoplasmic RNA sedimenting at 16s and 19s as well as a high molecular weight heterogeneous RNA containing both host and viral RNA sequences which is nuclear in origin (Weinberg et al., 1972). We assumed that the very great majority of translated products are directed by poly A containing RNA species derived from the cytoplasm, although an accurate estimation of the proportion (if any) of products directed by poly A containing nuclear RNA compared to cytoplasmic messenger RNA species has not been reported.

Poly A containing RNA from SV40 infected cells which was subsequently hybridized to and eluted from SV40 DNA immobilized on filters was active in directing the synthesis of polypeptides in the wheat germ system. Figure 2 is a series of densitometer tracings of autoradiograms of (^{35}S) methionine labeled polypeptides

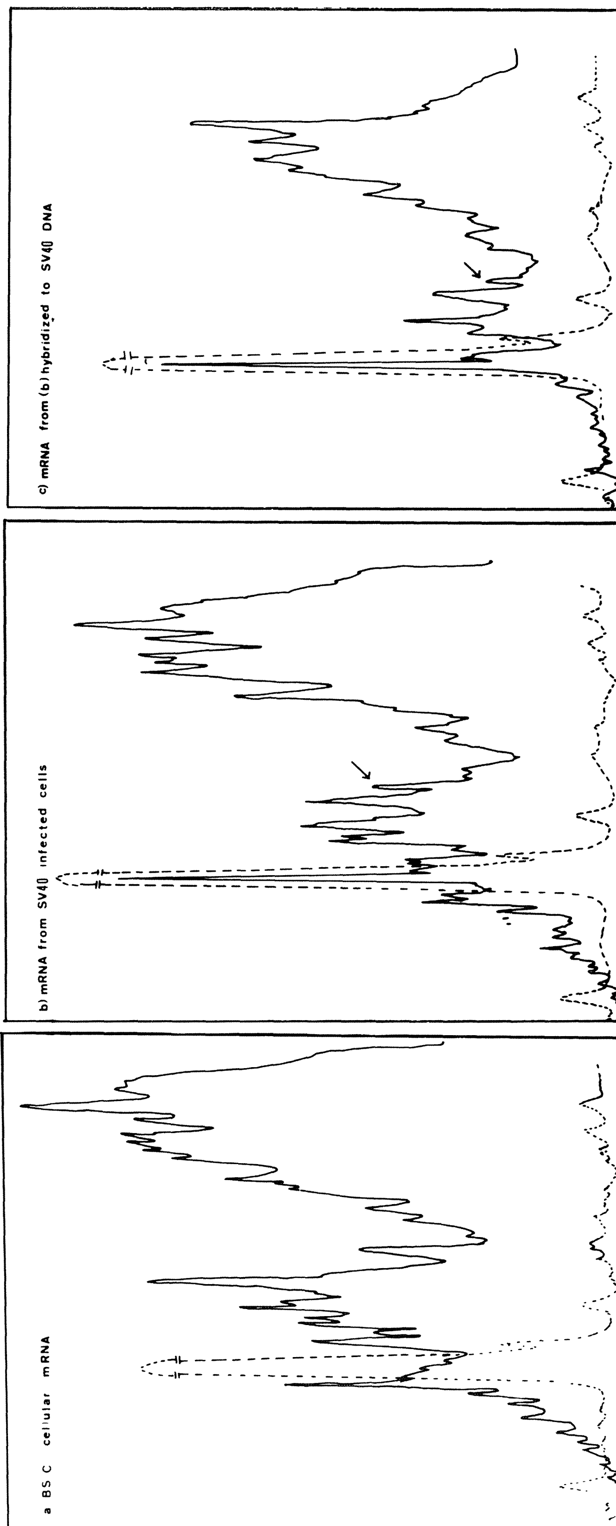
a BS C cellular mRNA
b) mRNA from SV40 infected cells
c) mRNA from (b) hybridized to SV40 DNA

FIG. 2. Polypeptides synthesized in response to mRNA from SV40 infected and noninfected cells and to SV40 selected mRNA.

Autoradiograms were converted to their corresponding tracings by a Gilford Recording Densitometer. The dotted line pattern in each tracing refers to the corresponding autoradiogram of ^{35}S methionine labeled SV40 virions; the major peak of which is the VP-1 polypeptide.

a) densitometer tracing of autoradiogram of (e) of Figure 1.

b) densitometer tracing of autoradiogram of (c) of Figure 1.

c) densitometer tracings of autoradiogram of polypeptides synthesized in response to SV40 specific RNA isolated by hybridization of poly A containing RNA from SV40 infected cells to SV40 DNA immobilized on nitrocellulose filters. Preparative selective hybridization of SV40 RNA for translation was carried out as described by Prives _et al_. (1974). The arrow refers to the small (35,000 dalton) polypeptides which are not seen in uninfected cell free products.

synthesized *in vitro* and subjected to polyacrylamide gel electrophoresis. In each case the relative migration of the SV40 VP-1 marker is indicated by a dotted line. While not as sensitive to the appearance of minor components, densitometer tracings provide a more accurate assessment of the relative proportions of the various products.

It can be seen that poly A containing mRNA from SV40 infected cells gives rise to a large spectrum of polypeptides, of which the most intensely labeled were the smaller size class (approximately 15,000-25,000 daltons). It is considered likely that at least some of these smaller polypeptides represent prematurely terminated peptide chains of larger products (B. Roberts and B. Paterson, personal communication). Products larger in size than 25,000 daltons are more likely to be completed products. The novel polypeptide which comigrates with SV40 VP-1 represents approximately 10% of these larger polypeptides.

SV40 specific RNA obtained by preparative hybridization directed the synthesis of a somewhat reduced number of polypeptides but there was also a preponderance of products with smaller molecular weight. The product which comigrates with SV40 VP-1 comprises approximately 25% of the greater-than-25,000-daltons class of polypeptides. Other polypeptides synthesized in response to this RNA may include prematurely terminated polypeptide chains as mentioned above, as well as additional viral structural and nonstructural proteins. The arrow in these tracings represents the second novel 35,000 dalton polypeptide which was also present in products directed by SV40 specific RNA.

SV40 specific RNA prepared as described above, was rehybridized to SV40 DNA by two different methods in order to assess its purity. Firstly, more than 90% of SV40 RNA was rehybridized in SV40 DNA immobilized on nitrocellulose filters under the hybridization conditions described above and in the legend to Figure 2 for 72 hours. The increased degree of hybridization obtained over previously reported values (Rosenblatt and Winocour, 1972) was presumably due to increased time of annealing. Secondly, SV40 RNA was hybridized to a vast (hundredfold) excess of SV40 DNA in solution and the values obtained were similar to those observed with comparable hybridization of complementary RNA obtained by the transcription of SV40 DNA (component I) by the *E. coli* RNA polymerase (T. Seebeck, personal communication); both hybridized to SV40 DNA to the extent of greater than 90%.

Despite the high degree of purity of SV40 specific mRNA, the pattern of products directed by mRNA from SV40 infected cells and SV40 selected mRNA are somewhat similar. As noted above in Figure 1, several host mRNA directed products are diminished in quantity

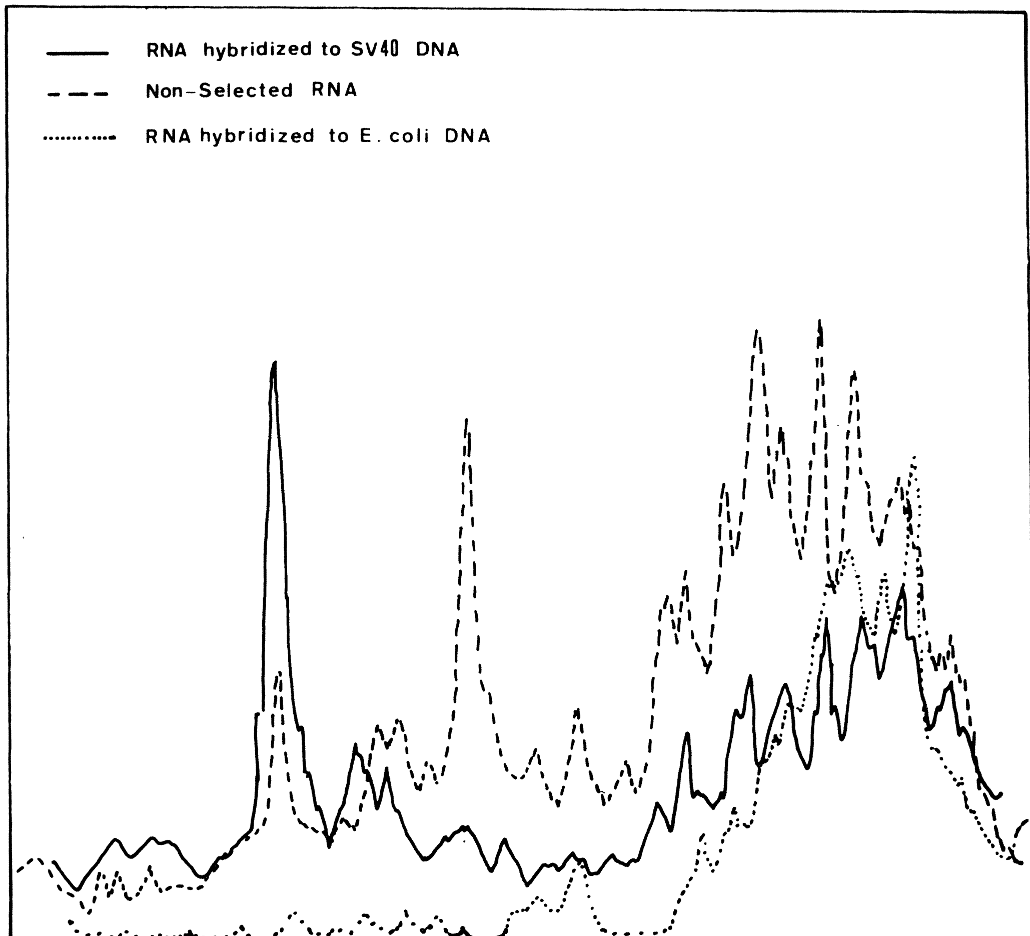

FIG. 3. Polypeptides directed by SV40 selected and non-selected RNA.

mRNA from SV40 infected cells was subjected to low temperature formamide hybridization to SV40 DNA immobilized on nitrocellulose filters as described by Prives et al. (1974). RNA remaining in solution after hybridization was collected and added to a wheat germ translation system, as was the hybridized RNA which has been eluted from the filters.

Densitometer tracing represent autoradiograms of polypeptides synthesized in response to (-)SV40 selected RNA; (---)SV40 non-selected RNA; and (...)mRNA from SV40 infected cells which was hybridized to equivalent quantities of E. coli DNA immobilized on nitrocellulose filters in identical fashion to techniques described for SV40 DNA-RNA hybridization.

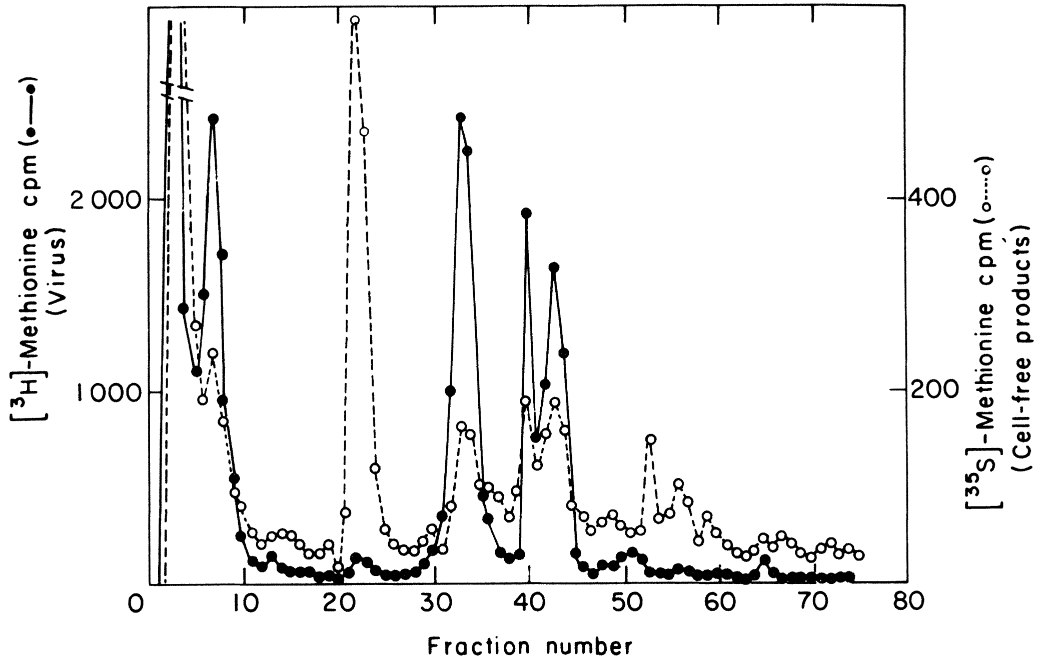

FIG. 4. Tryptic peptide analysis of SV40 virions and SV40 RNA cell free products.

(^{3}H)-methionine labeled purified SV40 virions prepared as described (Lavi and Winocour, 1972) and (^{35}S) methionine labeled polypeptides synthesized in the wheat germ system in response to SV40 selected mRNA were dialysed against 1% ammonium bicarbonate, lyophilized and then oxidized and trypsinized as described (Prives et al., 1974). ^{35}S methionine (55,000 cpm) and ^{3}H methionine (337,000 cpm) labeled tryptic peptides were simultaneously separated on a 0.9 x 2.0 cm column of Aminex A-7 (Biorad) and eluted by a gradient of pyridine acetate buffer (0.05 M, pH 2.5 to 1.2 M pH 5.0) at 55°C. Two ml fractions were collected and counted with appropriate corrections for (^{35}S) spillover.

late in infection. As infection proceeds possibly the viral RNA is preferentially translated. RNA in the process of translation is more likely to provide a source of biologically active messenger RNA when extracted from the cells than mRNA which is no longer associated with polysomes and may be somewhat degraded. Thus, there may be a greater proportion of functional SV40 specific mRNA in the poly A containing mRNA of infected cells than the estimated 10% (Table 1) and this would explain similar polypeptide patterns directed by SV40 selected mRNA and total cellular mRNA.

Another approach towards assessment of the relative purification of SV40 RNA was to compare the translation products of hybridized and non-hybridized RNA, or that RNA which remains in solution after hybridization. It can be seen from Figure 3 that the class of RNA which does not hybridize to SV40 DNA is also active as a messenger in the wheat germ system. There is a clear alteration in pattern of the polypeptides directed by SV40 selected and non-selected RNA with some reduction in complexity of the pattern of polypeptides synthesized in response to the former. Non-selected RNA directs a reduced but not totally diminished amount of the polypeptide which comigrates with VP-1 indicating that under the hybridization conditions employed, the yield of virus specific RNA is not 100%.

It is also noteworthy that RNA extracted from SV40 infected cells and hybridized to E. coli DNA immobilized on nitrocellulose filters as described above also directs the synthesis of a range of polypeptides which are small in size (Figure 3). These products may account for some of the smaller molecular weight polypeptides obtained in response to the SV40 selected RNA added to the wheat germ system. We concluded that selection of SV40 specific RNA by hybridization to SV40 DNA provides a method of obtaining purified viral messenger RNA. The resulting increase in virus specific completed products, specifically SV40 VP-1, before and after selection is two- to threefold.

Analysis of Tryptic Peptides of Cell-Free Products and Virion Proteins

Further identification of the major products specified by SV40 mRNA as the virion capsid protein was afforded by a comparison of the tryptic peptides of purified SV40 virions and cell free products directed by SV40 RNA. Tryptic digest products were analyzed by cation exchange column chromatography (see legend to Figure 4). From Figure 4 it can be seen that SV40 virions yield 4 major methionine-containing polypeptides which bind to the column and one or more which do not (tubes 1 through 5 represent the non-bound fraction). The tryptic digest of the cell-free products contain

methionine containing peptides which co-elute with the major virion peptides as well as 3-4 which do not, of which 1 is predominant. As the VP-1 capsid protein of SV40 comprises 70-80% of the virion, one may assume that the tryptic peptides of the virus are derived mainly from this protein. The number of methionine containing peptides in the SV40 virion obtained in this analysis is consistent with an estimated 5-6 methionine residues present in the SV40 capsid derived from the amino acid analysis of SV40 (Greenway and LeVine, 1973). The additional peptides present in the cell-free product may be the result of additional viral structural proteins or peptides derived from viral products which must undergo cleavage *in vivo* prior to viral assembly. From the agreement of the 5 major tryptic peptides seen after cation exchange column chromatography as well as their similar electrophoretic mobility, we have concluded that the SV40 major capsid polypeptide and the major cell-free product directed by SV40 RNA are the same protein.

CONCLUSIONS

We have been able to isolate virus specific RNA from SV40 infected cells by selective hybridization to SV40 DNA and to demonstrate that it can direct the cell-free synthesis of the major capsid protein VP-1. This approach can lead to understanding of several aspects of SV40 gene expression. Using purified viral messenger RNA it may be possible to assess control mechanisms operating at the level of translation. It may also become feasible through this approach to study the proteins encoded by SV40 DNA derived from various substitution and deletion mutants of the virus.

SV40 specific RNA can be divided into several classes. These include: (1) early 19s SV40 RNA isolated both from permissive cells during the initial stages of lytic infection and from SV40 transformed cells; (2) late SV40 RNA species consisting of 16s and 19s RNA components isolated from the cytoplasm of lytically infected cells late in infection; (3) SV40 RNA derived from the nuclear portions of lytically infected and transformed cells (Weinberg *et al.*, 1972). It should eventually be possible to isolate these different classes of RNA and to determine which products they specify. Progress in this direction is under way with separated late 16s and 19s cytoplasmic SV40 RNA species. These purified viral messenger RNA species and their polypeptide products can eventually be assigned to specific regions of the SV40 genome by the use of the specific fragments of SV40 DNA generated by restriction endonucleases. The techniques described herein can therefore provide the basis for a system for identification of all virus specified products from permissive and transformed cells.

ACKNOWLEDGEMENTS

I am grateful to H. Aviv, M. Revel and E. Winocour for assistance and advice at all stages of this work. The excellent technical assistance of B. Danovitch is acknowledged. This work was supported by the National Cancer Institute, Contract, NO 1 CP 33220.

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STUDIES ON NON-TRANSFORMING HOST RANGE MUTANTS OF POLYOMA VIRUS

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INTRODUCTION AND REVIEW

Two kinds of conditional lethal mutants of polyoma virus have been isolated and partially characterized. The most widely studied are the temperature-sensitive mutants which comprise at least four distinct groups based on complementation and other physiological tests. The second kind of mutant to be isolated is of the host range type; these mutants are selected to grow on polyoma-transformed mouse cells but not on normal mouse cells. Table 1 gives the selected properties of these two general classes of mutants. Progress in the characterization of temperature-sensitive and host range mutants has recently been reviewed (Benjamin, 1972).

Only some of the mutants selected as conditional lethals are affected in their ability to transform cells. With respect to gene functions essential to transformation, two types of functions based on temperature-sensitive mutants have been described. One is essential early in the transformation process; the action of this viral gene, required only transiently, leads apparently to a stable

TABLE 1. Selected properties of conditional lethal polyoma virus mutants

Mutant	Normal mouse fibroblast line (3T3) 31°C	Normal mouse fibroblast line (3T3) 38°C	Polyoma virus transformed mouse fibroblast line (Py-3T3) 37°C
Temperature sensitive	+	-	
Host Range		- (37°C)	+

+ = permissive for virus growth; - = non-permissive for virus growth

association of viral and cellular genes. The second kind of viral gene function essential for transformation is required continuously, and leads to phenotypic changes in cell growth patterns and other characteristics of transformed cells. Cells transformed under permissive conditions (31°C) by mutants of the first or "initiation" class remain transformed under non-permissive conditions (39°C), while cells transformed by mutants in the second or "maintenance" class revert to a normal state in at least some of the properties associated with transformation. All of the mutants isolated by the transformed cell-dependent host range selections are non-transforming; they resemble a maintenance-defective mutant of the temperature-sensitive type with respect to their inability to cause cell agglutinability by lectins concanavalin A or wheat germ agglutinin (Benjamin and Burger, 1970; Eckhart, Dulbecco and Burger, 1971).

The maintenance function can apparently be expressed independently of the initiation function as judged by the fact that ts-a, a mutant of the initiation class, can abortively transform cells (Stoker and Dulbecco, 1969). (The abortive transformation response measures the ability of a virus to cause a transient loss in anchorage dependence of cell growth.) In addition to being able to carry out abortive transformation, mutants of the initiation class are also able to stimulate cellular DNA synthesis and cause agglutinability at the non-permissive temperature. The ability of mutants of the maintenance class to cause abortive transformation has not been extensively reported; mutants of this class uniformly fail to induce agglutinability, but show variable responses in inducing cellular DNA synthesis.

CURRENT INVESTIGATIONS OF A NON-TRANSFORMING HOST RANGE MUTANT (WORK BY L. NORKIN AND T. L. BENJAMIN)

Abortive Transformation

The ability of NG-18 to induce abortive transformation in a continuous line of rat embryo cells has been investigated. A careful analysis of the frequency and size distribution of clones in semi-solid methyl cellulose suspension culture showed a lack in ability of NG-18 to induce this transient growth response; at multiplicities of infection comparable to wild type, NG-18 was at least two orders of magnitude less effective (Benjamin and Norkin, 1972). Similar results were obtained using the BHK line of hamster fibroblasts as the host.

The retention of ability by NG-18 to stimulate cellular DNA synthesis [shown previously in normal mouse cells (Benjamin, 1971)] is somewhat surprising in view of its failure to induce abortive transformation. Among many possible explanations, the following might be considered: 1) the requirement for induction of cellular DNA synthesis for cells in suspension is different (and more stringent) than for cells on monolayers; 2) the induction of cell DNA synthesis is necessary but not sufficient for abortive transformation; the membrane change of agglutinability (in which NG-18 is defective) is also required; 3) differences in the host species are such that mouse, but not rat or hamster, cells allow the induction of cellular DNA synthesis by NG-18 in monolayer cultures.

Evidence supporting the third possibility was obtained in the following way. Normal rat embryo cells ($RECl_3$) were arrested in G-1 by incubation in 0.5% serum for 72 hours, then infected by wild type or NG-18, and incubated for 48 hours in serum-free medium containing 2 μC/ml ^{3}H-thymidine. The number of cells synthesizing DNA under the influence of each virus was determined by autoradiography. The results shown in Table 2 demonstrate that at an

TABLE 2. Induction of cellular DNA synthesis in serum-starved rat cells*

Virus (moi)	Fraction of Labeled Nuclei	(%)
Control	21/507	(4.1)
NG-18 (10-20)	43/516	(8.3)
Wild Type (10-20)	283/508	(55.7)

*Continuous labeling 0-48 hours after infection, and incubated in serum-free medium.

input multiplicity of infection of 10 to 20 pfu/cell, wild type virus is at least ten times more effective than NG-18 at inducing cellular DNA synthesis. Under identical conditions of infection and pre-incubation in low serum, wild type virus was still able to induce abortive transformation while NG-18 was not. Under these conditions, only about 5 to 10% of cells stimulated by the virus to enter "S" register as abortive transformants. Both wild type and NG-18 proved to be equally effective in inducing the polyoma-specific tumor (T) antigen in the serum-starved rat cells. Thus, whereas NG-18 was previously found to be as effective as wild type in inducing cellular DNA synthesis in monolayers of normal mouse cells, it appears to be considerably less effective than wild type when tested in rat cells. The inability to cause abortive transformation is most likely related to the failure to induce cellular DNA synthesis, and to bring about the cell surface change which has been shown to be dependent on cellular DNA synthesis (Benjamin and Burger, 1970).

Relationship Between the Transformed Phenotype and Permissivity for NG-18

The failure of NG-18 to transform cells (stably or abortively) correlates with the failure to cause the cell surface change from the non-agglutinable to the agglutinable state (Benjamin and Burger, 1970). The permissive host for NG-18 expresses agglutinability as a constitutive property; however, the relationship between the ability to support the growth of the "membrane defective" mutant NG-18 and the expression of the membrane change is not clear. The expression of the cell surface change *per se* would not appear to be essential for growth of the mutant.

The block to growth of NG-18 in normal 3T3 cells appears to be intracellular; the mutant effectively induces the T antigen(s) and yet is unable to produce a normal burst of progeny virus when either whole virus or viral DNA is used to infect 3T3 cells (Benjamin, 1970).

The relationship between agglutinability and permissivity was tested directly by isolation of phenotypic revertants of permissive polyoma-transformed 3T3 cells which have regained normal growth properties. Two spontaneous (unselected) revertants and one obtained by FUdR selection (Pollack, Green and Todaro, 1968) were tested for agglutinability by concanavalin A and permissivity for wild type and NG-18. The results in Table 3 show that the properties of agglutinability and permissivity can readily be dissociated from one another. In all three cases, the membrane in the revertants was restored to the agglutinable level characteristic of normal 3T3 cells; at the same time, the cells remained largely permissive, being no less than half as permissive as Py-6 and at least ten-fold more permissive than 3T3.

TABLE 3. Agglutinability and permissivity for polyoma mutant NG-18 in transformed and revertant cell lines

Cell Line	Description	Agglutinability* Con A	Output/Input† NG-18	Wild Type	Permissivity ζ
3T3 (Swiss)	Normal	1500	5	200	.03
Derivatives:					
Py-6	Transf. by polyoma	100-200	150	250	0.8
Py-6-R-1	FUdr revertant	1500	100	120	0.8
Py-6-R-3	Spontaneous revertant	1500	100	300	0.3
Py-6-R-6	Spontaneous revertant	1500	65	250	0.3

*Half-maximal agglutinin concentrations (γ/ml).

†Approximate average burst size determined after single cycle-growth at low multiplicity of infection.

ζRatio of Output/Input for NG-18 to Output/Input for wild type.

Relationship Between Agglutinability and Permissivity in Non-Polyoma Transformed Cells

The data in the previous experiment showed that either a cell-associated viral function or a viral-induced cellular function in permissive polyoma transformed cells can complement NG-18 without necessarily leading to the membrane change. Both the origin (cellular versus viral) of the permissive function and its mode of action in allowing NG-18 to grow remain to be clarified. Attempts are being made to examine whether cells expressing agglutinability following transformation by means other than polyoma infection can be permissive for NG-18. Preliminary data of this kind are shown in Table 4 in which a series of transformants derived from the A-31 clone of Balb-3T3 are tested. It is clear that a substantial enhancement of permissivity can accompany transformation of normal cells by murine or avian sarcoma virus; however, not all transformants expressing agglutinability become permissive as shown by the cases of the SV-40 and spontaneous transformants.

TABLE 4. Agglutinability and permissivity for polyoma mutant NG-18 in various normal and transformed cell lines

Cell Line	Description	Agglutin-ability* Con A	Output/Input† NG-18	Output/Input† Wild Type	Permissivity$^{\zeta}$
A-31 (Balb-3T3)	Normal	1500	2.5	50	.05
Derivatives:					
PyA-31	Transf. by polyoma	100-200	100	100	1.0
SVT2	Transf. by SV40	100-200	5	100	.05
71EAC1-3	Transf. by MSV	400	75	300	0.3
B77-A-31	Transf. by avian sarcoma virus B77	200-300	80	400	0.2
3T12	Transformed spontaneously	1200	10	600	.02

*Half-maximal agglutinin concentrations (γ/ml)

†Approximate average burst size determined after single cycle-growth at low multiplicity of infection.

$^{\zeta}$Ratio of Output/Input for NG-18 to Output/Input for wild type.

From these preliminary results, it appears that 1) both the agglutinable state and the permissive function are expressions of cellular genetic information capable of being triggered by different transforming viruses and that 2) since permissivity and agglutinability can be dissociated from one another, several steps must be involved between the expression of cell-associated viral genes and the cell surface change.

ACKNOWLEDGEMENTS

Work in Dr. Thomas Benjamin's laboratory has been supported by Grant R01CA11473 from the National Cancer Institute, National Institutes of Health, Grant VC-2 from the American Cancer Society and Contract 72-2028 from the Cancer Virus Program of the National Cancer Institute, National Institutes of Health. Dr. Benjamin is recipient of a Scholar Award from the Leukemia Society of America, Inc.; Dr. Goldman is recipient of a postdoctoral fellowship from

The Damon Runyon Society. Work in Dr. Leonard Norkin's laboratory is supported by Grant CA12948-02 from the National Institutes of Health.

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THE PROPERTIES OF A POLYOMA VIRUS MUTANT, ts_3

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I would like to describe briefly the properties of a polyoma virus mutant, ts_3, which we feel is probably related to the host range (hr) mutants described by Benjamin (this volume), which cannot transform. The results I am going to describe were obtained by Dr. Walter Eckhart and myself.

The ts_3 mutant is temperature-sensitive both in lytic infection and transformation. I will first describe its behavior in transformation because it is simpler. Using growth in agar to differentiate the transformed cells, we have found that the mutant will transform BHK cells both at permissive (32°C) and non-permissive (39°C) temperature. However, the growth and surface properties of the transformed cells are different at the two temperatures. Thus at low temperature the topoinhibition parameter of the cells is very low and their agglutinability by lectins is high, as in cells transformed by wild type virus, whereas at high temperature topoinhibition is higher and agglutination low, as in regular BHK cells (Dulbecco and Eckhart, 1970; Eckhart, Dulbecco, and Burger, 1971). The changes in topoinhibition reflect changes for serum requirement in a crowded cell layer (Dulbecco and Elkington, 1973). The morphology of the cells also changes with the temperature. It appears that the main properties of transformed cells, except growth in agar, are temperature reversible, suggesting that the mutation is in a gene whose continued expression is required for transformation.

The properties of the mutant in lytic infection at high temperature show many deviations from those of wild type polyoma virus in certain cells, in other cells the mutant behaves nearly like wild type virus. Thus the first property of the mutant is that temperature-sensitivity of growth is cell dependent. The ratio of viral yield at 32°C over that at 39°C (in plaque-forming units) is of several hundreds in BALB c/3T3 cells, but about 2 in PY6 cells, which are Swiss 3T3 cells transformed by polyoma virus. In mouse embryo cells the temperature sensitivity is very low (a ratio of 2-6) somewhat higher in primary mouse kidney cultures (10-20). These differences apply also to the production of infectious viral DNA. In BALB/c3T3 cells at high temperatures the mutant fails to induce cellular DNA synthesis or increased agglutinability by lectins. The different behavior of ts3 virus in different cells could be due either to the physiological state or to the genetic organization of the cells. Concerning their physiology, it seems that temperature sensitivity is related to the ability of the cells to enter a resting stage, with BALB c/3T3 cells at one end and PY6 at the other. From the genetic point of view PY6 cells contain a polyoma virus genome, whereas the other cells do not. However, there are certainly other differences related to expression of cellular genes between these cells.

The possibility of a physiological mechanism is suggested by the dependence of viral DNA replication on cellular DNA replication for both polyoma virus and SV40 (Thorne, 1973a and 1973b; Pages et al., 1973). This possibility was investigated by stimulating BALB c/3T3 with serum at various times before and after infection. A much lower ratio of the yield at 32°C to that at 39°C (22-25) was obtained when serum was added 16-8 hours before infection. Although temperature sensitivity was not abrogated, its reduction suggests that the physiological state of the cells may be important for the expression of this viral gene.

The second property of the ts3 mutant is that the infectivity of the purified DNA extracted from the virus is not temperature-sensitive in BALB c/3T3 cells. This result may implicate a virion protein. However, only a very small proportion of the cells can be infected by the viral DNA even at saturating concentrations, raising the possibility that there are special cells, in which temperature sensitivity is not expressed.

The third property, deduced from temperature shift experiments, is that the mutation is expressed early, after viral absorption, and that the stage blocked at non-permissive temperature takes place at permissive temperature in the presence of hydroxyurea to prevent DNA synthesis.

The fourth property is failure to complement with other polyoma

virus ts mutants, of any known complementation group in BALB c/3T3 cells at non-permissive temperature.

This result is not due to a dominant lethal effect, since the yield of wild type polyoma virus is not decreased by co-infection with ts3. This result again suggests that the mutation affects a virion protein which when altered prevents the function of the viral DNA, possibly by remaining bound to it. Since ts3 does not complement with tsa, which is affected in an early function (Fried, 1970), the cellular function involved would have to do with the transcription or translation of the early region of the genome.

The properties of the mutant in lytic infection resemble those of the 101 mutant of SV40 (Robb and Martin, 1972).

The interesting behavior of the mutant raises several questions:

1) Is it by chance a double mutant? Efforts to obtain a partial revertant have been so far negative.

2) If the mutation affects a virion protein, what is its nature and function?

3) Is complementation of the viral function by the cells due to the expression of cellular or viral (integrated) genes? Work directed at answering these questions is now proceding in both my laboratory and Eckhart's laboratory.

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ADENOVIRUS

INTRACELLULAR FORMS OF ADENOVIRUS DNA IN PRODUCTIVELY INFECTED CELLS: EVIDENCE FOR INTEGRATION OF THE VIRAL GENOME

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ABSTRACT

In order to understand the mechanism of replication of adenovirus DNA in productively infected human cells, the intracellular forms of newly synthesized DNA have been studied. KB cells growing in monolayers were inoculated with CsCl-purified adenovirus type 2 (Ad2) at a multiplicity of 100 PFU/cell. At various times after infection, the cells were labeled with ^{3}H-uridine or ^{3}H-thymidine. In some experiments the cells were prelabeled with ^{14}C-thymidine. The intracellular DNA was extracted after lysis of the cells with SDS or with alkali and was analyzed in dye-buoyant density gradients or by zonal centrifugation in neutral or alkaline sucrose gradients. The results of the experiments can be summarized as follows: 1) There is no evidence that parental or newly synthesized Ad2 DNA becomes supercoiled. 2) A virus-specific DNA-RNA complex can be isolated in dye-buoyant density gradients. This complex is probably involved in transcription. 3) In CsCl density gradients viral DNA of high buoyant density is observed which is a precursor to virion DNA as judged from pulse-chase experiments. These molecules are in part single-stranded. 4) In alkaline sucrose gradients viral DNA is detected which sediments at a rate of 50-90 S. The evidence suggests that this DNA may represent an integrated form of the viral genome.

INTRODUCTION

Adenovirus type 2 (Ad2) replicates to high titers in KB cells, a continuous line of human cells. Since a large number of viral gene copies are synthesized in each cell, this system is well suited for the detailed analysis of the mechanisms of viral DNA replication and transcription. Furthermore, this system allows one to investigate whether viral DNA or fragments of it become integrated into the DNA of the host cell even in lytic infection. The understanding of these events is contingent upon a complete knowledge of the various forms of viral DNA synthesized in productively infected cells.

A number of laboratories have begun to investigate the mechanism of replication of adenovirus DNA (Horwitz, 1971; Robin, Bourgaux-Ramoisy, and Bourgaux, 1973; Sussenbach _et al._, 1972; van der Eb, 1973). A partly single-stranded DNA molecule seems to be a precursor in viral DNA replication. A model for the replication of the DNA of adenovirus type 5 has been derived from studies carried out in a system using isolated nuclei (Jansz _et al._, this symposium; Sussenbach _et al._, 1972). According to this model, DNA replication proceeds by replacement of one of the two strands. A great deal of work still is required to gain complete understanding of the mechanism of adenovirus DNA replication.

In baby hamster kidney (BHK21) cells abortively infected with adenovirus type 12 (Ad12) viral DNA does not replicate (Doerfler, 1969). The parental viral DNA is fragmented to pieces of approximately 15-18 S (Burlingham and Doerfler, 1971) and fragments of Ad12 DNA are covalently linked to cellular DNA (Doerfler, 1968 and 1970). Moreover, late in infection, fragments of cellular DNA are observed (Doerfler, 1969). It is unclear how the fragmentation of cellular DNA is correlated to the "pulverization of chromosomes" described in the BHK Ad12 system (Stich and Yohn, 1967).

There is mounting evidence that the DNA of adenoviruses can become integrated by covalent linkage into cellular DNA. Integration of fragments of viral DNA has been shown for Ad12 DNA in abortively infected BHK21 cells (Doerfler, 1968 and 1970). In Ad2 transformed rat cells Ad2 DNA persists and continues to be transcribed (Green, 1970; Lindberg and Darnell, 1970; Pettersson and Sambrook, 1973). In fact, integration may be a general phenomenon in cells infected or transformed by DNA tumor viruses. Integrated genomes have been demonstrated for Simian Virus 40 (SV40) both in transformed cells (Collins and Sauer, 1972; Hirai, Lehman, and Defendi, 1971; Sambrook _et al._, 1968) and in lytically infected cells (Hirai and Defendi, 1972; Sauer _et al._, this symposium) and in cells lytically infected with polyoma virus (Ralph and Colter, 1972). Fragments of the Herpesvirus genome may be integrated in

cells found in certain cases of cervical cancer (Frenkel *et al.*, 1972).

In this study, some of the intracellular forms of Ad2 DNA found in productively infected KB cells will be described. An alkali stable form of Ad2 DNA sedimenting at a rate of 50-90 S and with a buoyant density in alkaline CsCl gradients intermediate between that of viral and cellular DNA (Burger and Doerfler, unpublished results) probably represents integrated viral DNA. In the second part of this paper, evidence will be presented for a DNA-RNA complex which is involved in the transcription of the viral genome.

Lastly, experimental data will be described which implicate partly single-stranded viral DNA molecules as possible precursors in viral DNA replication.

The *Materials and Methods* used in this study have been described in two recent publications (Burger and Doerfler, 1974; Doerfler *et al.*, 1973), and will not be repeated here. Experimental details necessary to understand the significance of the experiments presented will be given in the figure legends.

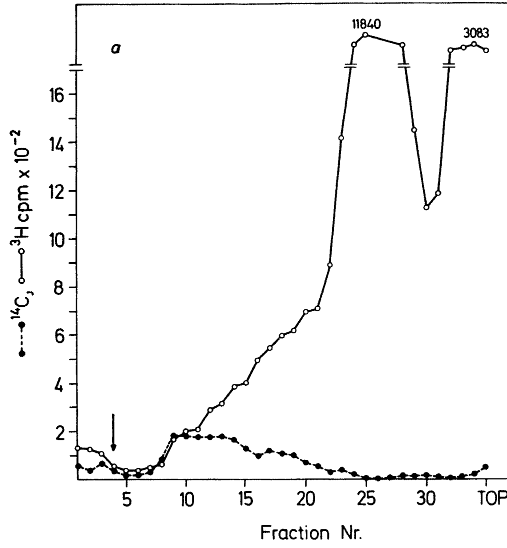
RESULTS AND CONCLUSIONS

I) Fast Sedimenting, Alkali Stable DNA in Ad2-Infected KB Cells--An Integrated Form of Ad2 DNA?

In earlier work (Burlingham and Doerfler, 1971) fast sedimenting, alkali-stable DNA was found in Ad2-infected KB cells, however, only transiently and as a minor component. It now appears that only small amounts of fast sedimenting DNA were seen because of the procedure used to extract DNA at that time. This procedure consisted of extraction by SDS-lysis, pronase treatment of the lysate and phenolization, a procedure that could easily have fragmented high molecular weight DNA. To avoid fragmentation, a more gentle method of extraction of the intracellular DNA has been applied (Doerfler, 1970; Lett *et al.*, 1967; McGrath and Williams, 1966): Lysis of intact cells or nuclei in alkali. The results presented in Figure 1A demonstrate that after direct lysis of the Ad2-infected cells in 0.5 M NaOH, four size-classes of DNA can be separated by zonal sedimentation in alkaline sucrose density gradients:

1) DNA sedimenting very rapidly on to the cushion of alkaline CsCl on the bottom of the gradient. This class of DNA contains most of the ^{14}C-prelabel and represents predominantly cellular DNA.

2) DNA sedimenting in a broad region between 50 S and 90 S. This DNA contains only small amounts of prelabel, although some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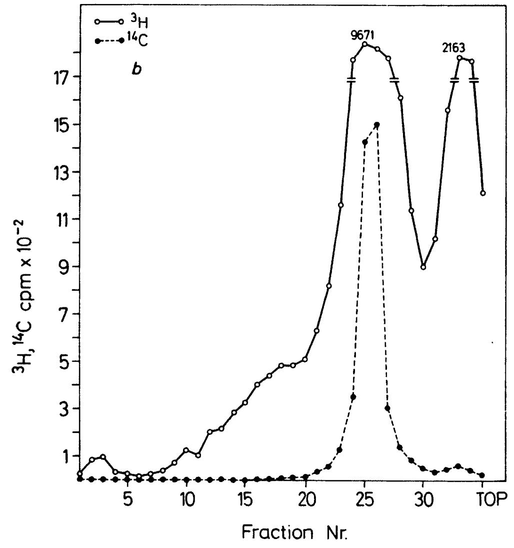
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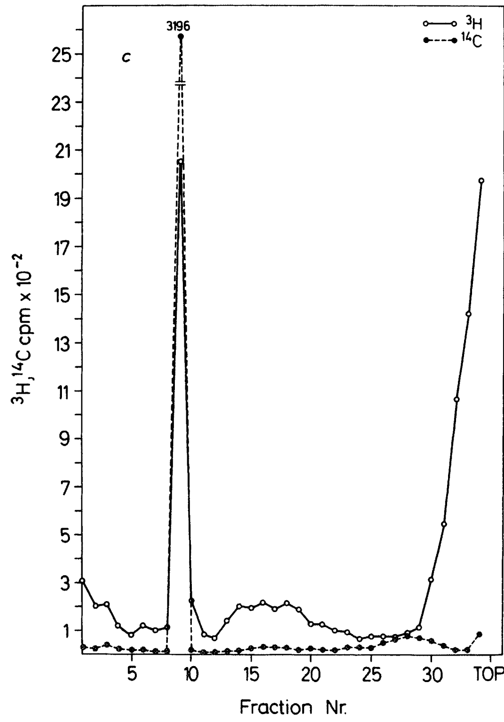
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FIG. 1. DNA synthesis in Ad2-infected KB cells. Analysis by zonal sedimentation in alkaline sucrose density gradients. KB cells growing in monolayers were infected with unlabeled CsCl-purified Ad2 at an m.o.i. of 100 PFU/ml or were mock-infected with PBS. In the experiments illustrated in parts A and C the KB cells were prelabeled by adding ^{14}C-thymidine (0.4 μCi/ml) to the medium and maintaining the cells under these conditions for 3 days prior to infection. In the experiment shown in part B the cells were not prelabeled. In experiments A and C the cells were washed several times with PBS immediately prior to infection, in order to remove the ^{14}C prelabel. At various times after infection, the newly synthesized DNA was labeled with ^{3}H-thymidine (30 μCi/ml of medium). At the end of the labeling period, the cells were washed several times with PBS and were lysed for 18 hours on top of an alkaline sucrose gradient.

a. KB cells prelabeled with ^{14}C-thymidine were infected with Ad2 and the newly synthesized DNA was labeled with ^{3}H-thymidine from 14-17 hours post infection. Centrifugation was at 22,000 rpm for 380 minutes at 4°C in the SW 27 rotor of the L2-65B ultracentrifuge.

b. KB cells were infected with Ad2 and the newly synthesized DNA was labeled with ^{3}H-thymidine from 14-17 hours post infection. The sample was lysed in alkali for 18 hours together with ^{14}C-labeled Ad2 marker DNA and then centrifuged in the SW 41 rotor at 35,000 rpm for 140 minutes at 4°C.

c. KB cells prelabeled with ^{14}C-thymidine were mock-infected with PBS and labeled with ^{3}H-thymidine from 16-18 hours after mock-infection. The cells were lysed in alkali and centrifuged at 23,000 rpm for 430 minutes at 4°C in the SW 27 rotor.

experiments, such as that presented in Figure 2a, show more prelabel than seen in the experiment presented in Figure 1a.

3) The main peak of ^{3}H-labeled DNA, usually free of ^{14}C-prelabel, cosediments at 34 S (Doerfler and Kleinschmidt, 1970) with ^{14}C-labeled Ad2 marker DNA extracted from purified virions (Figure 1b). It should be emphasized that the reference DNA sediments as a sharp, symmetrical peak demonstrating that 34 S viral DNA is not dragged into faster sedimenting regions.

4) Slowly sedimenting DNA probably representing short pieces of viral DNA.

The present study deals with the characterization of the 50 S-90 S DNA. When mock-infected cells prelabeled with ^{14}C-thymidine are analyzed in the same way (Figure 1c), the bulk of the ^{3}H-label is found on top of the cushion together with the mass of ^{14}C-prelabeled cellular DNA. There is a minor peak heterogeneous in size, approximately corresponding to the 50-90S region in the gradient. Thus, this size-class of DNA is found in both uninfected and Ad2-infected KB cells.

DNA in the 50-90 S size class has been observed in KB cells after Ad2 infection at multiplicities of 1, 10, 100, and 1000 PFU/cell and in experiments in which the labeling period with ^{3}H-thymidine was as brief as 30 minutes. In short pulses (1-7 minutes) the 50-90 S peak is not evident.

The 50-90 S DNA is seen throughout the course of infection. It is detected from 8-10 hours post infection until late in infection.

Characterization of the 50-90 S DNA by DNA-DNA hybridization. Fast sedimenting DNA is present in both uninfected and Ad2-infected cells. In order to determine if the fast sedimenting DNA from infected cells contains viral sequences, it is necessary to hybridize it to viral DNA. In the experiment shown in Figure 2a, ^{14}C-prelabeled cells were infected with Ad2, were labeled with ^{3}H-thymidine from 14-17 hours post infection, and were analyzed by zonal sedimentation. After centrifugation, aliquots of every other fraction were hybridized to Ad2 and to cellular DNA. The data demonstrate that the 50-90 S DNA and the 34 S peak hybridize to viral but not to cellular DNA (Figure 2b).

Therefore, the fast sedimenting 50-90 S DNA is at least partly viral in nature. It is possible that the 50-90 S DNA also contains cellular DNA, since hybridization of cellular DNA is inefficient when the filter method of hybridization is used.

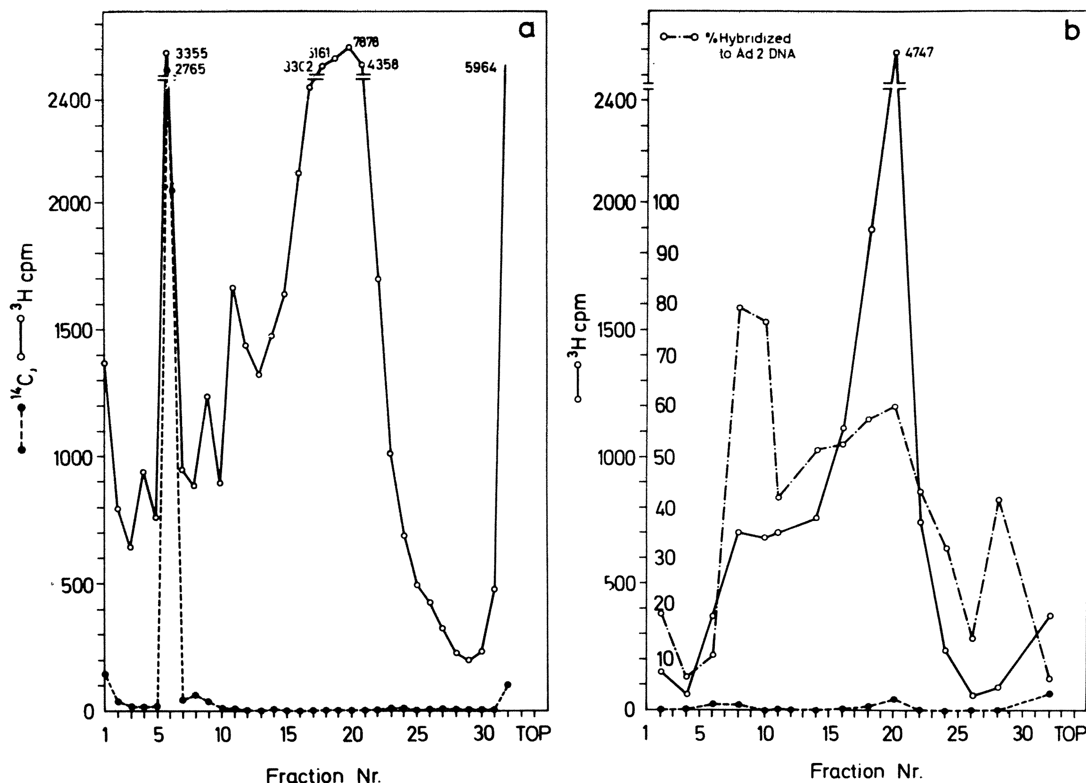

FIG. 2. Characterization of fast sedimenting DNA by DNA-DNA hybridization.

a. KB cells growing in monolayers were labeled with ^{14}C-thymidine (0.4 μCi/ml) for 4 days. The medium was then removed, the cells were carefully washed with PBS and infected with Ad2 at an m.o.i. of 100 PFU/cell. The cells were labeled with 50 μCi of ^{3}H-thymidine per milliliter of medium from 14-17 hours post infection. At the end of the labeling period, the cells were washed with PBS, scraped off the plastic dish and lysed on top of an alkaline sucrose gradient. The sample was centrifuged in the SW 27 rotor at 23,000 rpm for 7 hours at 4°C. Fractions were collected, 0.2 ml aliquots were acid precipitated and analyzed as described under Methods.

b. The ^{3}H- and ^{14}C-labeled DNA molecules in the fractions from the experiment described in a) were characterized by DNA-DNA hybridization. Five micrograms of unlabeled Ad2 DNA or 5 μg of unlabeled KB DNA were fixed to nitrocellulose filters. Every other fraction was analyzed. Aliquots of 0.2 ml were taken for hybridization. The aliquots were identical in volume to the aliquots taken in section a. o——o, ^{3}H-cpm hybridized to Ad2 DNA; •---•, ^{3}H-cpm hybridized to KB DNA; o-·-·-·-o, percent ^{3}H-cpm hybridized to Ad2 DNA.

Resedimentation of fast sedimenting viral DNA. The fast sedimenting viral DNA can be isolated from an alkaline sucrose density gradient and can be recentrifuged. It resediments at approximately 50-90 S (Figure 3). In order to achieve resedimentation at this rate, it is necessary to avoid shear breakage, pipetting and dialysis. The resedimentation at 50-90 S establishes the fast sedimenting DNA as a real and distinct class of viral DNA.

The fast sedimenting DNA is not an artifact of the lysis or sedimentation procedure. To determine if the fast sedimenting DNA could be a complex of RNA and viral DNA, a sample of ^{14}C-labeled ribosomal RNA from KB cells was lysed in alkali for 18 hours at 4°C as described above. After sedimentation, all the radioactivity was found on top of the gradient, showing that under the conditions used in this experiment RNA is hydrolyzed and thus cannot account for the high sedimentation rate of the 50-90 S viral DNA.

Digestion of protein during alkali lysis. The data in Figures 4a and 4b demonstrate that it is essential to lyse the Ad2-infected cells in 0.5 N NaOH at 4°C for many hours to remove protein efficiently from fast sedimenting viral DNA. After short incubation periods (1 hour), appreciable amounts of protein remain associated with the fast sedimenting DNA (Figure 4a). Preliminary analysis of this protein moiety by SDS-polyacrylamide gel electrophoresis suggests that it is homogeneous; however, its nature is unknown. Since the protein is effectively removed from the fast sedimenting DNA by long incubation in alkali, aggregation with protein cannot explain its high sedimentation rate.

Analysis of the fast sedimenting viral DNA in dye-buoyant density gradients. It is conceivable that the fast sedimenting viral DNA represents supercoiled circular molecules. This possibility has been eliminated by equilibrium sedimentation in dye-buoyant density gradients. When a mixture of ^{14}C-labeled Ad2 marker DNA and ^{3}H-labeled fast sedimenting viral DNA was denatured in alkali, neutralized, and sedimented to equilibrium in dye-buoyant density gradients, the fast sedimenting DNA cobands exactly with the Ad2 marker (Figures 5a and 5b). Therefore, the fast sedimenting DNA does not contain supercoiled stretches. It has previously been shown (Doerfler et al., 1973) that supercoiled, viral DNA cannot be isolated from Ad2-infected KB cells.

Conclusions. The presence of fast sedimenting, alkali stable viral DNA can be explained by one of the following possibilities:

1) mechanical drag of viral DNA into the fast sedimenting regions caused by the presence of cellular DNA
2) association of protein with DNA
3) complexing of RNA with DNA
4) supercoiled circular viral DNA

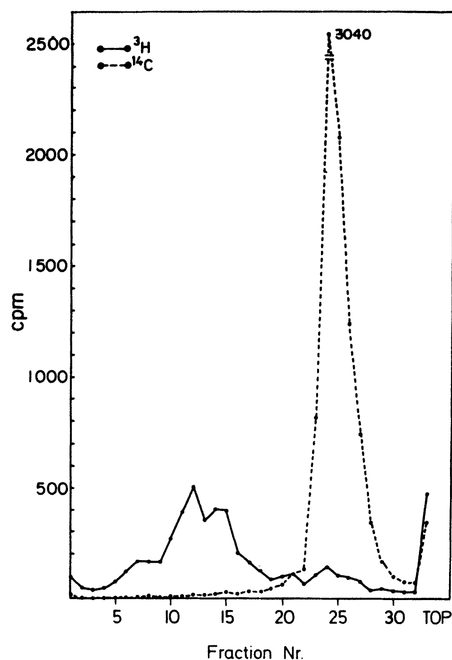

FIG. 3. Resedimentation of fast sedimenting viral DNA in alkaline sucrose density gradient. Fast sedimenting viral DNA was isolated in an experiment similar to the ones described in Figures 1a, 1b, and 2a. The fractions comprising the fast sedimenting peak were carefully poured on top of an alkaline sucrose density gradient (15-30%) together with ^{14}C-labeled Ad2 marker DNA. Pipetting of the samples was avoided to minimize shear breakage of the fast sedimenting DNA. Conditions of sedimentation: SW 27 rotor, 23,000 rpm for 10 hours at 4°C. The DNA in fractions 8 to 16 was calculated to have approximate S values of 50 to 90.

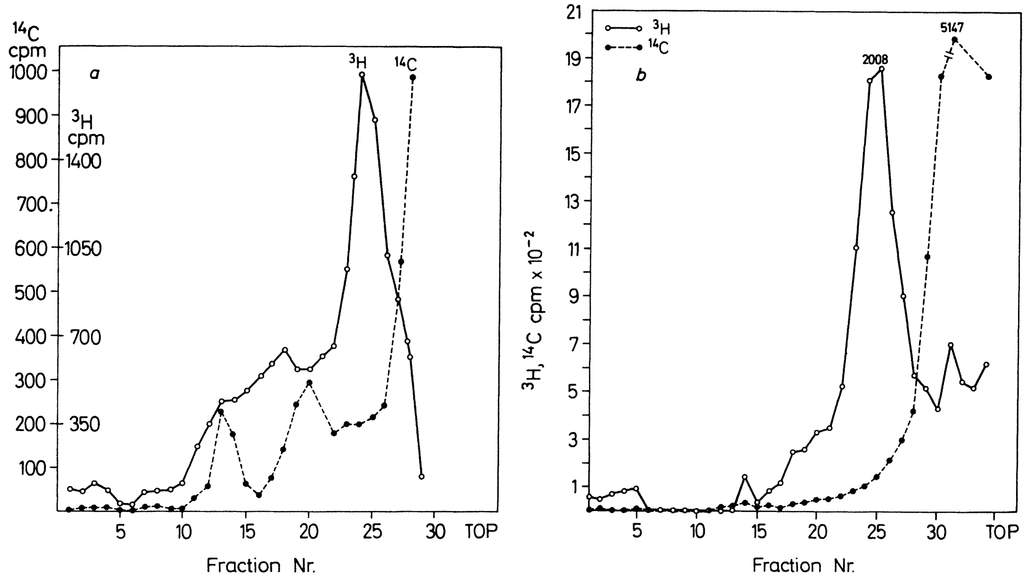

FIG. 4. Release of protein from fast sedimenting DNA by extensive alkali treatment. KB cells were infected with unlabeled, CsCl-purified Ad2 at an m.o.i. of 100 PFU/cell. At 13 hours post infection, the Eagle's medium containing 10% calf serum was removed and the medium was changed to: 25% Eagle's medium containing 10% calf serum, and 75% reinforced Eagle's medium without amino acids containing 10% calf serum. In addition, the new medium contained ^{14}C-labeled protein hydrolysate (10 μCi/ml) that was concentrated approximately tenfold by lyophilization. At 14 hours post infection, ^{3}H-thymidine (30 μCi/ml) was added to the medium. At 17 hours post infection, the cells were washed and lysed in alkali.

a. Alkali lysis at 4°C proceeded for 1 hour. The sample was centrifuged in the SW 27 rotor at 22,000 rpm for 375 minutes at 4°C.

b. Alkali lysis was extended to 17 hours at 4°C. The sample was centrifuged in the SW 27 rotor at 22,000 rpm for 375 minutes at 4°C.

5) Linear or circular oligomeric viral DNA
6) Viral DNA integrated by covalent linkage into cellular DNA.

The present studies show that the last possibility, covalent integration of viral DNA into the cellular genome, appears to be the most likely one. The possibility of mechanical trapping of viral DNA in cellular DNA can be excluded for several reasons. The first is evidence from resedimentation. When fractions containing fast sedimenting DNA are pooled and recentrifuged on alkaline sucrose gradients, the fast sedimenting DNA resediments in the same 50-90 S position (Figure 3). Secondly, viral marker DNA does not show any drag (Figure 1b). In addition, the use of less than 1 μg of DNA per gradient makes mechanical drag of the DNA unlikely. Furthermore, even when alkaline gradients are overloaded, the sedimentation patterns are unaltered.

It is very unlikely that protein remains associated with DNA after extensive alkaline hydrolysis (Figure 4). After a short term incubation of one hour in alkali, proteins, possibily specific polypeptides, remain associated with the fast sedimenting DNA. The nature of these polypeptides still has to be determined. However, after a seventeen-hour incubation in alkali, the labeled proteins no longer sediment with the fast sedimenting DNA. Similar experiments demonstrate that RNA is digested by the same long-term incubation in alkali, and therefore complexing of RNA to fast sedimenting DNA is unlikely as well.

The results of equilibrium centrifugation of the fast sedimenting viral DNA in dye-buoyant density gradients indicate that this DNA does not consist of supercoiled, covalently closed molecules (Figure 5). Other recently published results employing similar methods (Doerfler et al., 1973) demonstrate that covalently closed supercoiled circular viral DNA cannot be detected in productively or abortively infected cells. Although covalently closed supercoiled circular viral DNA is not found, it is possible that protein or other intracellular structures maintain the viral DNA in a circular configuration within the cell.

It is not possible to determine unequivocally at the present time whether the fast sedimenting DNA is oligomeric viral DNA or viral DNA integrated into the cellular genome. The fast sedimenting DNA represents a heterogeneous population of molecules since it sediments in a region extending from 50-90 S. This heterogeneity may result from fragmentation of the molecules and is consistent with the possibilities of both oligomeric and integrated viral DNA. The results of equilibrium centrifugation of fast sedimenting viral DNA in alkaline CsCl gradients (Burger and Doerfler, unpublished results) suggest that the fast sedimenting DNA has a buoyant density between that of the viral and the cellular genomes and therefore

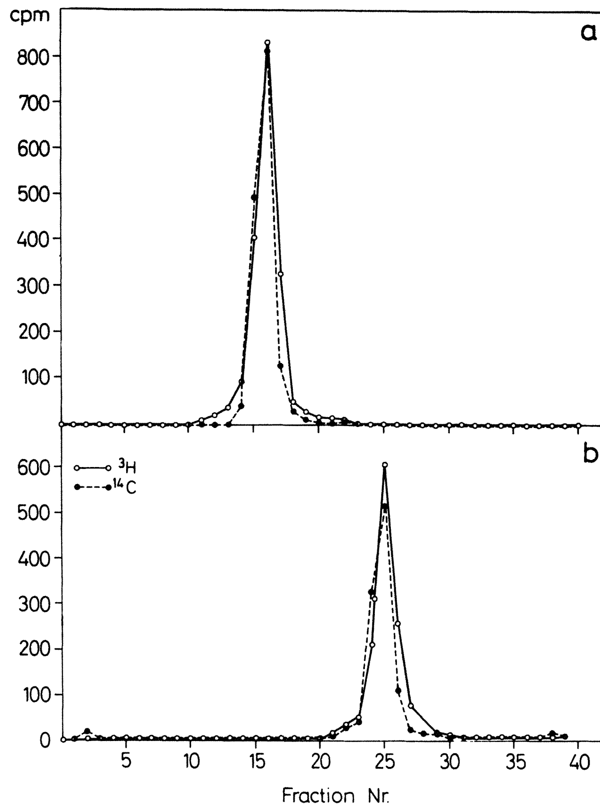

FIG. 5. Analysis of the fast sedimenting viral DNA by equilibrium centrifugation in dye-buoyant density gradients. The fast sedimenting viral DNA was isolated in an experiment similar to the one described in the legend to Figure 1b. ^{14}C-labeled Ad2 marker DNA was added to the sample, the mixture was adjusted to 0.1 N NaOH and was incubated at 4°C for 15 minutes. The sample was then neutralized by adding a predetermined amount of 1 N HCl and 1 M Tris-hydrochloride, pH 7.5. The DNA was then analyzed either directly by equilibrium centrifugation in a dye-buoyant density gradient (a) or after dialysis into 0.01 M Tris-hydrochloride pH 7.2, 0.001 M EDTA, 0.2 M NaCl (b). The samples were centrifuged to equilibrium in the SW 56 rotor at 40,000 rpm for 43 hours at 20°C. After centrifugation five-drop fractions were collected and directly analyzed in TM solution. In the figures the densities increase to the left.

must contain an integrated form of viral DNA.

If integration of Ad2 genetic material into cellular DNA does indeed occur in productive infection, the biological significance of this event has yet to be determined. Further experiments should explore the possibility whether integration may be required for efficient late transcription of viral genes using cellular promoter sites. Such promoters may be preferred by the cellular and/or viral polymerase systems. It must also be determined if integration is a chance event perhaps carrying serious consequences for the cell but no advantage to the virus.

All the evidence available to date may warrant the generalization that integration is important not only for the fixation and expression of the viral genome in a latently infected or a transformed cell, but has a function also in productive infection. This functional role that integration plays in lytic infection is hardly understood at present. The effects of viral integration on the cell have yet to be explored.

II) A DNA-RNA Complex Isolated from Ad2-Infected KB Cells

The newly synthesized DNA in KB cells productively infected with Ad2 was analyzed in dye-buoyant density gradients (Hudson *et al.*, 1969), in order to investigate whether newly synthesized DNA occurs in the form of covalently closed supercoiled circular molecules.

Analysis in dye-buoyant density gradients of the newly synthesized DNA in Ad2-infected KB cells. At late times after infection, the DNA synthesized in cells productively infected with Ad2 is almost exclusively viral (Doerfler, 1969; Green, 1962; Piña and Green, 1969; Takahashi *et al.*, 1969). Thus, it is possible to label predominantly viral DNA or intracellular forms of viral DNA involved in the replication of adenovirus DNA when Ad2-infected KB cells are maintained between 24 and 30 hours post infection in medium supplemented with ^{3}H-thymidine. Since in cells productively infected with Ad2 a large number of copies of viral DNA are synthesized (Doerfler, 1969; Green, 1962) even a minor, metabolically important component of viral DNA may be detectable in this system.

The newly synthesized DNA in Ad2-infected KB cells has been analyzed in dye-buoyant density gradients (Figure 6). A relatively small fraction (up to 15.2% [see Figure 10]) of the newly synthesized DNA bands in a higher buoyant density stratum in dye-buoyant density gradients than the bulk of the DNA which cosediments with the ^{14}C-labeled Ad2 marker DNA (not shown in Figure 2, but see Figure 7, bottom).

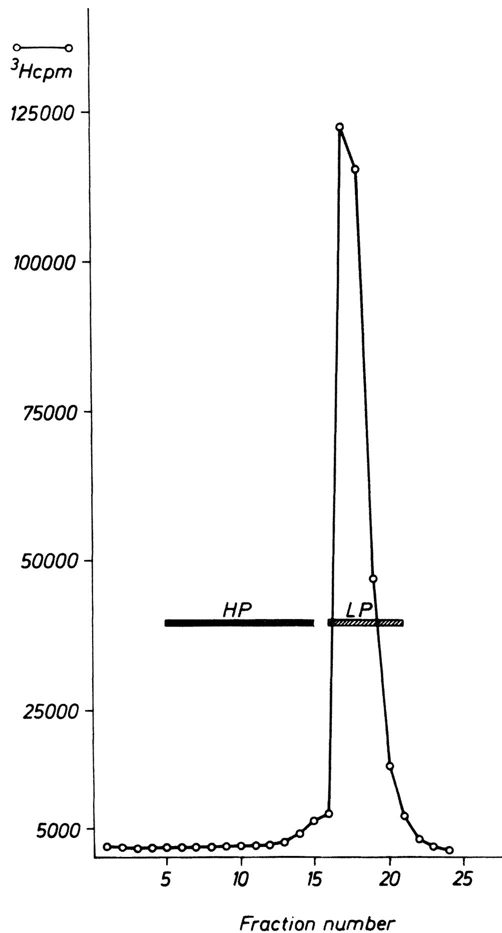

FIG. 6. Analysis of the newly synthesized DNA in Ad2-infected KB cells in dye-buoyant density gradients. Monolayers of KB cells were inoculated with a 1:50 dilution in PBS of a crude extract of Ad2-infected KB cells. After a 2 hour adsorption period at 37°C, the inoculum was removed and 5 ml of MEMC was added. At 24 hours post infection, ^{3}H-thymidine (20 μCi/ml) was added to the medium and at 30 hours post infection, the total intracellular nucleic acid was extracted and analyzed in dye-buoyant density gradients. Centrifugation was performed in an SW 56 rotor at 40,000 rpm for 41 hours at 20°C. The abbreviations HP and LP refer to the heavy and light peak fractions, respectively. The fractions indicated by the bars were pooled and analyzed by recentrifugation in a dye-buoyant density gradient (see Figure 7).

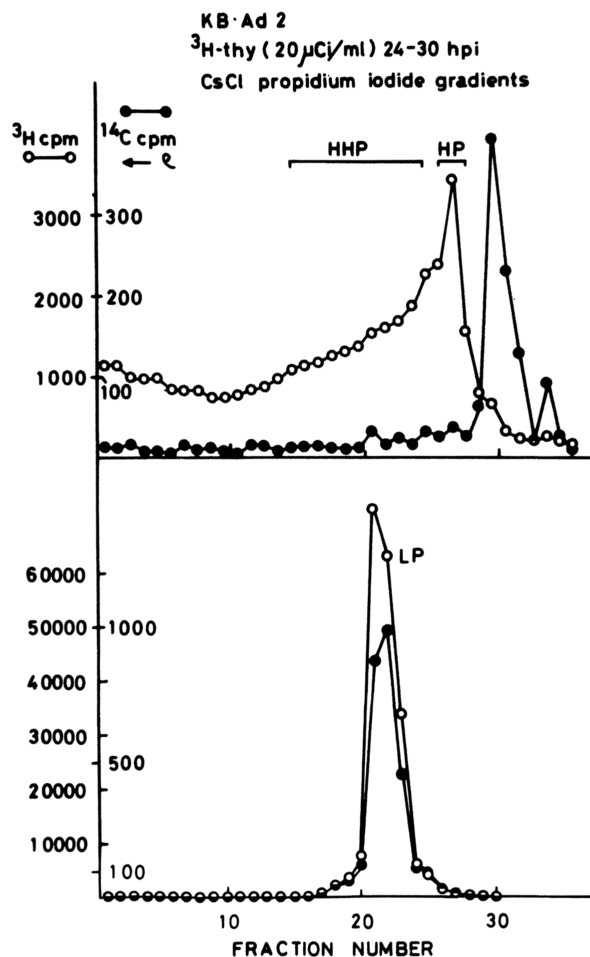

FIG. 7. Resedimentation of ^{3}H-labeled DNA in dye-buoyant density gradients. In the experiment described in the legend to Figure 6, fractions 5-15 (HP) and 16-21 (LP) were combined and resedimented in dye-buoyant density gradients. To each gradient ^{14}C-labeled Ad2 DNA was added as density marker. HHP, HP, and LP refer operationally to heavy-heavy, heavy and light peaks, respectively, indicating DNA fractions of increasing buoyant density, relative to LP which has the buoyant density of viral (Ad2) DNA complexed with propidium iodide. The limit between the HHP- and HP-fractions was drawn arbitrarily.

The DNA fractions of high buoyant density are designated HP (heavy peak), those cosedimenting with marker DNA are referred to as LP (light peak). The HP DNA is observed also when ^{3}H-thymidine is added to the medium between 8 and 13 or between 16 and 22 hours post infection. The HP DNA is found also when extracts of uninfected KB cells are analyzed in dye-buoyant density gradients.

In the experiment described in Figure 7, the HP- and LP-fractions from the experiment shown in Figure 6 have been pooled and resedimented in dye-buoyant density gradients. It is apparent that each DNA fraction resediments in the proper density stratum, the HP-fraction in a relatively wide region which has been subdivided arbitrarily into the HHP- and HP-fractions.

Further characterization of the HHP- and HP-DNA fractions. a) Rate zonal sedimentation: The combined HHP- and HP-DNA and the LP-fractions have been dialyzed extensively. Subsequently, the DNA from these fractions has been analyzed further by rate zonal sedimentation experiments in neutral (pH 7.6) and alkaline (pH 12.5) sucrose density gradients (Figure 8). At pH 7.6, the HHP/HP fractions sediment in a double peak at 59 and 39 S (Figure 8A) and the LP-fractions in a double peak of 45 and 32 S (Figure 8B). At pH 12.5, the HHP/HP-fractions sediment in a single peak at 34 S (Figure 8C), i.e. together with the ^{14}C-labeled Ad2 DNA used as marker.

b) DNA-DNA hybridization: The data in Table 1 demonstrate that the DNA in the HHP-, HP- and LP-fractions is predominantly viral DNA. The extent of the hybridization of these DNA-fractions to cellular (KB) DNA lies significantly above background. It is concluded that the DNA in the HHP-, HP- and LP-fractions is predominantly Ad2 DNA and sediments at 59 and 39 S in neutral, at 34 S in alkaline sucrose density gradients (Doerfler and Kleinschmidt, 1970). Cellular DNA synthesis is not completely shut off between 24 and 30 hours post infection and cellular DNA in small amounts is present in both HHP/HP- and LP-fractions.

Is the DNA from the HHP- and HP-fractions associated with RNA? The possibility has to be considered that the DNA banding in the HHP- and HP- regions in dye-buoyant density gradients represents supercoiled circular molecules (Hudson et al., 1969). However, the results of the rate zonal sedimentation experiments in neutral and alkaline sucrose density gradients are not consistent with the presence of supercoiled DNA molecules. Moreover, electron microscopic examination of the DNA in the HHP- and HP-fractions does not reveal circular structures.

Analysis in Cs_2SO_4 density gradients. The ^{3}H-labeled DNA from the HHP-, HP- and LP-fractions has been centrifuged to equilibrium in Cs_2SO_4 density gradients before and after treatment with pan-

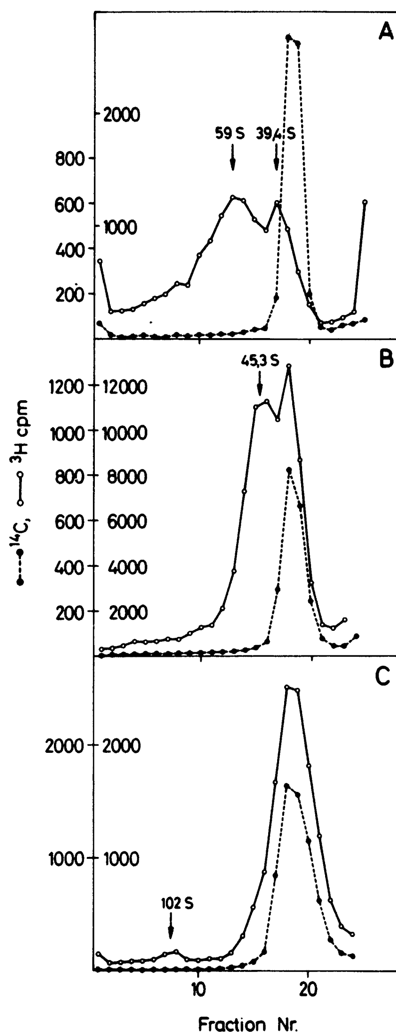

FIG. 8. Zonal sedimentation of HP DNA and LP DNA in neutral and alkaline sucrose density gradients. KB cells were infected with unlabeled Ad2 and the infected complexes were labeled with ^{3}H-thymidine between 12 and 24 hours post infection. The HP- and LP-DNA fractions were isolated by two cycles of equilibrium centrifugation in dye-buoyant density gradients. Subsequently, the dye was removed, the fractions were dialyzed against TE, and analyzed by zonal sedimentation in neutral or alkaline sucrose density gradients. A small amount of ^{14}C-labeled Ad2 DNA was used as marker. The samples were centrifuged in an SW 56 rotor at 45,000 rpm for 80 minutes at 4°C. At the end of centrifugation, six-drop fractions (0.2 ml) were collected and counted.

A. HP in neutral sucrose density gradient.
B. LP in neutral sucrose density gradient.
C. HP in alkaline sucrose density gradient.

TABLE 1. Characterization of the DNA from the HHP-, HP-, and LP-fractions (Figure 3) by DNA-DNA hybridization

Experiment	^3H DNA from	Input (counts/min)	Counts/min hybridized to KB DNA	Counts/min hybridized to Ad2 DNA	% of input hybridized to KB DNA	% of input hybridized to Ad2 DNA
1	HHP	8,224	111	3,039	1.3	37.0
	HP	10,209	148	5,008	1.4	49.1
	LP	61,761	2,402	57,657	3.9	93.4
	Ad2 virus (control)	125,278	161	56,460	0.1	45.1
2	HP	36,730	-	16,586	-	45.1
	LP	301,152	-	124,733	-	41.4
	Ad2 virus (control)	12,020	-	5,870	-	48.8

Experiment 1: Aliquots of the fractions designated HHP, HP and LP were dialyzed against 5 M NaCl in TE and against TE, were treated with a mixed bed resin (Bio-Rex RG 501-x8), and were hybridized to KB DNA and Ad2 DNA (each 5 micrograms) fixed to nitrocellulose filters (see Materials and Methods). The figures represent the mean of double determinations. Control experiments were performed with ^3H-labeled Ad2 DNA.

Experiment 2: KB cells were infected with unlabeled Ad2 and were labeled with 20 μCi of ^3H-thymidine per milliliter from 15 to 20 hours post infection. The HP- and LP- fractions were purified over two cycles of equilibrium centrifugation in dye-buoyant density gradients. The dye was removed by extensive dialysis versus 5M NaCl in TE and TE. Aliquots of the ^3H-labeled HP- and LP-fractions and of the ^3H-labeled Ad2 DNA were boiled in 0.27 N NaOH for 10 minutes to denature the DNA and destroy the RNA. Subsequently, the samples were chilled, rapidly neutralized and added to the hybridization mixture. Each value represents the mean of double determinations.

creatic ribonuclease at low (0.005 M Tris-hydrochloride, pH 7.5) and high (0.2 M NaCl in 0.005 M Tris-hydrochloride, pH 7.5) salt concentrations to determine whether the viral DNA in these fractions is associated with RNA. The results of this experiment clearly demonstrate (Figure 9) that--prior to digestion with ribonuclease--the DNA in the HHP- and HP-fractions from dye-buoyant density gradients sediments in Cs_2SO_4 gradients in a density stratum characteristic for DNA-RNA complexes. The data indicate that the DNA from the HHP-region is associated with larger amounts of RNA than

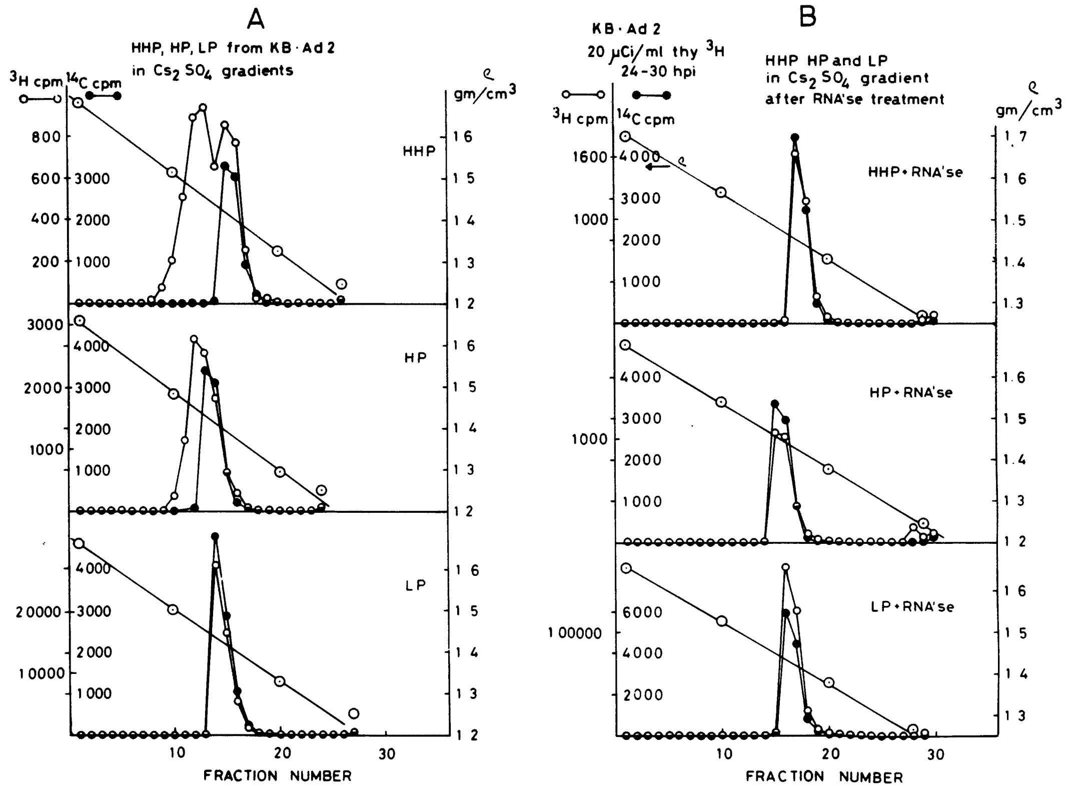

FIG. 9. Analysis of the HHP-, HP-, and LP-fractions by equilibrium sedimentation in Cs_2SO_4 density gradients.

A. Aliquots of the HHP-, HP-, and LP-fractions were dialyzed against 5M NaCl in TE and against TE for a total of 54 hours, and were subsequently centrifuged to equilibrium in Cs_2SO_4 density gradients. Each gradient contained 6.4 µg of ^{14}C-labeled Ad2 DNA as density marker. The gradients were centrifuged for 44 hours at 35,000 rpm and at 20°C. In every tenth fraction the refractive indices were measured and the densities were calculated according to the equation of Vinograd and Hearst (1962).

B. In this series of experiments the samples from the HHP-, HP-, and LP-fractions were incubated with pancreatic ribonuclease prior to equilibrium sedimentation. Aliquots (0.1 ml) of the HHP-, HP-, and LP-fractions were diluted with 0.4 ml of 0.005 M Tris-hydrochloride and were incubated with pancreatic ribonuclease (18 µg/ml) at 37°C. After 80 minutes, the mixtures were extracted with twice the volume of phenol saturated with 1 M Tris-hydrochloride, pH 7.5. The phenol was removed by diethylether. Finally, the samples were flushed with N_2 and prepared for equilibrium sedimentation in Cs_2SO_4 density gradients.

the DNA from the HP-region. Both fractions of DNA contain structures which apparently lose their RNA components during removal of the propidium iodide prior to equilibrium centrifugation in Cs_2SO_4 density gradients since some of the DNA cobands with the ^{14}C-labeled Ad2 marker DNA in Cs_2SO_4 density gradients. As expected, the DNA from the LP-region in the dye-buoyant density gradients cobands with the marker DNA in Cs_2SO_4 density gradients. When the DNA in the HHP-, HP- and LP-fractions is treated at high or low salt concentrations with pancreatic ribonuclease (previously heated to 90°C for 5 minutes) and then sedimented to equilibrium in Cs_2SO_4 density gradients, all of the ^3H-activity cobands with the marker DNA (Figure 9B).

These results suggest that a fraction of the newly synthesized viral DNA in Ad2-infected KB cells is associated with RNA in such a way that the RNA component of the complex becomes susceptible to digestion by pancreatic ribonuclease at high or low salt concentration. This finding can be explained best by assuming that an RNA chain is hydrogen-bonded to DNA by only part of its sequences and that the major part of the RNA chain remains free. The DNA complex can be separated from "free" viral DNA by equilibrium centrifugation in CsCl-propidium iodide density gradients.

The RNA-moiety of the HP-fraction is partly liberated before or during the final centrifugation procedure since there is free RNA present in the gradient even prior to thermal denaturation of the HP-fraction. This RNA is possibly liberated from the complex due to the shift from solutions of high to low to high salt concentration. After heat denaturation of the HP-fraction and banding in Cs_2SO_4 density gradients the label in the density stratum characteristic for a DNA-RNA complex shifts to the density position of free RNA.

In a control experiment Ad2-infected KB cells were double labeled with ^3H-uridine and ^{14}C-thymidine and the HP- and LP-fractions were separated by equilibrium centrifugation in CsCl-propidium iodide and Cs_2SO_4 density gradients. The ^3H- and ^{14}C-activity profiles of the LP-fractions coincide exactly in CsCl-propidium iodide gradients (Figure 10).

<u>Characterization of the RNA component associated with viral DNA. a) Isolation by Cs_2SO_4 density gradient centrifugation</u>. The DNA-RNA complex has been labeled with both ^{14}C-thymidine and ^3H-uridine. After equilibrium sedimentation in dye-buoyant density gradients, both the HP-region (fractions 6-14) and the LP-fractions (fractions 16-17) in Figure 10 contain ^{14}C- and ^3H-activities. The results of an experiment in which the HP- and LP-fractions have been recentrifuged in a second CsCl-propidium iodide gradient are shown in Figure 11. The HP-fraction gives rise to a peak of

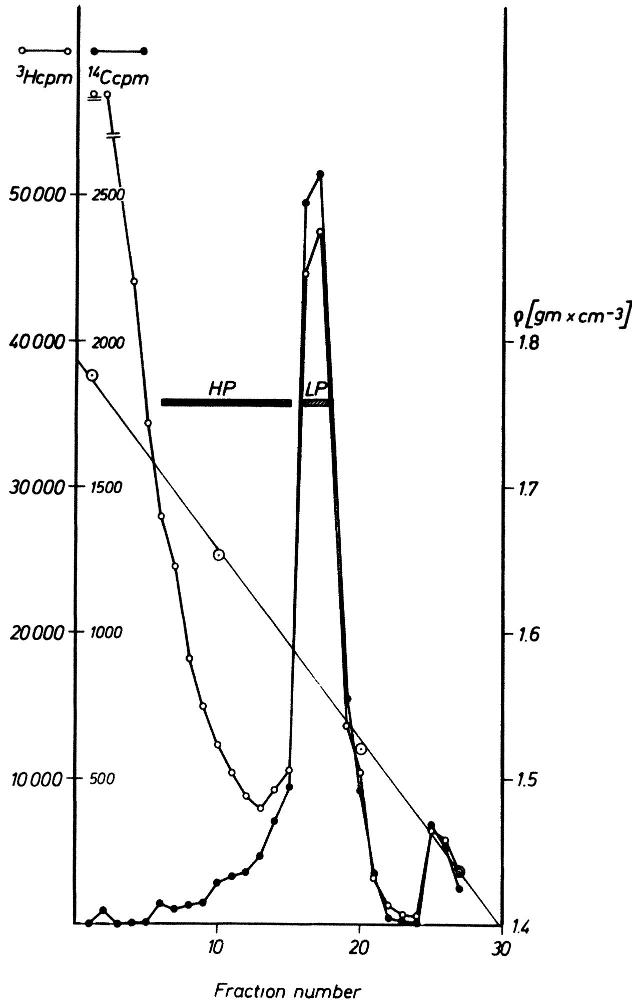

FIG. 10. Analysis of DNA from Ad2-infected KB cells labeled with ^{3}H-uridine and ^{14}C-thymidine. KB cells (4.9 x 10^{6} cells per petri dish) growing in monolayers were inoculated with CsCl-purified Ad2 (ca. 10^{3} PFU/cell). At 16 hours post infection, 4 μCi of ^{3}H-uridine and 0.5 μCi of ^{14}C-thymidine were added per milliliter of medium. At 22 hours post infection, the cells were washed with PBS-d and the intracellular DNA was extracted and analyzed in a dye-buoyant density gradient using CsCl and propidium iodide. Aliquots of all fractions were precipitated with TCA and the radioactivity was determined to yield the distribution of the ^{3}H- and ^{14}C-activities. Fractions 6 to 15 were combined to the HP (heavy peak) fraction, fractions 16-18 to the LP (light peak) fraction. Densities were calculated from the refractive indices as described by Vinograd and Hearst (1962).

free RNA (fractions 1-5) and to at least two peaks of RNA-DNA (fractions 7-11) and DNA-RNA (fractions 13-18) complexes containing different amounts of DNA, whereas the LP-fractions resediment as a sharp, symmetrical peak of DNA in which the ^3H- and ^{14}C-activity profiles are matched exactly (see above).

The fractions characterized by the experiment in Figure 11 have been purified further by equilibrium centrifugation in Cs_2SO_4 density gradients. It can be shown that the free RNA component and the RNA-DNA complex can be clearly resolved in these gradients. Again, it is observed that the ^3H- and ^{14}C-activity profiles in the free DNA component are completely congruent.

b) RNA-DNA hybridization: The RNA components of the RNA-DNA and DNA-RNA fractions as well as free RNA as characterized by the experiment described in Figure 11 have been analyzed by RNA-DNA hybridization (Table 2, Experiment 1). In one experiment (Table 2, Experiment 2) the RNA component of the complexes was isolated in Cs_2SO_4 density gradients after heat denaturation of the complexes and then used in RNA-DNA hybridization experiments. All RNA-fractions hybridize predominantly to viral DNA. There is no difference in the extent of hybridization to viral DNA between the RNA isolated from the complexes and the RNA still in the complex. There is little hybridization to cellular DNA.

Conclusions. The intracellular DNA of KB cells productively infected with adenovirus type 2 has been analyzed by equilibrium sedimentation in dye-buoyant density gradients to investigate whether Ad2 DNA can become circularized in the process of viral DNA replication. Although as much as 15 percent of the newly synthesized viral DNA has a buoyant density in CsCl-propidium iodide gradients which is characteristic of supercoiled circular DNA, further analysis of this fraction of viral DNA does not support the notion that Ad2 DNA becomes circularized in the cell. Electron microscopic examination of the material from the higher buoyant density regions does not reveal circular structures (unpublished data). Furthermore, the results of rate zonal analysis in sucrose density gradients at pH 7.6 and 12.5 are not compatible with the presence of supercoiled circular Ad2 DNA molecules (Figure 8).

The experimental evidence presented is in agreement with the conclusion that an Ad2 specific DNA-RNA complex can be separated from the bulk of the newly synthesized DNA due to the increased buoyant density of the complex in CsCl propidium iodide density gradients. Preliminary data (Ortin and Doerfler, unpublished results) indicate that the bulk of the RNA isolated from the complexes carries sequences of polyadenylic acid since more than 80% of the RNA binds to polyuridylic acid-sepharose under suitable conditions. This finding further supports the notion that the DNA-RNA complex is indeed involved in transcription.

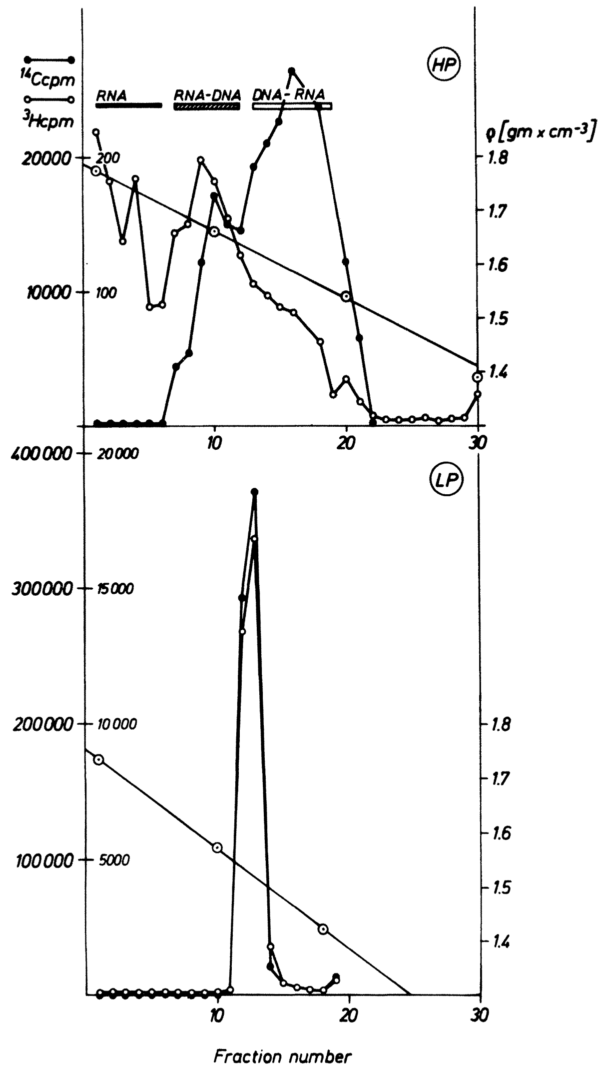

FIG. 11. Resedimentation of the HP- and LP-fractions in CsCl propidium iodide gradients in dye-buoyant density gradients. The HP material (top frame) can be resolved into the RNA-, RNA-DNA-, and DNA-RNA-fractions. Judging from the buoyant densities of the fractions, free viral DNA would be expected to band between fractions 20 and 22 in the HP resedimentation experiment. The LP material resediments in the density stratum of free DNA (bottom frame). The experimental conditions are the same as described in the legend to Figure 10.

TABLE 2. Characterization of the RNA moiety from the RNA-, RNA-DNA, and DNA-RNA fractions by RNA-DNA hybridization

Experiment	^{3}H-labeled RNA	Input (counts/min)	Hybridized counts/min KB DNA	Ad2 DNA	% of input hybridized KB DNA	Ad2 DNA
1	RNA fraction	41,131	102	4,639	0.25	11.3
	RNA-DNA fraction	9,582	134	3,745	1.4	39.1
	DNA-RNA fraction	1,026	6	1,430	0.6	100
2	HP-heated	3,159	-	473	-	15.0
	HP-unheated	673	-	325	-	48.3

Experiment 1: The fractions after the second cycle of equilibrium sedimentation in dye-buoyant density gradients (Figure 11) were analyzed by the DNA-RNA annealing procedure. To each filter 5 micrograms of ^{14}C-labeled Ad2 DNA or KB DNA were fixed. By using ^{14}C-labeled DNA on the filter, it was possible to standardize the results to a unit amount of DNA on the filter. In these experiments, the RNA-DNA and DNA-RNA fractions were added directly to the hybridization mixture without previous denaturation. The values represent the mean of double determinations.

Experiment 2: Ad2-infected KB cells were labeled with ^{3}H-uridine (20 μCi/ml) and ^{14}C-thymidine (0.1 μCi/ml) from 16-23 hours post infection. The HP- and LP-fractions were isolated by two cycles of equilibrium sedimentation in dye-buoyant density gradients. The dye was removed by dialysis and the HP-fractions were resedimented in Cs_2SO_4 density gradients. Prior to equilibrium sedimentation in Cs_2SO_4 density gradients, one half of the HP-material was heat denatured (100° for 5 minutes, then chilled). For the DNA-RNA hybridization reaction the ^{3}H-labeled material from the density position of free RNA (HP-heated) and of the DNA-RNA hybrid (HP-unheated) was used.

The data obtained so far do not provide information on the question whether some of the RNA in this complex is covalently linked (Sugino, Hirose, and Okazaki, 1972; Wickner et al., 1972) to viral DNA. It has to be emphasized that the ^{3}H-uridine label incorporated into DNA (in the LP structure) is alkali stable, and hence not in RNA. A number of laboratories have reported that a partly

single-stranded DNA structure may play a role in the replication of adenovirus DNA (Sussenbach et al., 1972; van der Eb, 1973; van der Vliet and Sussenbach, 1972).

In short pulses of ^{3}H-thymidine of 5, 10, and 15 minutes duration given at 16 hours post infection, 36.4%, 43.4% and 32.6%, respectively, of the total label incorporated into DNA is found in the HP-fraction. A high proportion of this label can be chased (30 minutes, 100 μg of cold thymidine per milliliter) into the LP-position of free viral DNA. Thus, it is likely that a part of the label found in the HP-position after short pulses represents partly single-stranded DNA which may be involved in viral DNA replication. When a six hour labeling period is used the bulk of the ^{3}H-label in the HP-region is due to the DNA-RNA complex.

ACKNOWLEDGEMENTS

H. B. is a graduate fellow of the Rockefeller University. During part of this work W. D. was a Career Scientist of the Health Research Council of the City of New York (I-620). This research was supported by grants from the American Cancer Society, VC 14A and the Deutsche Forschungsgemeinschaft, SFB 74 to W. D.

We thank Almut Latscha and Elisabeth Marczinski-Verheugt for technical assistance.

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THE MECHANISM OF REPLICATION OF ADENOVIRUS TYPE 5 DNA

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INTRODUCTION

The virions of adenovirus type 5 (Ad5) DNA contain a linear double-stranded DNA molecule with a length of 11 μm corresponding to a molecular weight of 23 million daltons (Eb, Kesteren, and Bruggen, 1969; Green et al., 1967). Pearson and Hanawalt (1971) have shown that by labeling adenovirus-infected HeLa cells with ^{3}H-thymidine for 5 minutes, the major fraction of the label entered into a fast-sedimenting "replication complex." The label could be chased from this complex into mature adenovirus DNA. The DNA isolated from these complexes showed an increased buoyant density in CsCl as compared to mature adenovirus DNA. This suggested the presence of single-stranded DNA or RNA in the replicative forms of adenovirus DNA.

We have further characterized the replicative intermediates employing a system of isolated nuclei derived from Ad5-infected KB cells (Sussenbach and Vliet, 1972; Vliet and Sussenbach, 1972). These nuclei continue to support viral DNA synthesis from the four deoxyribonucleoside triphosphates (dNTP's). A similar system has been developed by Reichard and collaborators for polyoma virus (Winnacker, Magnusson, and Reichard, 1971). The nuclear system has several advantages over intact infected cells, e.g., for labeling of DNA the direct precursors of DNA synthesis (dNTP's) can be used, which allows for rapid pulse and density shifts. This facilitates the identification and isolation of replicative intermediates. Our investigations so far have mainly been concerned with the structure

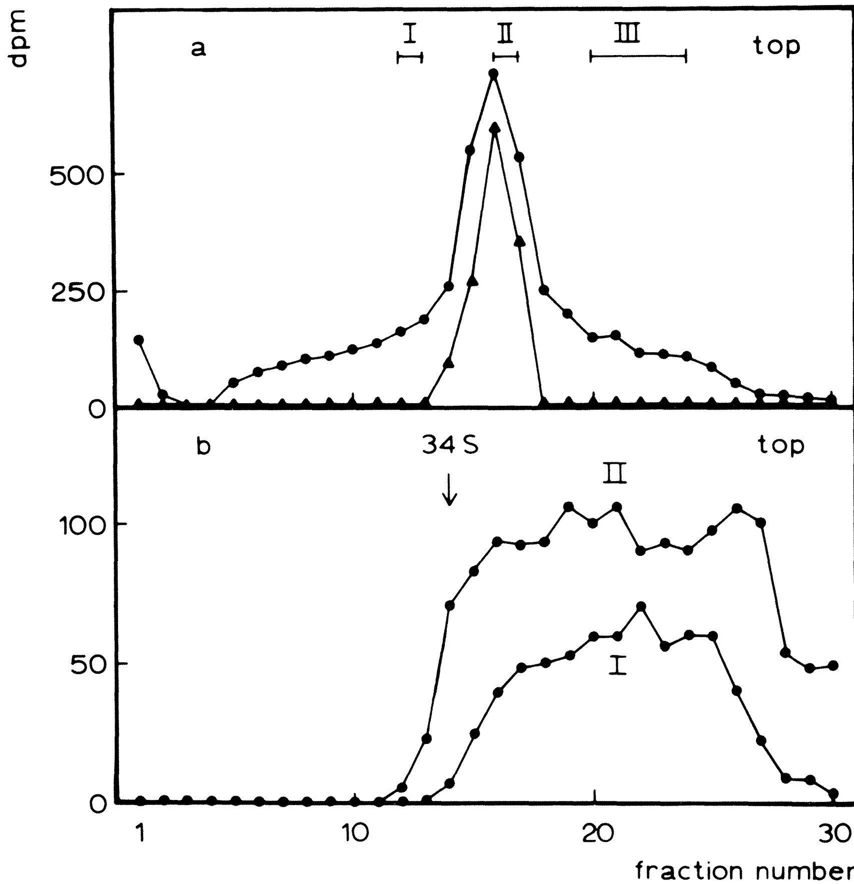

FIG. 1. A) Sucrose gradient centrifugation of new ^{3}H-DNA (●——●) on an isokinetic sucrose gradient synthesized in the nuclear system. Centrifugation was performed for 16 hours at 19,000 rpm in the Spinco SW25. ^{14}C-labeled mature Ad5 DNA (▲——▲) was used as marker (31S).

B) Alkaline sucrose gradient centrifugations of fractions I and II on isokinetic sucrose gradients at 37,000 rpm for 4 hours in the Spinco SW41 rotor with mature Ad5 DNA as 34S marker.

of the replicative intermediates of Ad5 DNA and the interpretation of such structures in terms of a mechanism of DNA replication. In this paper we present experiments which support a displacement mechanism for the replication of the Ad5 DNA involving λ-shaped intermediates and which will be referred to as the λ mechanism in order to distinguish it from the well-known θ and σ mechanism for circular DNA replication.

RESULTS

For the isolation and characterization of replicative intermediates we have developed a system of isolated infected nuclei. Cells in suspension culture were infected with Ad5 at a M.O.I. of 3,000 physical particles per cell. At 18 hours after infection cells were harvested and resuspended in a hypotonic Hepes buffer. At that time cellular DNA synthesis is considerably reduced, while active viral DNA synthesis occurs. Subsequently the cells were ruptured by a Potter homogenizer, and the nuclei were isolated by centrifugation. In order to study viral DNA synthesis, nuclei were incubated in a synthetic medium containing 40 mM Hepes pH 7.1, 90 mM NaCl, 5 mM ATP, 8 mM $MgCl_2$, 0.4 mM $CaCl_2$, 8 mM phosphoenolpyruvate, 0.05 mM each of dGTP, dCTP, dATP, 0.001 mM 6-^{3}H-dTTP (7 Ci/mM) and 1 U/ml pyruvate kinase. After incubation DNA was isolated using a Hirt extraction procedure (Sussenbach and Vliet, 1972; Vliet and Sussenbach, 1972).

Figure 1A represents the result of a sucrose gradient centrifugation of DNA extracted from isolated infected nuclei which were incubated for 60 minutes. This incubation period corresponds to a pulse of approximately 3 minutes in intact cells. Three classes of new DNA were observed, viz. one sedimenting faster than mature Ad5 DNA (I), one sedimenting slower (III), and one sedimenting with the same velocity (II).

There is agreement among several groups working in this field about the interpretation of a sedimentation profile as shown in Figure 1A. Eb (1973), Petterson (in press), and Vlak (unpublished results) obtained similar results with Ad2- or Ad5-infected intact cells, which were pulse-labeled with ^{3}H-thymidine for 1-3 minutes at 14 hours after infection. Bellett and Younghusband obtained similar results with CELO virus (1972).

The material sedimenting faster than mature Ad5 DNA (I) and the material at the same position as mature Ad5 DNA (II) contain replicative intermediates. The labeled material sedimenting slower than mature DNA (III) consists for a considerable part of cellular DNA, while the other part represents fragments of replicative intermediates produced by shear.

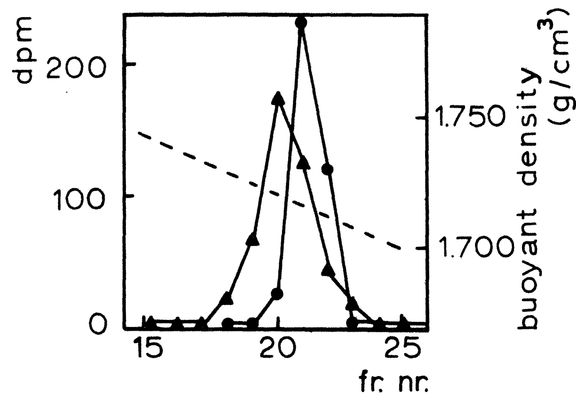

FIG. 2. Equilibrium density gradient centrifugation of ^{3}H-DNA (▲—▲) (fraction I from Figure 1A) in neutral CsCl gradients. Centrifugation was performed at 38,000 rpm for 76 hours in a Spinco Ti50 rotor with ^{14}C-labeled Ad5 DNA as density marker (●—●).

The evidence that fractions I and II contain replicative intermediates can be summarized as follows:

1. It has been shown that during short pulse-labeling of new DNA in intact infected cells, the label is incorporated in fast-sedimenting molecules with sedimentation coefficients between 31 and 80S (Eb, 1973; own unpublished results). During a subsequent chase period all pulse-label can be converted into material sedimenting identical to mature adenovirus DNA (31S).

2. The DNA present in the fast-sedimenting material hybridized to a high extent to adenovirus DNA and not to cellular DNA, indicating its viral origin (Sussenbach and Vliet, 1972; Vliet and Sussenbach, 1972).

These results are compatible with the assumption that the fast-sedimenting molecules represent replicating adenovirus DNA. An extended study of the replicative intermediates, isolated from nuclei, has led to the following conclusions:

1. Replicative intermediates contain new DNA of genome length or shorter. Centrifugation of the replicative intermediates in fraction II (Figure 1A) in alkaline sucrose gradients shows that this fraction contains new DNA strands of genome size (34S) or shorter molecules (Figure 1B). The fast-sedimenting molecules (fraction I), however, contain only new strands shorter than genome length. Single-stranded new DNA longer than genome length as expected for a rolling circle type of replication has never been observed.

2. Replicative intermediates contain single-stranded DNA. The fast-sedimenting DNA, observed in the nuclear system, was further studied by centrifugation in neutral CsCl gradients. Figure 2 shows that this DNA has a higher buoyant density (1.716-1.724 g/ml) than mature Ad5 DNA (1.716 g/ml) (Sussenbach and Vliet, 1972). The high density of the replicative intermediates suggests the presence of considerable regions of single-stranded DNA or RNA. The absence of RNA was concluded from the observation that digestion of the fast-sedimenting DNA with 20 μg/ml pancreatic RNase and 0.25 U/ml T_1RNase for 2 hours under low salt conditions did not influence the buoyant density in CsCl. The presence of single-stranded DNA was established by binding experiments with this DNA to benzoylated naphthoylated DEAE-cellulose (BND-cellulose). Almost all fast-sedimenting DNA behaved as single-stranded DNA containing molecules (Sussenbach *et al.*, 1972).

3. The new DNA in replicative intermediates is not covalently linked to parental DNA. To investigate a possible linkage between parental and new DNA, nuclei were isolated from cells infected with

Ad5 containing ^{32}P-DNA. Subsequently the nuclei were incubated in a synthetic medium containing dBTP and ^{3}H-dATP. Under these conditions heavy new ^{3}H-DNA is synthesized. The absence or presence of a covalent linkage was studied by centrifugation of the replicative intermediates in alkaline CsCl gradients. It was observed that no linkage existed between the light ^{32}P-DNA and the heavy ^{3}H-labeled new DNA, indicating the absence of repair synthesis (Vliet and Sussenbach, 1972).

4. Replicative intermediates have a linear structure. Electron microscopy of isolated intermediates revealed the existence of branched and unbranched linear molecules containing considerable regions of single-stranded DNA (Sussenbach et al., 1972). A collection of several types of these molecules is shown in Figure 3. Also completely single-stranded molecules of genome length were observed.

These observations have led to a model for the replication of Ad5 DNA (Figure 4), which will be referred to as the λ model. In this model DNA synthesis starts at the end of the linear duplex on one strand displacing the other strand. This process can continue until one strand is completely displaced explaining the presence of single strands of genome size. Displacement synthesis thus produces one completed double-stranded daughter molecule and a single strand. This single strand is converted into the other daughter molecule by discontinuous synthesis of a complementary strand, explaining the single-stranded molecules with double-stranded regions. Finally and alternatively, this complementary strand synthesis may already start in the branched structure ultimately leading to the same result.

Recently we have studied in more detail several questions raised by the λ model. This model predicts that the template strand is in a double-stranded form. To study this prediction, KB cells were infected with ^{3}H-labeled Ad5 and 18 hours after infection viral DNA was isolated. Figure 5 shows the result of a sucrose gradient centrifugation of this isolated DNA. A part of the parental viral DNA sediments at the position of replicative intermediates. Several fractions pooled as indicated in Figure 5 were analyzed for the ratio of single- over double-stranded ^{3}H-DNA by BND-cellulose chromatography. Before the BND-cellulose chromatography the DNA was sonicated to fragments of 300,000 daltons. The percentages of ^{3}H-DNA, which was found in a single-stranded form, have been indicated in Figure 5. This analysis shows that a considerable fraction of the parental DNA in replicative intermediates is single-stranded.

In contrast we have found that new DNA was in a double-stranded form. To investigate this aspect, it is necessary to have a system in which DNA replication does not exceed one round of replication.

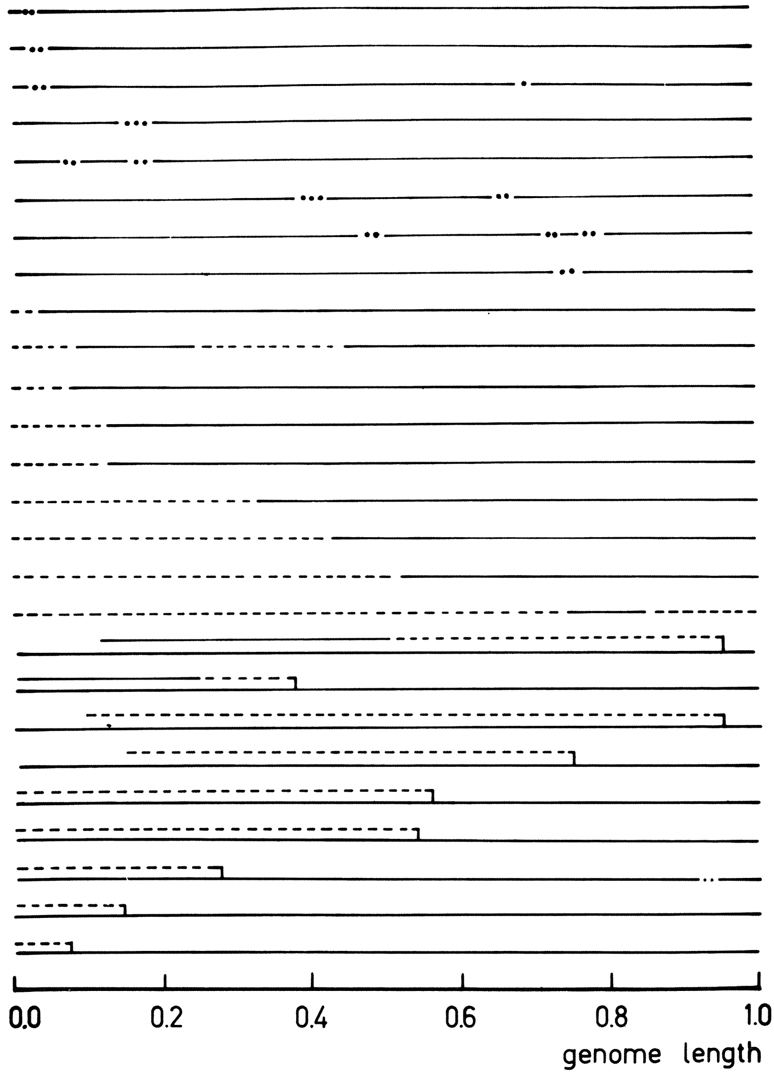

FIG. 3. An array of replicating intermediates of Ad5 DNA observed by electron microscopy of replicating Ad5 DNA. Molecules were normalized to genome length. The solid and dashed lines represent double-stranded and single-stranded regions, respectively.

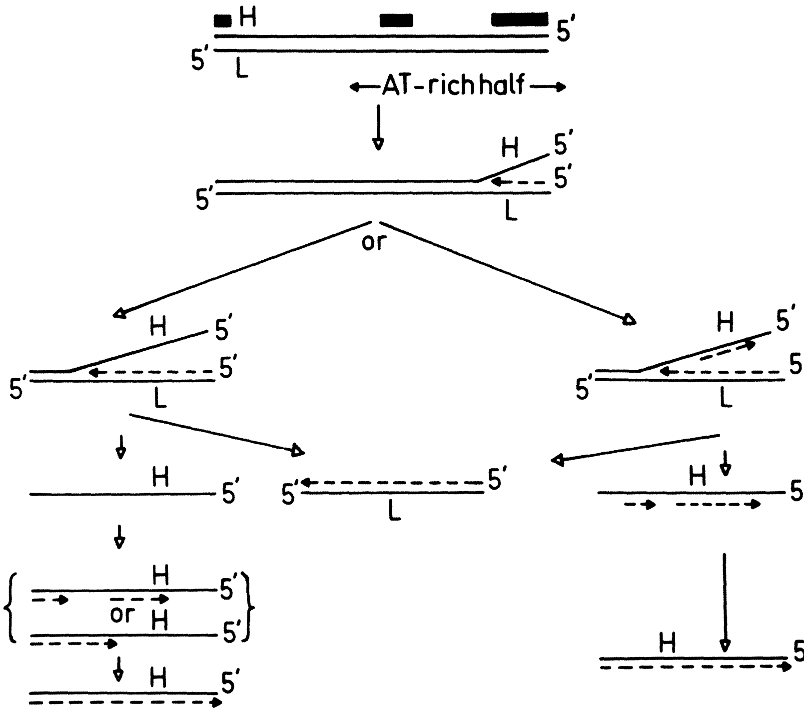

FIG. 4. λ model for the replication of Ad5 DNA. Parental strands have been drawn as solid lines and new daughter DNA has been indicated with broken lines. The black bars in the upper part of this figure represent the three prominent denaturation sites. The complementary strands with the lower and higher equilibrium density in alkaline CsCl have been indicated with L and H, respectively.

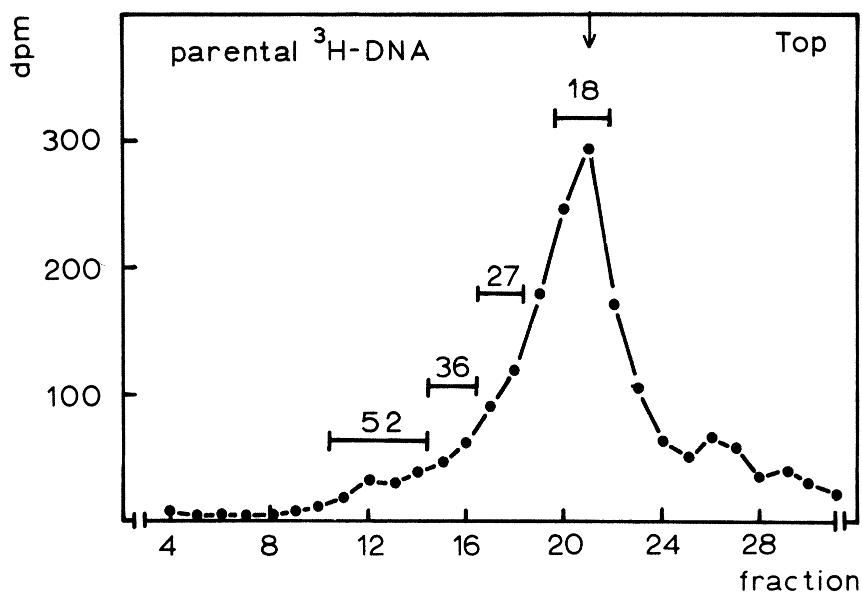

FIG. 5. Neutral sucrose gradient centrifugation of ^{3}H-DNA isolated from cells after infection with Ad5 containing ^{3}H-DNA. Centrifugation was performed for 16 hours at 19,000 rpm in the Spinco SW25 rotor. Fractions were pooled as indicated and the percentage of single-stranded DNA was determined by BND-cellulose chromatography. The results are shown in the figure. The arrow indicates the position of mature DNA (31S).

Consequently, we have used the nuclear system, in which no new initiations occur but forked molecules are completed (Vliet and Sussenbach, 1972). Isolated nuclei were incubated for 2 hours in the presence of ^{3}H-dTTP. The isolated viral DNA was centrifuged in neutral sucrose gradients, and the replicative intermediates sedimenting at 31S and 50S, respectively, were analyzed by BND-cellulose chromatography after sonication. More than 95% of the newly synthesized DNA in both fractions behaved as double-stranded DNA.

Another question raised by the λ model concerns the nature of the single-stranded DNA in replicative intermediates, viz. whether it represents only the viral H-strand or only the viral L-strand or both strands. To solve this question single-stranded DNA isolated from replicative intermediates was hybridized to separated strands of Ad5 DNA.

Fractions 9-20 from the sucrose gradient presented in Figure 5 were pooled, sonicated, and fractionated on BND-cellulose to separate double-stranded from single-stranded material. The single-stranded fragments were then hybridized to filters containing the separated H- and L-strand. These separated strands were obtained by repeated alkaline CsCl gradient centrifugations and self-annealing followed by hydroxyapatite chromatography. Although there is only a small difference in density (4mg/ml) between the viral L(ight) and the viral H(eavy) strand (the strands with the lower and higher equilibrium buoyant density in alkaline CsCl, respectively), this procedure leads to a satisfactory separation of the complementary strands. Hybridization of 0.08 μg of the single-stranded fragments with 0.02 μg of the separated H- and L-strand, respectively, which were bound to filters, revealed that 4% of the added fragments hybridized to the H-strand and 15% to the L-strand. This indicates that the single-stranded material hybridized preferentially to the viral L-strand, suggesting that the displaced strand is of the heavy type. This conclusion was confirmed by using hydroxyurea. We have infected KB cells with Ad5 in the presence of an inhibitor of DNA synthesis, hydroxyurea, in an attempt to synchronize the start of DNA replication. When the inhibitor was removed, viral DNA replication began. Under these conditions displacement synthesis occurred but the synthesis of the complementary strand was retarded, leading to an accumulation of single-stranded viral DNA (Sussenbach and Vliet, 1973). Hybridization of the displaced single strands with the separated strands showed duplex formation with the viral L-strand exclusively (results not shown here).

The preferential displacement of the viral H-strand implies that displacement synthesis starts mainly from the same molecular end of the linear Ad5 duplex. To determine from which end

FIG. 6. Partial denaturation pattern of Ad5 DNA obtained by denaturation in 85% formamide, 10 mM Tris, 1 mM EDTA pH 8.5, 21°C. The boxes represent denaturation sites.

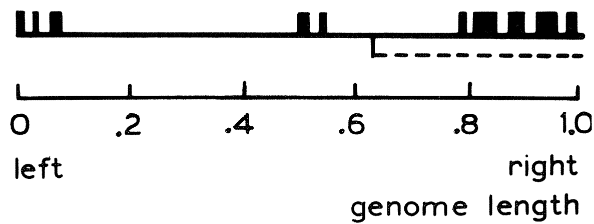

FIG. 7. Partial denaturation pattern of replicating Ad5 DNA. This example is representative for 22 analyzed branched intermediates. The boxes represent the denaturation sites; the dotted line corresponds to the single-stranded branch.

replication starts, the orientation of the replication point in the replicative intermediates relative to regions of partial denaturation was further investigated.

Partial denaturation of Ad5 DNA by formamide shows three prominent denaturation sites (as has also been found by Doerfler and Kleinschmidt for Ad2 DNA [1970]) between 0.0-0.1, 0.5-0.6, and 0.8-1.0 on a scale of fractional length (Figure 6). The region at 0.8-1.0 is defined as the molecular right end. The asymmetric denaturation pattern allows an easy distinction of the molecular ends in replicative intermediates. Replicating molecules were prepared in the nuclear system and isolated as described earlier (Sussenbach et al., 1972). After isolation, the DNA was partially denatured by formamide and studied by electron microscopy. Analysis of 22 Y-shaped replicative intermediates which contained a single-stranded and a double-stranded arm of equal length showed that in all cases the double-stranded arm contained the typical denaturation of the molecular right side (Figure 7). These preliminary results indicate that replication of Ad5 DNA starts always at the molecular right end of the linear duplex displacing the viral heavy strand (Figure 4). This implies that the 5'-end of the viral H-strand is located at the molecular right end and that this end is recognized by an initiation factor or DNA polymerase.

CONCLUSION

Based on the study of the structure of replicative intermediates of Ad5 DNA, we have proposed a model for the replication of this DNA. Several predictions of this model have been confirmed as shown in this paper and others (Sussenbach et al., 1972; Sussenbach and Vliet, 1973). Although this model explains the existence of different types of intermediates, the detailed processes involved in replication are still unknown. We do not know which DNA polymerases are involved in the displacement and the complementary strand syntheses, why and how replication starts from one specific molecular end, and how the molecular ends are conserved during replication. Experiments to solve several of these problems are in progress.

ACKNOWLEDGEMENTS

The authors thank M. G. Kuijk, A. T. Mulder, and G. van Dam for their expert technical assistance. This study was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for the Advancement of Pure Research.

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THE ISOLATION AND CHARACTERIZATION OF DNA BINDING PROTEINS SPECIFIC FOR ADENOVIRUS INFECTED CELLS

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INTRODUCTION

Adenoviruses have been isolated from a number of different species including man (33 serotypes), monkeys, mice, dogs, cows, birds (Green, 1970), and frogs (Clark *et al.*, 1973). The most extensively studied adenoviruses are those of human origin. In particular, we now know a great deal about the structure and replication of the DNA from human adenoviruses types 2 and 5. For this reason this review will be restricted to a discussion of these two viruses.

The Structure of Adenovirus DNA Isolated from Virions

Viral DNA is most commonly isolated from purified virions with the aid of pronase and sodium dodecylsulfate followed by phenol extraction. This method yields linear DNA with a molecular weight of about 23 x 10^6 daltons (Green *et al.*, 1967; Eb, Kesteren, and Bruggen, 1969). Denaturation of this DNA followed by renaturation does not result in the formation of double stranded circles, as is the case with the bacteriophages T-2 or T-4, indicating the absence of a circularly permuted nucleotide sequence (Thomas and MacHattie, 1967). Treatment of linear native adenovirus DNA with *E. coli* exonuclease III followed by renaturation does not result in the formation of double stranded circles, as it does with T-2 or T-7 DNA, indicating the absence of that type of terminally redundant

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polynucleotide sequence (Green, 1970; Thomas and MacHattie, 1967). However, when low concentrations of denatured DNA from human adenoviruses are subject to annealing conditions, single stranded circular molecules are observed with a high frequency (Garon, Berry, and Rose, 1972; Wolfson and Dressler, 1972). These single stranded circles are sensitive to exonuclease III treatment and if double stranded linear adenovirus DNA is treated with exonuclease III, circles will not be formed by subsequent annealing of the single strands. This previously unobserved polynucleotide arrangement has been explained by an inverted terminal repeat of about 0.5-1.5% of the polynucleotide sequences of adenovirus DNA.

Denaturation mapping of types two (Doerfler and Kleinschmidt, 1970) and five (Ellens, Sussenbach, and Jansz, 1973) adenoviruses shows that one half of the molecule is predominantly rich in A-T sequences and this half has been called the right half of the molecule by analogy with lambda DNA (Davidson and Szybalski, 1971). The unique denaturation map of Ad2 and Ad5 DNA from molecule to molecule also eliminates any possible circular permutations of polynucleotide sequences. The DNA's from types 2 and 5 adenovirus show extensive homology with each other (about 85%) based on DNA-DNA hybridization experiments (Green, 1970). Heteroduplexes formed between these molecules (Garon et al., 1973) show two prominently single stranded or mismatched regions specifically located in the right half of the molecule at 0.50-0.65 and 0.78-0.92 fractions of a unit length. By convention the left end of the molecule is taken as 0.00 and the right end (A-T rich end) as 1.00.

The restriction endonuclease $R \cdot R_I$ cleaves Ad2 DNA into six fragments called A-F in order of decreasing size (Pettersson et al., 1973). By analysis of partial digest products, the sequence of these fragments has been established to be A-B-F-D-E-C with A on the left-hand side of the molecule (higher G+C side) and C on the right-hand side (P. Sharp, personal communication). The same restriction enzyme yields 3 fragments for Ad5 DNA (C. Mulder, personal communication). The three additional endo $R \cdot R_I$ restriction enzyme sites found in Ad2 DNA when compared to Ad5 DNA appear to lie in the regions of nonhomology found in the Ad2:Ad5 heteroduplex maps. There are then two common Endo $R \cdot R_I$ sites between Ad2 and Ad5 DNA's.

The adenovirus-SV40 nondefective hybrids (Lewis et al., 1973; Levin et al., 1971), called $Ad2^{+}ND_{1-5}$, have between 5 and 7% of the Ad2 DNA, in the D-E region of the molecule, deleted. This is replaced by varying lengths of SV40 DNA (Morrow and Berg, 1972; Kelly and Lewis, 1973; Lebowitz et al., 1973). The ability of these nondefective hybrids to replicate normally in human cells suggests that Ad2 DNA may contain some information (in the D-E region) nonessential for lytic growth. On the other hand, SV40

information might replace the deleted adenovirus functions required for lytic infection.

The Replication of Adenovirus DNA

During lytic infection of human cells by adenovirus the synthesis of cellular DNA is suppressed (Ginsberg, Bello, and Levine, 1967), so that late in infection most of the newly synthesized DNA is of viral origin. The viral replicative intermediates as well as the newly synthesized mature DNA can be selectively separated from cellular DNA (Hirt, 1967). Thus far, studies concerning the mechanism of replication of adenovirus DNA have dealt with the isolation and characterization of the replicative intermediate.

Replicating adenovirus DNA has been isolated from intact infected cells (Pearson and Hanawalt, 1971; Bellett and Younghusband, 1972; Eb, 1973; U. Pettersson, personal communication) as well as from infected cell nuclei incubated *in vitro* in a DNA synthesizing system (Sussenbach *et al.*, 1972; Vliet and Sussenbach, 1972). In the *in vitro* system, cellular DNA synthesis remains suppressed while viral DNA is replicated (Sussenbach and Vliet, 1972). Viral DNA synthesis in isolated nuclei is semiconservative and only those replicative intermediates preexisting in infected cells take part in the *in vitro* reaction. New rounds of DNA synthesis are not initiated (Vliet and Sussenbach, 1972). Synthesis of DNA in this system then appears to reflect the physiological state of the cells prior to the preparation of the nuclei.

The replicative intermediates, isolated from intact cells as well as from infected cell nuclei, sediment faster than mature adenovirus DNA (31S) in neutral sucrose gradients. The majority of this replicating DNA has a sedimentation value of 40 to 60S but values up to 200S have been reported (U. Pettersson, personal communication) which may be the result of aggregation. The density of replicating DNA in neutral pH CsCl density gradients is generally 5/10 mg/ml greater than that of mature viral DNA and sometimes approaches the density of single stranded viral DNA. This increased density is not caused by the presence of extensive levels of ribonucleotides associated with the replicative intermediate, but appears to be due to large single stranded regions present in the DNA (Sussenbach *et al.*, 1972). Treatment of the high density DNA with a single strand specific nuclease from *N. crassa* eliminated the density difference between replicating and mature viral DNA, while RNase treatment did not alter the density of replicating DNA (U. Pettersson, personal communication; Sussenbach *et al.*, 1972). The presence of single stranded regions of DNA in the replicating intermediate was also demonstrated by chromatography on benzoylated-naphthoylated DEAE cellulose columns (Eb, 1973; Sussenbach *et al.*,

1972; Bellett and Younghusband, 1972). The high sedimentation values of replicating DNA in neutral sucrose gradients (40-60S) are then most likely explained by the presence of single stranded regions of DNA which have a more compact configuration than double stranded DNA at high salt concentrations and neutral pH's. Consistent with this is the observation that the sedimentation rate of replicating DNA is strongly dependent on the ionic strength at neutral pH as is the case with single stranded DNA alone (Vliet, unpublished results).

The replicative intermediates have been observed in the electron microscope. Linear branched molecules were found in which one of the arms was either totally or partially single stranded. Linear nonbranched molecules with single stranded regions and linear single and double stranded molecules of unit genome length were also observed (Sussenbach et al., 1972; Eb, 1973). Based upon the different types of molecules observed in the electron microscope and the physical properties of the replicative intermediates Sussenbach et al. (1972) have proposed a model for type 5 adenovirus DNA replication. DNA synthesis starts at one end of genome displacing the other strand (displacement synthesis). This generates a single strand that is later converted into double stranded DNA either before or after completion of the displacement synthesis. This model predicts a unidirectional, highly asymmetric DNA replication involving linear intermediates. A similar replication scheme involving circular intermediates has been described for mitochondrial DNA (Robberson, Kasamatsu, and Vinograd, 1972; Kasamatsu and Vinograd, 1973).

Recently several aspects of this model have been confirmed. Sussenbach and Vliet (1973) infected human cells with type 5 adenovirus in the presence of an inhibitor of DNA synthesis, hydroxyurea, in an attempt to synchronize the start of DNA replication. When the inhibitor was removed, viral DNA replication began. Under these conditions displacement synthesis occurred preferentially leading to an accumulation of single-strand viral DNA. Hybridization of this preferentially displaced strand with the separated strands of type 5 adenovirus DNA showed exclusive duplex formation (hybridization) with the viral L strand (the light strand after separation in alkaline CsCl gradients). The same result was obtained with single stranded DNA isolated from sheared replicating molecules. These experiments demonstrate that the displaced strand is the one that bands at a heavier density in alkaline CsCl equilibrium gradients. These data confirm the unidirectional displacement replication model and demonstrate that replication always begins at one unique end of the molecule (since all known polymerases synthesize in a 5' to 3' direction). Denaturation mapping of replicating molecules showed that the unique end, where DNA replication begins, was the right end or high A+T end of the genome (J. S. Sussenbach, personal communication).

RESULTS

While the properties of the adenovirus replicative intermediate are well characterized and a reasonable model for replication is at hand, the details of the initiation of DNA synthesis and the steps in polynucleotide chain propagation have yet to be understood. In particular not a single protein involved in adenovirus DNA replication has been isolated or characterized. The observation that replicating adenovirus DNA contained extensive single stranded regions led us to look for proteins, specific for infected cells, that bind only to single stranded DNA and may be involved in adenovirus DNA replication. Such a class of proteins had originally been described by Alberts and Frey (1970) in T-4 infected *E. coli* and have now been detected in a number of prokaryotic systems (Sigal *et al*., 1972; Alberts, Frey, and Delius, 1972) as well as in mammalian cells (Tsai and Green, 1973; Herrick, 1973). At least in the case of T-4 gene 32 protein (Alberts and Frey, 1970) this single strand specific DNA binding protein is required for DNA replication and genetic recombination. In order to eliminate the large excess of adenovirus coat proteins, synthesized in human cells after infection, which are known to bind to DNA (Levine and Ginsberg, 1968), we employed African green monkey kidney cells as a host. In these cells adenovirus DNA synthesis occurs at a normal rate but late viral capsid proteins are not synthesized or are produced in very small amounts (Baum, Horwitz, and Maizel, 1972).

In this paper, two adenovirus infected cell specific DNA binding proteins with molecular weights of 72,000 and 48,000 daltons have been isolated. Neither protein can be detected in preparations of purified virions and the synthesis of these proteins is not blocked by inhibitors of DNA replication. Both proteins can be detected in extracts prepared from cells infected with a DNA negative temperature sensitive mutant Ad5 ts36 (Wilkie, Ustacelebi, and Williams, 1973) grown at the permissive or nonpermissive temperatures. Neither protein can be detected in cell extracts prepared from cells infected with a DNA negative temperature sensitive mutant Ad5 ts225 (Ensinger and Ginsberg, 1972) grown at the nonpermissive temperature. Thirty percent of the normal yield of these proteins are present in Ad5 ts225 infected cell extracts prepared at the permissive temperature. Ad5 ts225 and Ad5 ts36 are representatives of the two different classes of adenovirus type 5 DNA negative mutants. These two mutants complement each other (Ginsberg and Williams, personal communication).

The 72,000 and 48,000 dalton proteins have been partially purified. They bind to single stranded DNA and the replicative intermediate of adenovirus DNA but not detectably to double stranded DNA. No binding of the 48,000 dalton protein to *E. coli* ribosomal RNA has been detected.

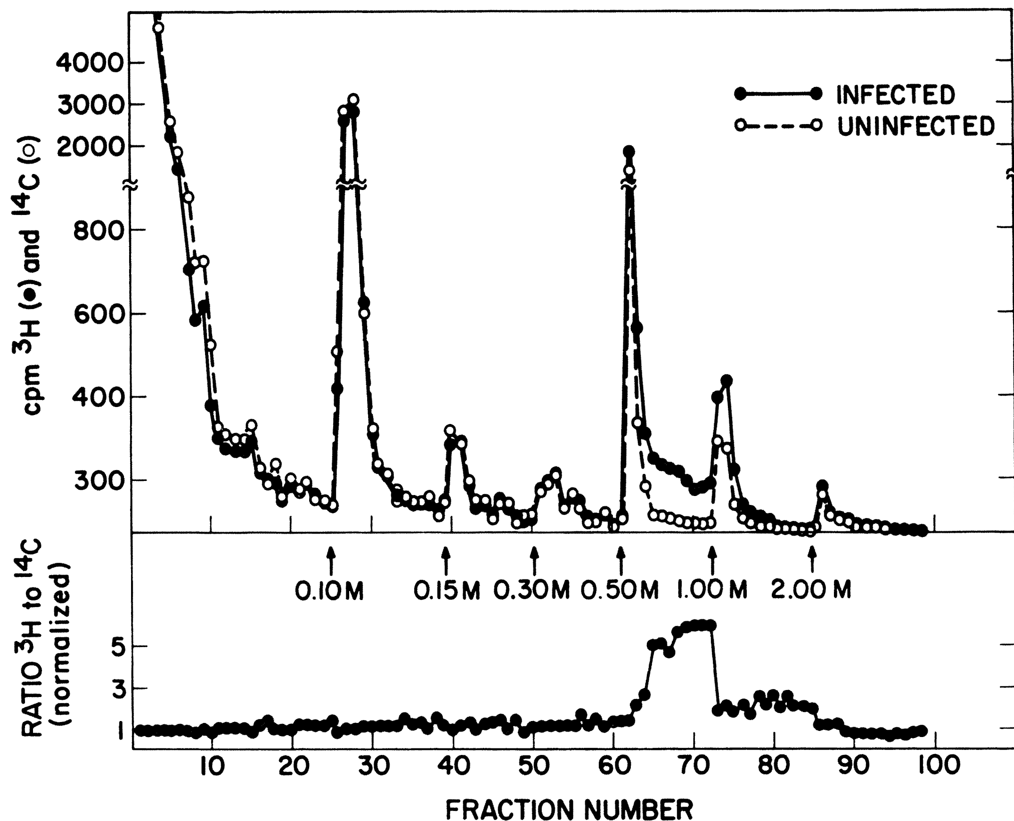

FIG. 1. Fractionation of infected cell proteins that bind to single stranded DNA cellulose columns. Protein extracts labeled with ^{3}H-leucine for adenovirus infected cells and ^{14}C-leucine for mock infected cultures were prepared as described in the text. The mixed extracts were passed over a DNA cellulose column at 2 ml/hour. The proteins that bound to the column were eluted stepwise with buffers of increasing NaCl concentrations and 2 ml fractions were collected and analysed.

Top: radioactivity eluted from the DNA cellulose column. •——•, ^{3}H-labeled infected cell proteins; o——o, ^{14}C-labeled mock infected cell proteins. Bottom: The ratio of ^{3}H-cpm/^{14}C-cpm normalized to 1.0 for the crude lysate.

The kinetics of synthesis of both proteins in infected monkey cells are similar to that of viral DNA synthesis. It is estimated that both proteins are synthesized in large amounts in that the techniques employed for their isolation detect 1-10 x 10^6 molecules per cell. All of these properties closely resemble those of T-4 gene 32 product first described by Alberts and Frey (1970).

Isolation of the Adenovirus Infected Cell Specific DNA Binding Proteins

Monolayer cultures of AGMK cells were infected with type 5 adenovirus (moi 50 PFU's/cell) in Dulbecco's modified Eagle's medium containing one hundredth the normal concentration of leucine. In this low leucine medium viral DNA synthesis proceeds at the same rate as in regular medium. Two hours later ^{3}H-leucine (10 μc/ml) was added to the infected cells and ^{14}C-leucine (1 μc/ml) was added to an equal number of mock infected cultures. At 24 hours after infection both sets of cultures were washed with phosphate buffered saline and harvested in a hypotonic buffer (0.02 M Tris HCl, pH 7.6; 0.01 M NaCl, 1.5 mM $MgCl_2$ and 2 mM β-mercaptoethanol). 500 μg/ml of bovine serum albumin was added and the cells were disrupted by sonication for 15 seconds. EDTA was then added to 5 mM and NaCl to 1.7 M and the lysate was kept at 0°C for 10 minutes after which 30% polyethylene glycol in 1.7 M NaCl was added to a 10% final concentration (weight/volume). Thirty minutes later the mixture was centrifuged at 15,000 x g for 20 minutes. This procedure eliminates DNA from the soluble extract (Alberts and Herrick, 1971). The supernatant was dialysed against 0.01 M Tris HCl pH 7.4, 0.05 M NaCl, 1 mM EDTA, 2 mM β-mercaptoethanol and 10% glycerol. The protein extract was then passed over a 1 ml cellulose column containing 24 OD_{260} units of single stranded calf thymus DNA (Alberts and Herrick, 1971). Ninety-four to ninety-seven percent of the labeled proteins passed through this column with the void volume. The bound proteins were eluted stepwise with buffers of increasing ionic strength (see Figure 1) and between 0.5 M and 1.0 M NaCl, a fraction was obtained that was four- to sixfold enriched for infected cell proteins. This fraction was then examined by SDS-polyacrylamide gel electrophoresis (Figure 2) where two prominent polypeptides, detected only in infected cells, were observed. The molecular weights of these proteins were estimated at 72,000 and 48,000 daltons. A third polypeptide, with a molecular weight of about 58,000 daltons, was consistently observed in both infected and uninfected cell extracts. Irregularly a 38,000 dalton infected cell specific protein could be observed. The ratio of the 72,000 MW protein to 48,000 MW protein was not always constant but appeared to vary from experiment to experiment possibly depending on the physiological state of the cells.

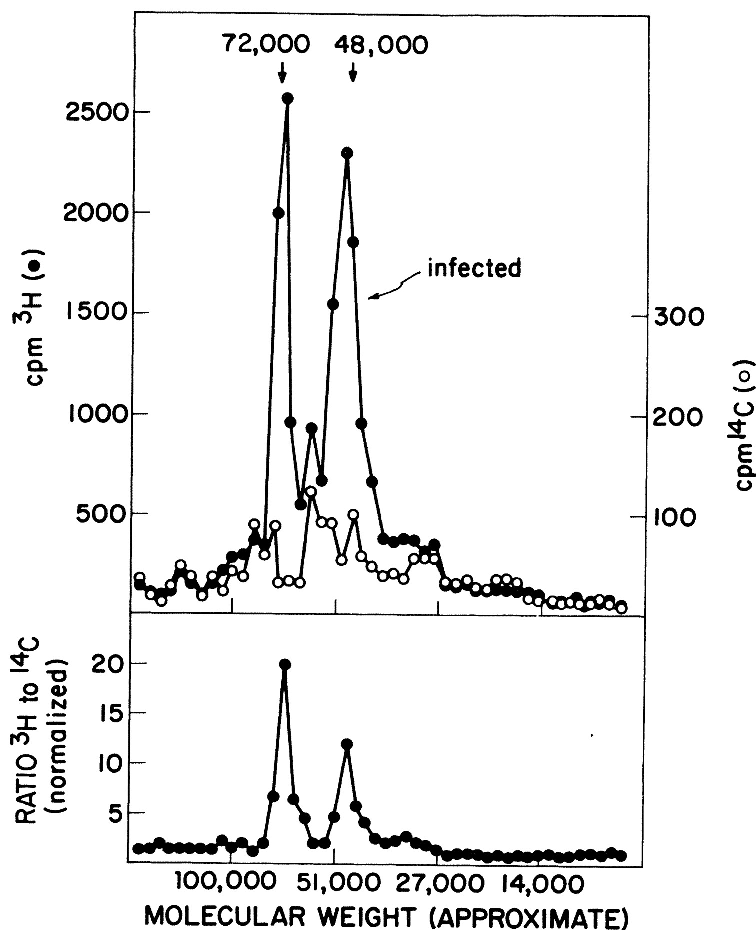

FIG. 2. SDS-polyacrylamide gel electrophoresis of the DNA binding proteins eluted at 1 M NaCl. Fractions 65-72 of Figure 1 were pooled and an aliquot was precipitated with 20% TCA. The precipitate was dissolved in 0.1 ml of 0.01 M sodium phosphate buffer (pH 7.4), 1% sodium dodecyl sulfate, 10% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue and boiled for two minutes. The 10 cm by 0.6 cm electrophoresis gels were made with 10% acrylamide and 0.3% ethylene diacrylate by polymerizing with 0.09% N,N,N',N'-tetramethylethylene diamine and 0.05% ammonium persulfate in 0.01 M sodium phosphate buffer pH 7.4 and 0.1% SDS. Samples were run at 50V and 12 mA per gel. The gels were frozen, sliced, and extracted for 24 hours at 37°C in 0.1% SDS and counted.

Top: ^{3}H-leucine (●——●) infected cell proteins and ^{14}C-leucine labeled uninfected proteins (o——o). Bottom: The ^{3}H to ^{14}C ratio normalized as in Figure 1.

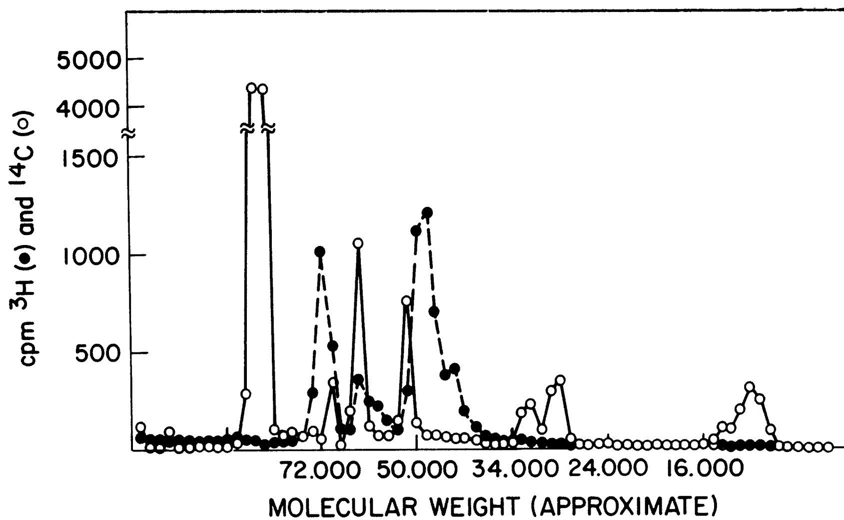

FIG. 3. SDS-polyacrylamide gel electrophoresis of a mixture of virion proteins and DNA binding proteins from infected cells. The ^{3}H-labeled DNA binding proteins were isolated as in Figure 1. The ^{14}C-leucine labeled virion proteins were obtained from purified virions grown in KB cells. The virus was purified as described by Sussenbach and Vliet (1973). o——o, ^{14}C-labeled virion proteins; •——•, ^{3}H-labeled 1 M NaCl eluted DNA binding proteins.

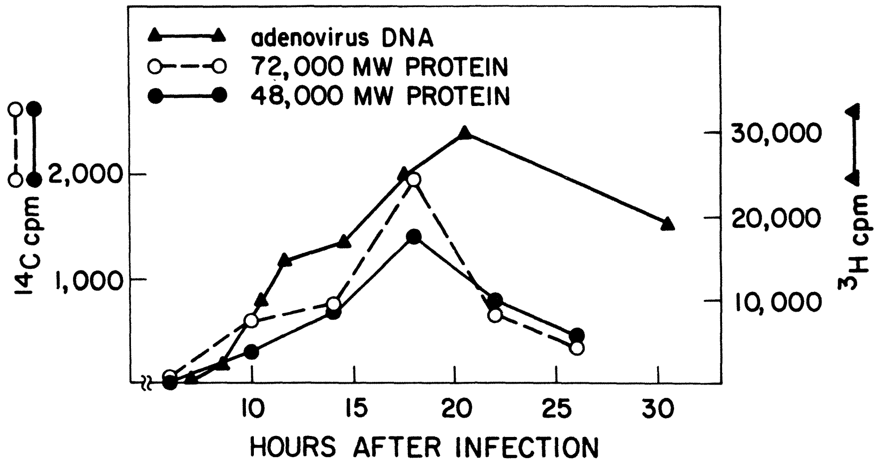

FIG. 4. The kinetics of synthesis of the two DNA binding proteins. Confluent AGMK cells were infected with type 5 adenovirus and labeled for 4 hour periods with ^{14}C-leucine (15 μc/3 x 10^6 cells). Protein extracts were prepared and analysed as in Figures 1 and 2. The open and closed circles, plotted at the mid-pulse time, represent the total radioactivity in each peak from 3 x 10^6 cells. Viral DNA synthesis (filled triangles) was monitored by a 2 hour labeling period with ^{3}H-thymidine. This DNA was selectively extracted (Hirt, 1967) and sedimented through a neutral sucrose gradient. The DNA that sedimented at 31S was taken as viral specific DNA.

The 72,000 and 48,000 dalton proteins contained between 0.3 and 0.5% of the total ^{3}H-leucine cpm found in labeled proteins from the infected cells. An estimate of the number of molecules of the 72,000 and 48,000 MW proteins synthesized in the infected cells was obtained by purifying these proteins by DNA cellulose columns followed by DEAE cellulose chromatography. Each protein was then run on SDS-polyacrylamide gels and stained with commasie blue. Known concentrations of bovine serum albumin run on parallel gels and stained with this same dye were employed as protein standards. From a comparison of the two staining intensities it was estimated that each protein was present in amounts between 1-10 x 10^{6} molecules per cell.

To determine if either of these proteins was present in adenovirus virions a mixture of ^{3}H-leucine labeled DNA binding proteins (eluted from DNA cellulose at 1M NaCl) and ^{14}C-leucine labeled purified virions were run together on SDS-polyacrylamide gels (Figure 3). Neither the 72,000 nor the 48,000 MW DNA binding proteins migrated with any of the detectable virion polypeptides. This was expected from the inability of type 5 adenovirus infected monkey cells to synthesize late virion proteins.

The kinetics of synthesis of these two DNA binding proteins was investigated by labeling adenovirus infected AGMK cells with ^{14}C-leucine (15 μc/3 x 10^{6} cells) for 4 hour periods at various times after infection. Protein extracts prepared from these cells were fractionated on DNA cellulose and analysed by SDS-polyacrylamide gel electrophoresis as described. Figure 4 shows the rate of synthesis of the 72,000 MW and 48,000 MW proteins. Both proteins were first detected at about 8 hours after infection and were synthesized at a maximal rate at about 18 hours after infection. The start of synthesis of these proteins correlates well with the onset of viral DNA replication in these cells (Figure 4). Viral DNA synthesis is not a necessary condition for the production of these two proteins, however. Normal levels of both DNA binding polypeptides were synthesized in infected AGMK cells in the presence of 40 μg/ml of cytosine arabinoside (Figure 5) or 10 mM hydroxyurea (figure not shown here).

In order to determine if an adenovirus specific function was required for the synthesis or induction of these proteins and to determine if these proteins were involved in viral DNA replication two different type 5 adenovirus temperature sensitive DNA negative mutants were employed. Assuming that the binding to single stranded DNA (DNA cellulose columns) is a functional test for these proteins, then it is possible to detect an adenovirus mutant that produces a nonfunctional protein at the nonpermissive temperature. These experiments were done in collaboration with Drs. M. Ensinger and H. S. Ginsberg. Monolayer cultures of AGMK cells were infected

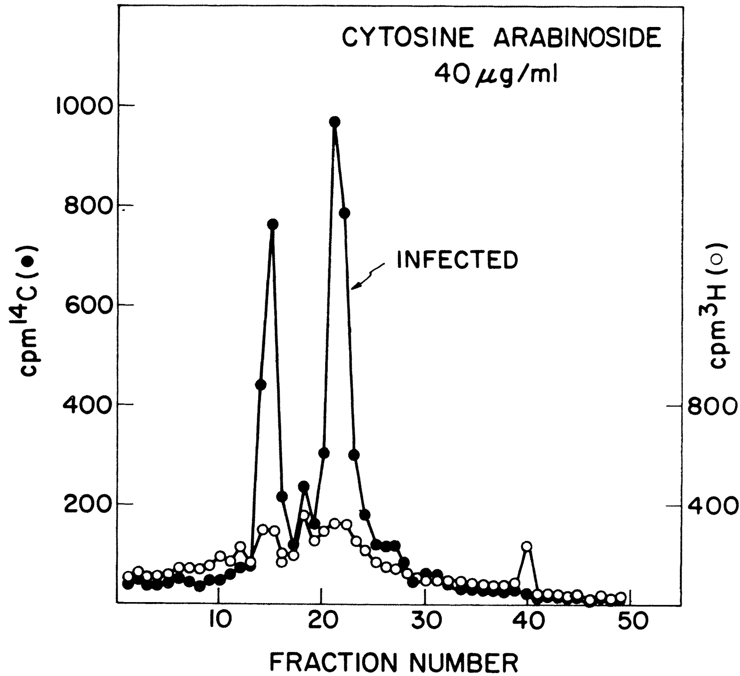

FIG. 5. DNA binding proteins synthesized in the presence of cytosine arabinoside. Monolayer cultures of AGMK cells were infected with type 5 adenovirus in the presence of cytosine arabinoside and labeled with ^{3}H-leucine. Mock infected cultures in the presence of this drug were labeled with ^{14}C-leucine. Protein extracts were mixed, prepared and fractionated as in Figure 1 on DNA cellulose. The 1 M NaCl eluted proteins were run on SDS polyacrylamide gels as in Figure 2. ●——●, ^{3}H-labeled infected cell proteins; o——o, ^{14}C-labeled mock infected cell proteins.

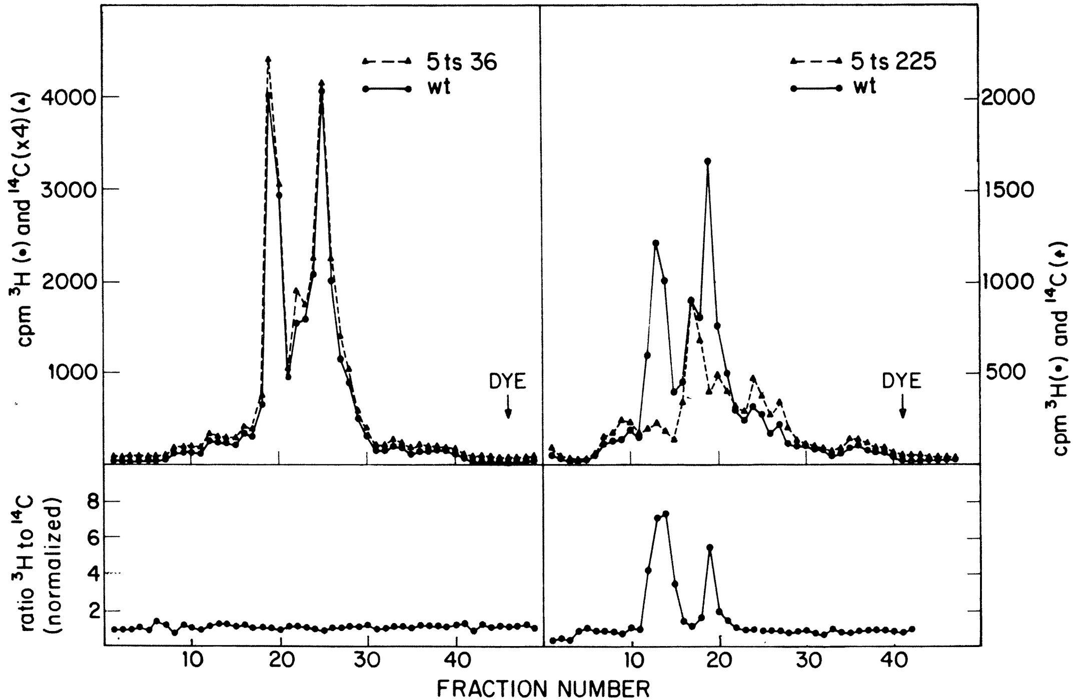

FIG. 6. DNA binding proteins from Ad5 wild type, Ad5 ts36 and Ad5 ts225 infected cell extracts prepared at 39°C (nonpermissive temperature). AGMK cells were infected with type 5 adenovirus wild type (^{14}C-leucine labeled), Ad5 ts36 or Ad5 ts225 (^{3}H-leucine labeled) at 39°C. Protein extracts were prepared as usual and chromatographed on DNA cellulose as described in Figure 1. The 1 M NaCl eluted proteins were run on SDS-polyacrylamide gels as in Figure 2.

Left panel: Top--^{14}C-leucine labeled Ad5 ts36 proteins (filled triangles); bottom--The ratio of ^{3}H to ^{14}C-cpm normalized as in Figure 1.

Right panel: Top--^{14}C-leucine labeled Ad5 wt proteins (filled circles) and ^{3}H-leucine labeled Ad5 ts225 proteins (filled triangles); bottom--The ratio of ^{3}H to ^{14}C-cpm normalized as in Figure 1.

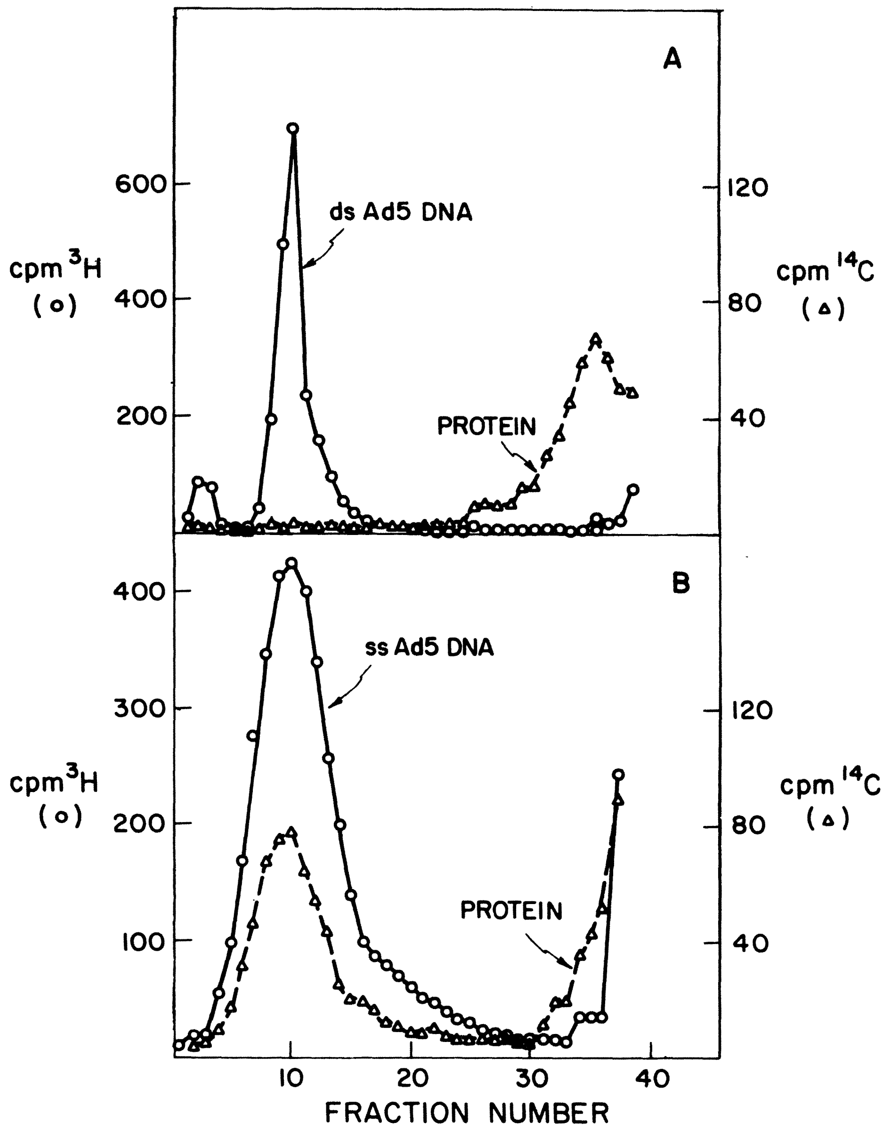

FIG. 7. Preferential binding of adenovirus infected cell specific proteins to single stranded DNA. Purified ^{14}C-labeled 48,000 MW (5 μg/ml) was mixed with 10 μg/ml of native ^{3}H-labeled adenovirus type 5 DNA (top panel) or 0.5 μg/ml of adenovirus type 5 denatured DNA (bottom panel). The mixture was then sedimented in a 5-20% neutral sucrose gradient for (top) 5 hours or (bottom) 2.5 hours in an SW 39 rotor. Fractions of 0.15 ml were collected. Identical results were obtained with the 72,000 MW protein.

Top: o——o, ^{3}H-labeled native Ad5 DNA; △—△, ^{14}C-labeled 48,000 MW protein. Bottom: o——o, ^{3}H-labeled single stranded Ad5 DNA; △—△, ^{14}C-labeled 48,000 MW protein.

with type 5 adenovirus wild type, or with each of two different temperature sensitive DNA negative mutants: Ad5 ts36 (Wilkie *et al.*, 1973) and Ad5 ts225 (Ensinger and Ginsberg, 1972). The cultures were kept at the nonpermissive temperature (39°C) for the entire infection. The adenovirus wild type infected cells were labeled with ^{3}H-leucine while each mutant was labeled with ^{14}C-leucine as described previously. At the end of the labeling period each mutant culture was mixed with a wild type culture and the DNA binding proteins were fractionated on DNA cellulose columns and analysed by SDS polyacrylamide gel electrophoresis. Figure 6 presents the polyacrylamide gel profiles obtained in this experiment. Normal levels of both the 72,000 and 48,000 MW proteins were detected in extracts infected with Ad5 ts36 made at the nonpermissive temperature (Figure 6). On the other hand neither the 72,000 nor the 48,000 MW proteins were detectable in extracts of Ad5 ts225 cells infected and kept at 39°C (Figure 6). Both DNA binding proteins were detected in Ad5 ts225 extracts of cells infected and kept at 33°C, although only 30% of the wild type yield at 33°C was obtained. The levels of these DNA binding proteins detected in Ad5 ts225 infected cells at the permissive temperature was well above the background levels observed at the nonpermissive temperature. When the DNA binding proteins were labeled in Ad5 ts225 infected cells at the permissive temperature and then the cultures were shifted up to 39°C (nonpermissive temperature) for 2 hours, the 72,000 and 48,000 MW proteins were no longer detectable in extracts prepared from such cells. These data suggest (but by themselves do not prove) that one or both of the DNA binding proteins detected here are coded for by the viral genome. Furthermore these experiments strongly suggest that one or both of these proteins are involved in adenovirus DNA replication.

The specificity of the binding of the 72,000 and 48,000 MW proteins to DNA was investigated by mixing ^{3}H-labeled adenovirus DNA and ^{14}C-labeled proteins and analyzing their interactions in neutral sucrose gradients. For this purpose each of these proteins was purified on DNA cellulose columns followed by DEAE cellulose chromatography. These columns were equilibrated with 0.01 M Tris HCl pH 7.2, 1 mM EDTA, 1 mM β-mercaptoethanol and 10% glycerol. The proteins were eluted between 0.16 M and 0.24 M NaCl by a linear 0.01 M to 0.5 M NaCl gradient. Analysis of these partially purified proteins on SDS-polyacrylamide gels indicated that more than 70% of the ^{14}C-leucine label was present in a single species of protein at either 72,000 or 48,000 daltons.

These preparations of ^{14}C-labeled 72,000 and 48,000 MW proteins were mixed with 10 μg/ml of ^{3}H-labeled adenovirus double stranded DNA or 0.5 μg/ml of adenovirus single stranded DNA. The mixtures were sedimented in a neutral sucrose gradient to detect binding of the proteins to DNA. Figure 7 shows that no binding of the 48,000

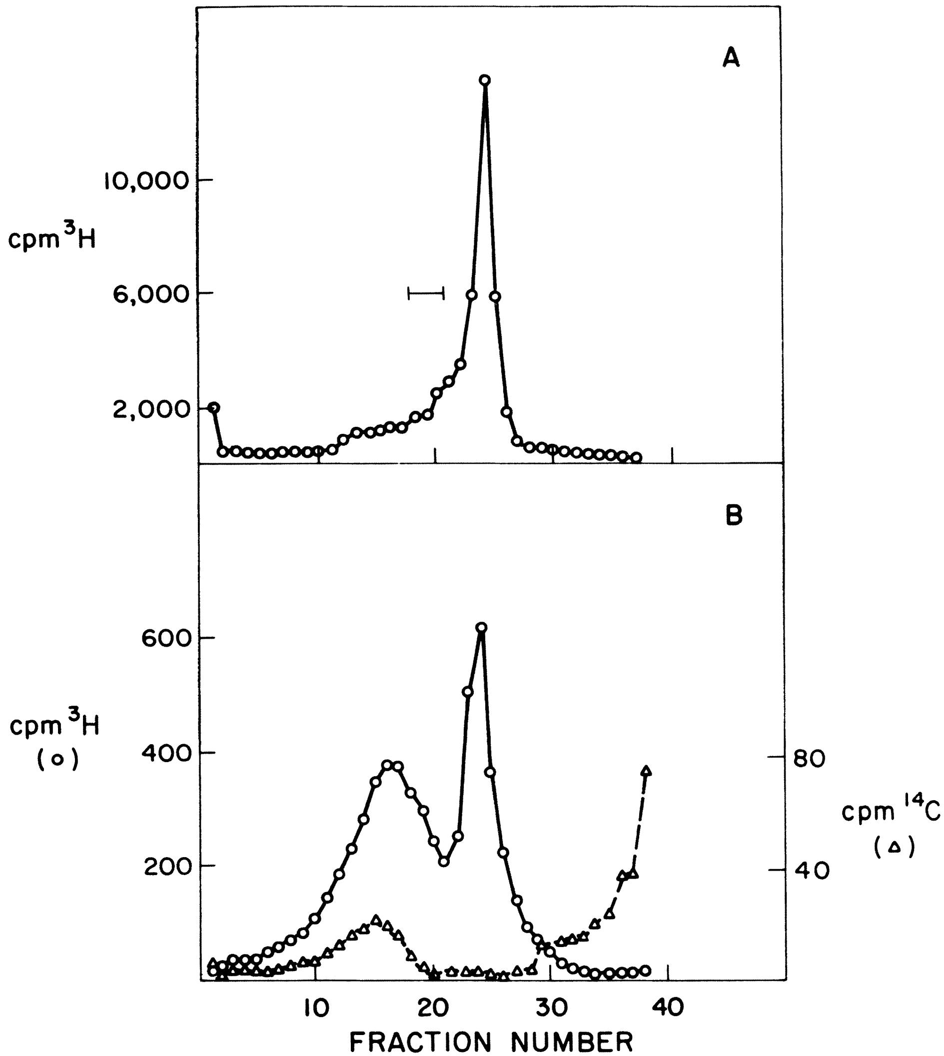

FIG. 8. Binding of the adenovirus infected cell specific proteins to replicating adenovirus DNA.

Top: AGMK cells infected with type 5 adenovirus were labeled with ^{3}H-thymidine from 16-18 hours after infection. Newly synthesized viral DNA was selectively extracted (Hirt, 1967) and centrifuged in a 5-20% neutral sucrose gradient for 3.5 hours in an SW 41 rotor at 38,000 rpm. Fractions of 0.3 ml were collected and counted. o——o, ^{3}H-cpm. Bottom: Fractions 18-20 from the sucrose gradient above were pooled, dialysed and mixed with ^{14}C-labeled 48,000 MW protein. The mixture was centrifuged in a 5-20% neutral sucrose gradient in an SW 50.1 rotor for 2 hours at 44,000 rpm. Fractions of 0.15 ml were collected and counted. o——o, ^{3}H-viral DNA; △—△, ^{14}C-labeled 48,000 MW protein. Identical results were obtained with the 72,000 MW protein.

MW protein could be demonstrated with native viral DNA while 75% of the labeled protein bound to single stranded adenovirus DNA. Identical results were obtained with the 72,000 MW protein. The binding to single stranded DNA was specific to polydeoxyribonucleotides since no binding of the 48,000 MW protein to 23S E. coli ribosomal RNA could be detected. The binding of these two proteins to single stranded DNA was not specific for adenovirus DNA since both proteins also bind to single stranded calf thymus and Fd DNA's.

The replicative intermediate of adenovirus DNA contains extensive single stranded regions and so one might expect these proteins to bind to this replicative form. To test this the replicative intermediate was isolated by labeling adenovirus infected AGMK cells with ^{3}H-thymidine (10 μc/ml) for 2 hours at 16 hours after infection. The adenovirus DNA was selectively extracted (Hirt, 1967) and sedimented through a neutral sucrose gradient. Figure 8A shows the sedimentation profile of 31S mature adenovirus DNA and the replicative intermediate sedimenting as a heterogeneous peak in front of 31S DNA. The fractions indicated by the bar in Figure 8A were pooled, dialysed and mixed with ^{14}C-labeled 48,000 MW protein. This mixture was then sedimented in a second sucrose gradient. Figure 8B demonstrates that the 48,000 dalton protein binds only to the adenovirus replicative intermediate and not to native 31S adenovirus DNA. Identical results were obtained with the 72,000 MW protein.

DISCUSSION

Two adenovirus infected cell specific DNA binding proteins have been isolated and characterized. The molecular weights of these proteins, as determined in SDS-polyacrylamide gels, are 72,000 and 48,000 daltons. Both proteins are synthesized early after infection and neither protein appears to be a detectable constituent of the adenovirus virion. The synthesis of these proteins is not restricted to adenovirus type 5 infected AGMK cells. Proteins with similar molecular weights have been detected in adenovirus types 2 and 12 infected monkey cells. Anderson, Baum, and Gesteland (1973) have observed a 71,000 dalton protein in extracts of adenovirus type 2 infected KB cells. This protein is not found in purified virions and exhibits similar kinetics of synthesis when compared to the 72,000 MW DNA binding protein described here.

The precise function of these proteins is at present unclear. In many ways these adenovirus infected cell specific proteins resemble T-4 gene 32 protein isolated by Alberts and Frey (1970). In both cases these protein bind to single stranded DNA but not to

double stranded DNA or RNA. In both T-4 infected E. coli and adenovirus infected monkey cells these early proteins are made in large amounts consistent with a stoichometric rather than a catalytic functional role. In both T-4 and adenovirus temperature sensitive mutants defective in DNA replication produce functionally altered DNA binding proteins when these mutants are grown at the nonpermissive temperature. Further experiments are needed however to determine if the adenovirus proteins belong to the class of "DNA unwinding proteins" described by Alberts and his collaborators (Alberts and Frey, 1970; Sigal et al., 1972; Herrick, 1973).

There appear to be three possible explanations of the observation that both the 72,000 and 48,000 dalton proteins cannot be detected in Ad5 ts225 infected cells grown at 39°C: 1) Ad5 ts225 is a double mutant defective in the genes coding for both polypeptides, 2) Ad5 ts225 codes for a function that induces both proteins (be they viral or cellular coded) or 3) the 48,000 dalton protein is a proteolytic breakdown product of the 72,000 protein. It is not clear whether this proteolytic event is essential for some function of the 48,000 MW protein or is an artifact arising in the extracts. While both proteins (72,000 and 48,000 MW) are functional for DNA binding it remains to be seen if they are functional for other properties as well. Experiments to test these alternatives are in progress.

ACKNOWLEDGEMENTS

We thank Ms. A. Teresky and C. Kaminski for their able technical assistance and Drs. B. Alberts and G. Herrick for helpful discussions. Dr. T. Henry kindly supplied the Fd DNA and J. Rabek obtained the results on the kinetics of DNA replication in AGMK cells. The mutant Ad5 ts36 was kindly supplied by Dr. J. F. Williams. The experiments with Ad5 ts225 were done in collaboration with Drs. Ensinger and Ginsberg. This work was supported by NCI grants CA11049-05 and CA12068-03 and American Cancer Society Grant E591. One of us (P.C.V.) was supported by the Netherlands Organization for the Advancement of Pure Research.

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ADENOVIRUS DNA: TRANSCRIPTION DURING PRODUCTIVE INFECTION, INTEGRATION IN TRANSFORMED CELLS, AND REPLICATION *IN VITRO*

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INTRODUCTION

The human adenoviruses provide excellent model systems for studying the molecular biology of the mammalian cell, i.e., the mechanism of DNA replication and RNA transcription and translation, and for analyzing the integration and function of viral genes in transformed cells. Adenoviruses are icosahedral particles that are 80 nm in diameter, weigh 175 million daltons, and contain 9-10 polypeptides and 12-13% DNA (Green, 1970). Adenovirus genomes are linear, duplex DNA molecules of molecular weight 20-25 x 10^6 (Green, 1970). The interaction of the adenovirus genome with the cell can result in either (i) productive infection (usually human cells) in which thousands of virus particles are replicated and the cell is killed, or (ii) cell transformation (usually rodent cells) in which no virus is formed, but a portion of the viral genome is integrated into cellular DNA, and growth properties and macromolecular synthesis are controlled in an unknown way by information from viral genes.

The synthesis of adenovirus macromolecules during productive infection of human KB cells mimics the synthesis of cell macromolecules with regard to basic mechanism and intracellular site of synthesis. Late after infection, viral DNA is replicated in the cell nucleus where it is transcribed to polycistronic RNA molecules that are processed and transported to the cytoplasm for translation

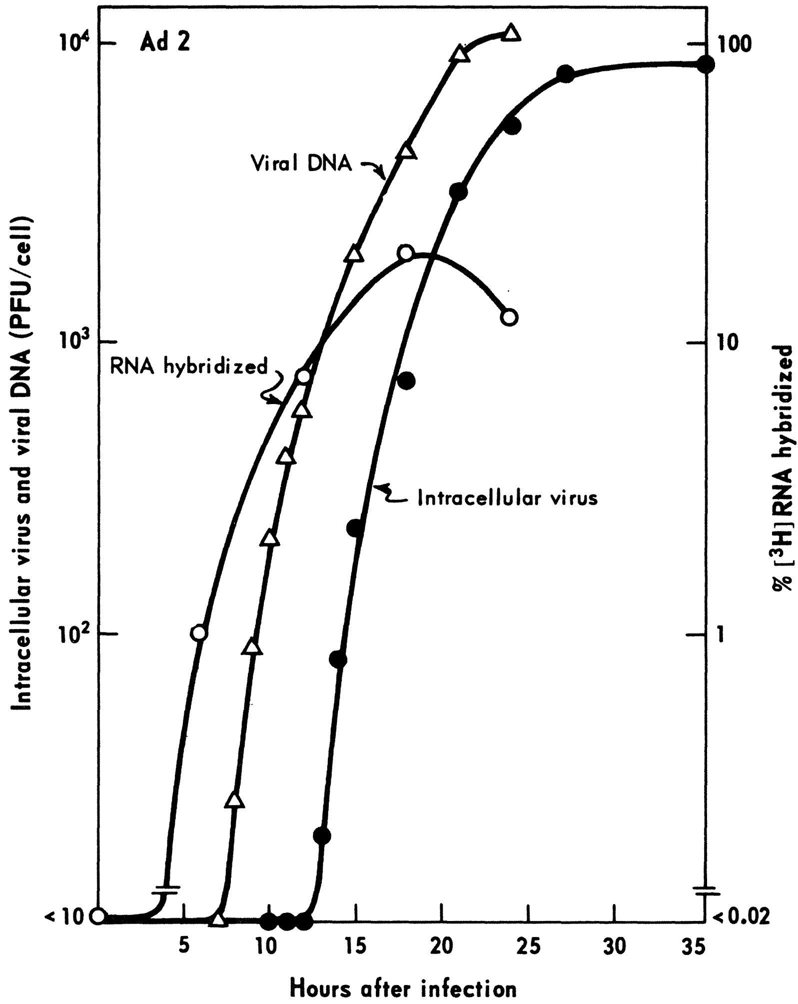

FIG. 1. Time course of formation of intracellular virus, viral DNA, and viral mRNA.

on polyribosomes. Early after infection, the transcription and processing of viral RNA occurs in the absence of protein synthesis, and thus must involve the RNA polymerase and transcription controls of the host cell (Parsons and Green, 1971). Because specific adenovirus RNA molecules can be identified by hybridization, early adenovirus-infected cells provide an excellent opportunity to analyze the regulation of gene expression by mammalian cells. At 18 hours after infection with adenovirus 2, viral DNA synthesis is maximal and cell DNA synthesis is blocked (Pina and Green, 1969), thus providing a model system to study viral DNA replication.

There are three groups of oncogenic human adenoviruses, A, B, and C, of which adenovirus types 12, 7, and 2, respectively, are the best studied serotypes. Adenovirus transformed cells provide important systems for analyzing viral DNA sequences integrated into mammalian cells and their role in cell transformation.

We describe below the results of some recent studies from our laboratory on (i) the control of gene transcription in adenovirus 2 infected KB cells, (ii) the integration of multiple copies of adenovirus 7 and 12 DNA fragments in transformed hamster cells, and (iii) the replication of adenovirus 2 DNA _in vivo_ and _in vitro_.

TRANSCRIPTION OF VIRAL GENES DURING PRODUCTIVE INFECTION OF KB CELLS BY ADENOVIRUS 2

Adenovirus 2-infected spinner cultures of human KB cells (Green and Daesch, 1961) have been the most frequently used model system to study the molecular events of adenovirus replication. In a one-step synchronous infection of KB cells with adenovirus 2, mature virus is formed between 13 and 24 hours and viral DNA synthesis begins at 6-7 hours after infection (Figure 1). Viral gene functions are classified as (1) early functions that involve viral RNA transcribed before viral DNA replication occurs, and (2) late functions that involve viral RNA transcribed after viral DNA synthesis has begun. Viral mRNA represents about 1-2% of newly synthesized RNA in polyribosomes early after infection and over 30% of newly synthesized RNA late after infection (Thomas and Green, 1966). In the presence of cycloheximide, early viral mRNA comprises nearly 20% of labeled polyribosomal RNA (Büttner, Veres-Molnar, and Green, in manuscript).

Fujinaga, Mak and Green (1968) showed by hybridization that 80-100% of the adenovirus 2 genome was transcribed by 18 hours after infection. By DNA-RNA hybridization competition experiments on filters, which detect "stable" long-lived RNA species, about 8-20% of viral RNA sequences present early after infection were detected late after infection (Fujinaga and Green, 1969). Viral

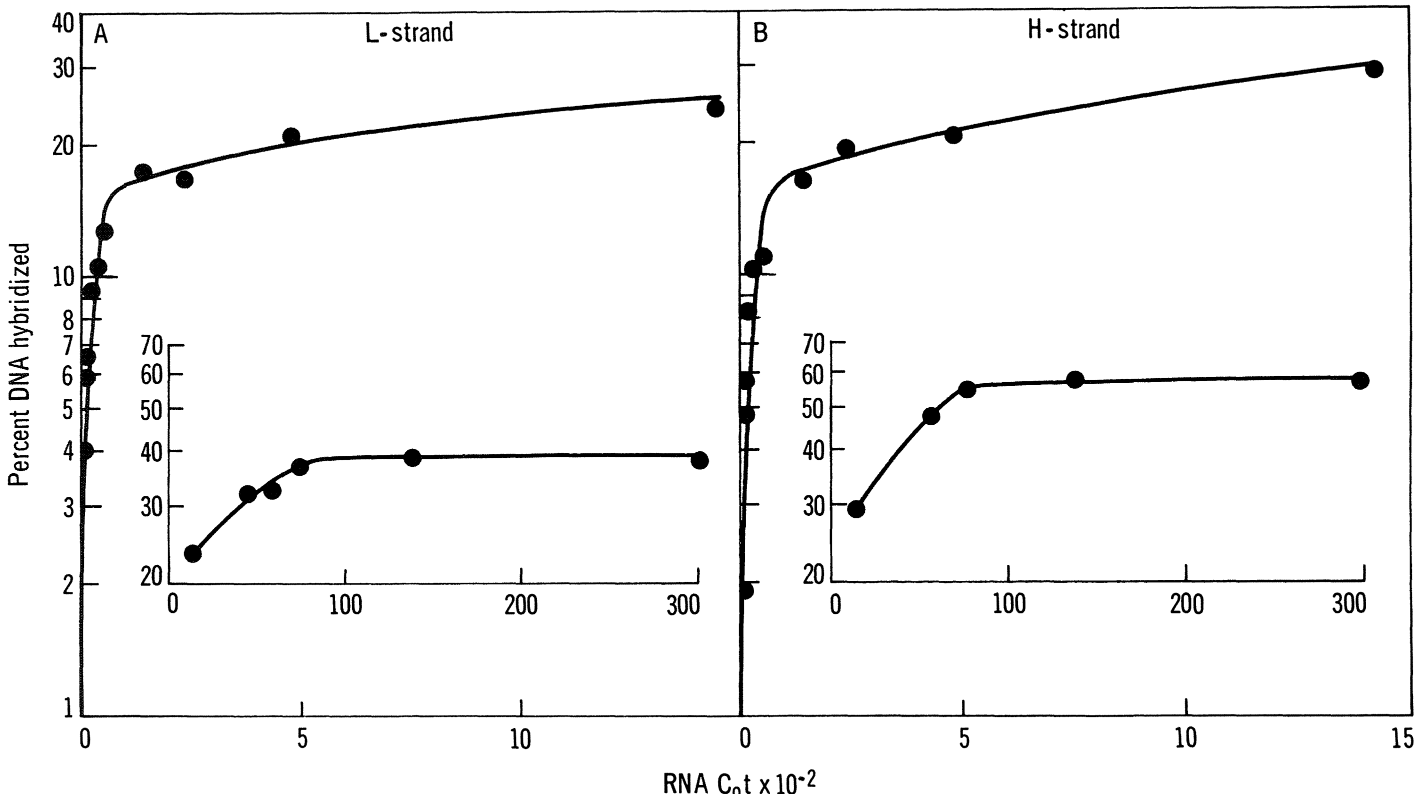

FIG. 2. Hybridization of ^{32}P-labeled H and L DNA strands with early (5 h) adeno 2-infected cell RNA.

RNA sequences in transformed cells were shown to be homologous to about one-half of stable early viral RNA sequences.

In order to determine precisely the fraction of the viral genome represented by stable viral RNA and to detect short-lived precursor RNA species, we performed very sensitive RNA-driven hybridization reactions between highly radioactive adenovirus 2 L and H DNA strands and excess RNA from adenovirus-infected cells. To prepare L and H DNA strands, adenovirus DNA was denatured and annealed with poly(U, G), and the DNA strand-complexes were resolved by equilibrium centrifugation in CsCl gradients, as previously described (Langraf-Leurs and Green, 1971). L and H ^{32}P-DNA strands were annealed with RNA isolated from KB cells 5 hr (early RNA) after infection with adenovirus 2. Hybridized DNA was quantitated by its resistance to the single-stranded nuclease, S-1. The data are plotted as logarithm of percent DNA hybridized against RNA C_ot, i.e. the initial concentration of RNA (moles-nucleotide/liter) multiplied by the time (seconds). Since with RNA in excess, the rate of the reaction is directly proportional to the concentration of the hybridizing RNA species, a straight line relationship is expected. The data of Figure 2 indicate that two classes of early virus-specific RNA are present early after infection: (i) RNA of high abundance which hybridized rapidly to RNA at C_ot values of about 50, and (ii) scarce RNA which requires RNA C_ot values of 10,000 for completion of hybridization. The abundant viral RNA species are transcribed from 14 to 16% of the L and H strand. At saturation, 55% of the H strand and nearly 40% of the L strand have hybridized (Green, Brackman, Cartas, and Devine, unpublished data). Thus the equivalent of almost the entire viral genome (95%) is transcribed early after infection. We believe that the two classes of early viral RNA species, abundant and scarce, represent stable viral mRNA molecules and RNA precursors of viral mRNA, respectively. Since the relative hybridization rates of abundant and scarce species are about 200 to 1, it appears that the scarce species represent the entire viral genome and are turned over rapidly. Most likely, the transcription of early adenovirus genes represents the transcription pattern regulated by cellular machinery, for it occurs in the presence of cycloheximide, an inhibitor of protein synthesis (Green, Brackman, Cartas, and Devine, unpublished data). Our data suggest that gene regulation in human KB cells may involve predominantly post-transcriptional processing (Darnell, Jelinek, and Molloy, 1973).

To isolate early viral mRNA species, we annealed RNA from polyribosomes labeled from 4 to 7 hours after infection in the presence of cycloheximide with L and H DNA strands under mild conditions. As shown in Figure 3, three size classes of early viral mRNA were isolated by hybridization with unfractionated viral DNA, a major 19-20S RNA plus minor 21-26S and 15-18S RNA peaks, as previously

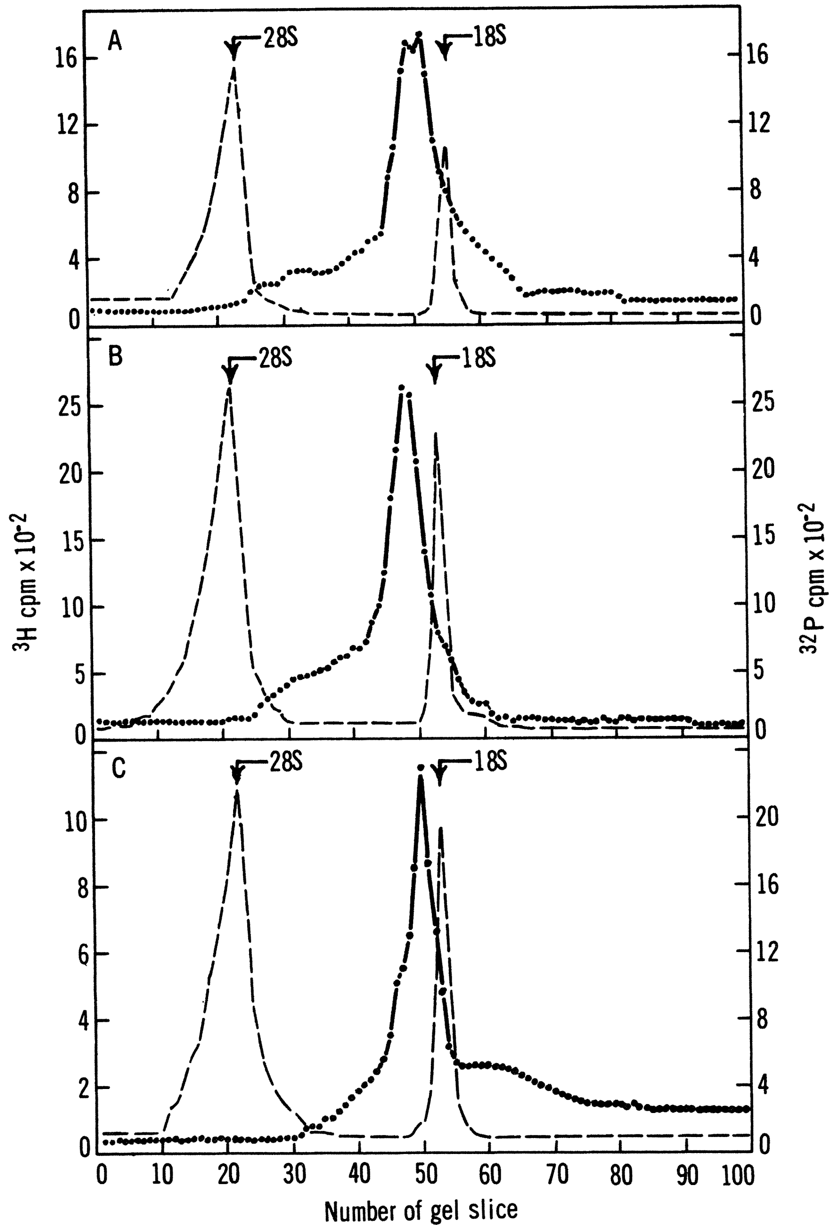

FIG. 3. Polyacrylamide gel electrophoresis of DNA strand-specific early viral mRNA molecules. Early polyribosomal RNA labeled with ^{3}H-uridine was annealed to unfractionated adenovirus 2 DNA (a), H-strand DNA (b), or L-strand DNA (c). Viral RNA was eluted and electrophoresed on agarose-polyacrylamide gels. ^{32}P-ribosomal RNA served as molecular markers ●—●: ^{3}H-cpm in virus-specific RNA, ------: ^{32}P-cpm in ribosomal marker RNA.

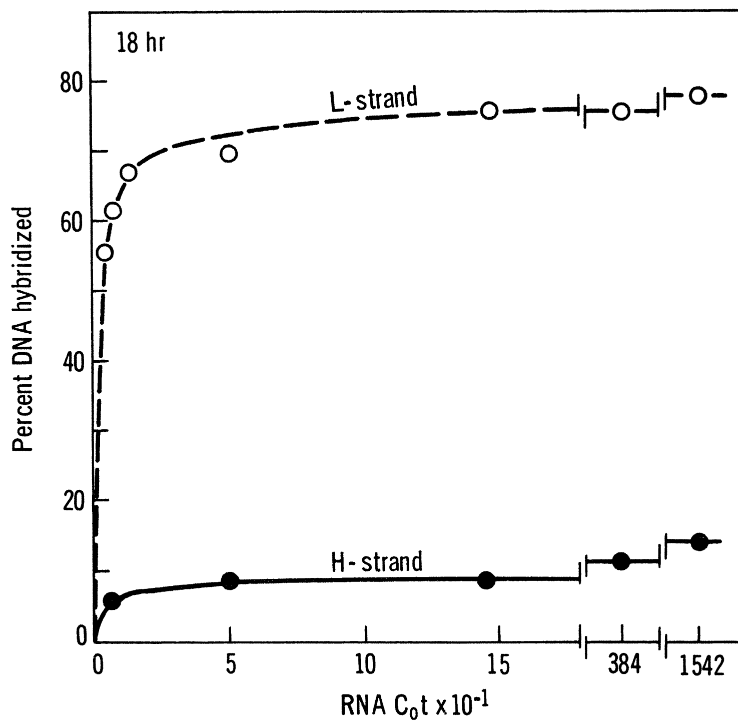

FIG. 4. Hybridization of ^{32}P-labeled H and L-strand DNA with late (18 h) adeno 2-infected cell RNA.

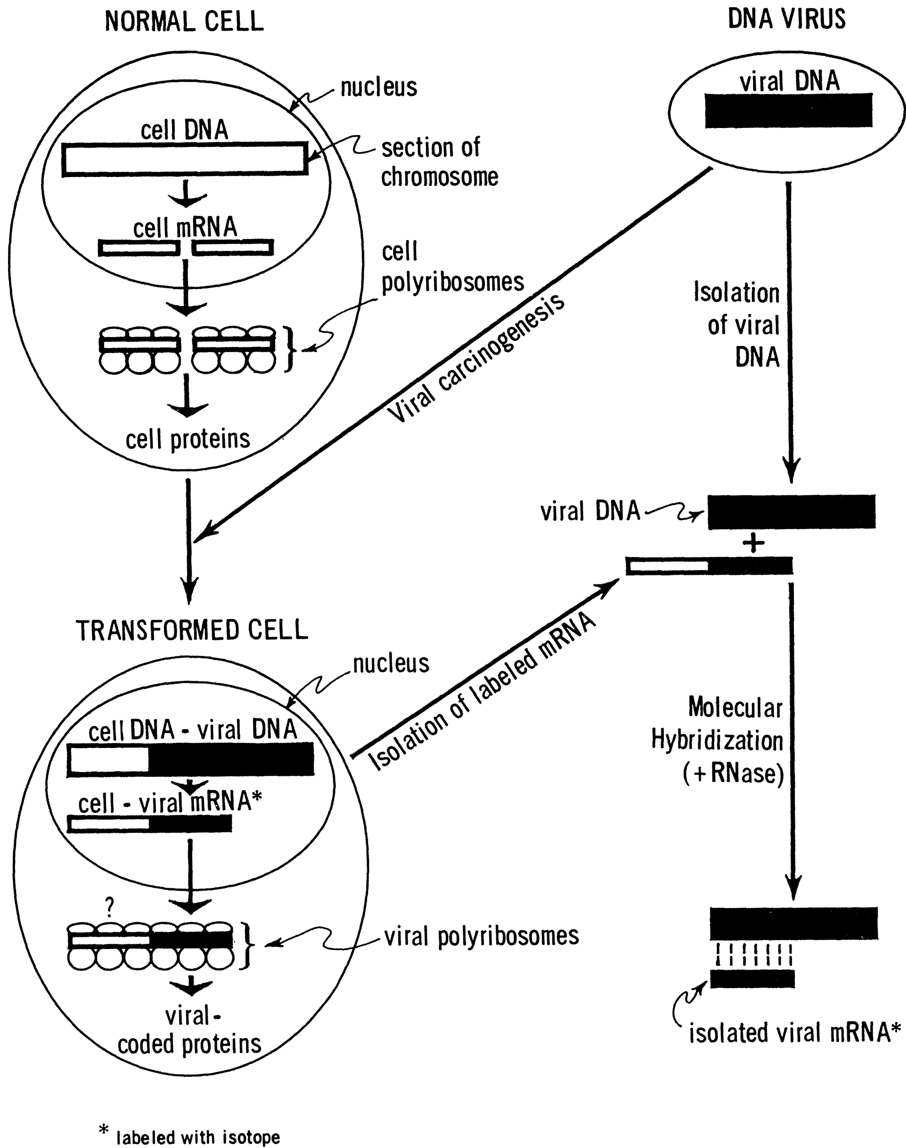

FIG. 5. Scheme illustrating mechanism of cell transformation by adenovirus.

observed by Parsons and Green (1971). Selection with individual DNA strands show that viral 19-20S RNA consists of two homogeneous RNA species with slightly different mobilities, L strand-specific mRNA of 0.74×10^6 daltons and H strand-specific mRNA of 0.77×10^6 daltons (Figure 3) (Büttner, Veres-Molnar, and Green, in manuscript). The 15-18S RNA is derived from the H strand and the 21-26S RNA from the L strand. The major 19 and 20S viral mRNA molecules can now be used for several interesting studies including mapping early viral genes by DNA-RNA hybridization and visualization in the electron microscope, and translation in cell-free protein synthesizing system. Only a small number of early viral proteins are coded by the viral genome, and among these could be the protein(s) responsible for maintaining the transformed state of the cell. Of further interest, the 19 and 20S mRNA are of the correct size to code for the 75,000 molecular weight proteins recently isolated from a nuclear membrane complex that synthesizes adenovirus DNA sequences *in vitro* (see later section).

A dramatic change in transcription occurs late after infection in that mainly the L strand is transcribed. As shown in Figure 4, total RNA from 18 hour-infected cells hybridized to nearly 80% of the L strand and to only 10% of the H strand. Similar strand utilization was measured at different times from 9 to 36 hours after infection. Virtually all the late RNA transcripts hybridize rapidly at low RNA C_0t values, and thus are predominantly viral RNA transcripts of high abundance. This change that occurs when late viral genes begin to function suggests that a viral induced mechanism results in both the predominant transcription of the L strand and its conservation as stable RNA. Most L strand transcripts are present in very low abundance early after infection, implying that they are rapidly turned over. The small fraction of the H strand DNA that is transcribed late after infection contrasts with that transcribed early after infection. These findings raise several questions concerning the mechanisms that block the transcription of the H strand and protect the L strand transcripts from degradation late after infection. The answer to these questions may illuminate basic mechanism of gene regulation in mammalian cells.

INTEGRATION OF ADENOVIRUS GENE SEQUENCES IN TRANSFORMED CELLS

A scheme depicting the mechanism of cell transformation by adenoviruses is given in Figure 5. Neither infectious virus nor intact viral DNA molecules have been detected in adenovirus transformed cells, nor has virus production been induced by cell fusion. However, cells transformed by human adenoviruses contain a part of the viral genome. We have previously shown that viral gene sequences are transcribed together with contiguous cell sequences as polycistronic RNA molecules, thus providing strong

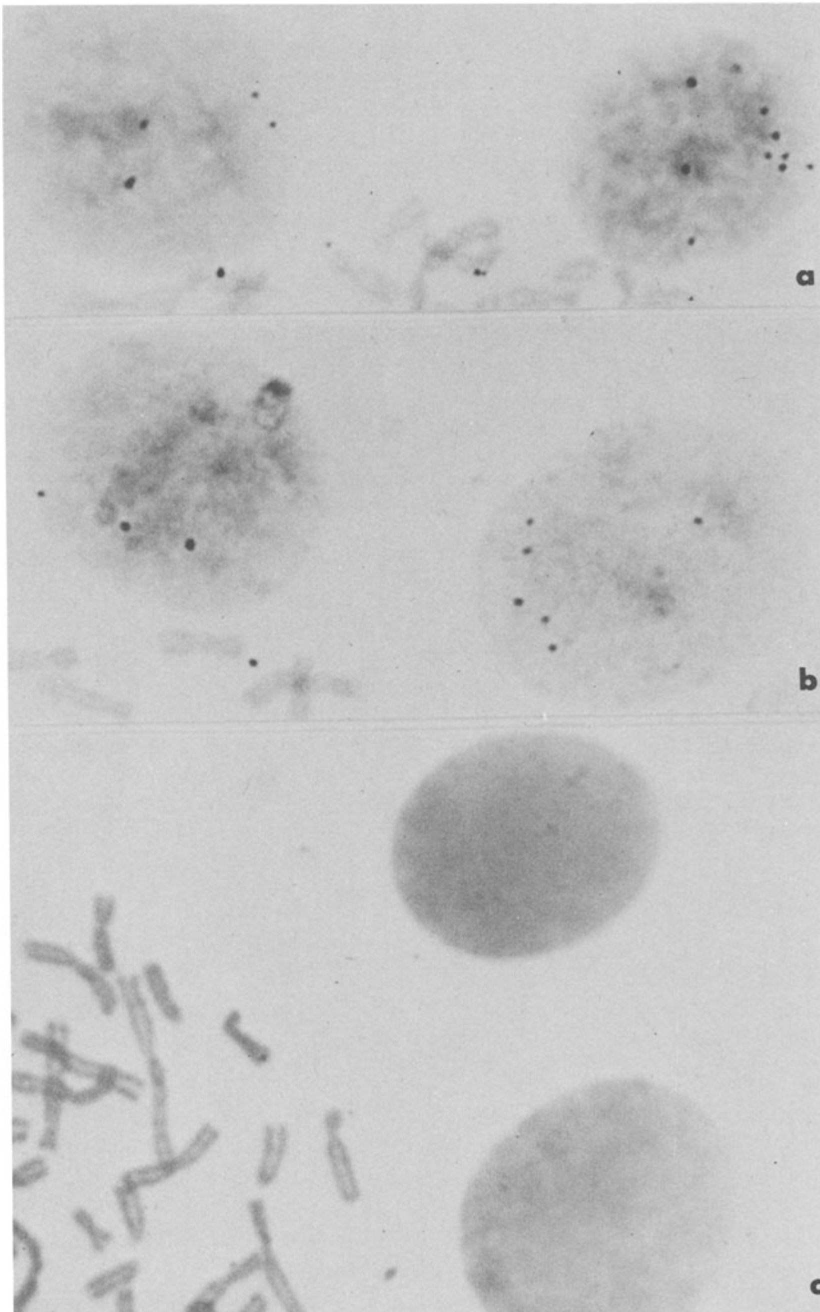

FIG. 6. Nuclei of adenovirus type 7-transformed hamster cells (a, b) and normal hamster cells (c) after <u>in situ</u> hybridization with adenovirus 7 ^{3}H-cRNA x 2,080.

evidence that viral DNA is integrated (Tsuei, Fujinaga, and Green, 1972). We have used three approaches to measure the number of viral gene equivalents and the fraction of the viral genome present in adenovirus transformed cells: (i) Viral cRNA hybridization--radioactive viral cRNA synthesized on an adenovirus DNA template by the *E. coli* RNA polymerase was annealed with DNA from transformed cells to detect viral DNA sequences. The computation of an exact copy number is difficult since the viral DNA sequence content of the cRNA probe and the transformed cells is unknown. Based on reconstruction experiments, approximately 14 to 100 DNA equivalents per cell were estimated for adenovirus 2, 7, and 12 transformed cells (Green *et al.*, 1970). (ii) *In situ* hybridization--Viral DNA sequences were demonstrated in adenovirus 2, 7, and 12 transformed cells by *in situ* hybridization. As shown in Figure 6, cytological preparations of adenovirus 7 transformed when annealed with adenovirus 7 ^{3}H-cRNA possessed an average of 6 grains per nucleus (Loni and Green, 1973). The number of integrated viral gene equivalents cannot be calculated from these data since the efficiency of *in situ* hybridization is unknown. (iii) Kinetic analysis of early reassociation kinetics--Measurements of DNA reassociation kinetics (Britten and Kohne, 1968) have been extremely valuable for determining the number of copies of specific cell and viral DNA sequences in a cell. However, the procedure cannot be used to calculate the number viral DNA equivalents in a cell when only a portion of the viral DNA probe is present. We have found that second order reaction kinetics are not followed when radioactive adenovirus DNA is denatured and re-annealed in the presence of transformed cell DNA (Fujinaga *et al.*, 1974a). Using the standard DNA reassociation plot to estimate copy number leads to serious error. For example, similar 50% reassociation values ($C_0t_{1/2}$) are obtained for 200 copies of 20% of the viral genome as for 5 copies of the entire adenovirus genome.

By measurement of the initial kinetics of reassociation, Fujinaga, Sekikawa, and Yamazaki (1974b) have developed a quantitative procedure which estimates both the number of viral DNA copies and the fraction of the viral genome present in transformed cells. When applied to adenovirus 7 transformed cells (Figure 7), the slope of the plot can be used to calculate the number of viral DNA fragments and the fraction of the viral genome represented by the DNA fragment (Fujinaga *et al.*, 1974a). From such data we have estimated that about 300 copies of 15-20% of the viral genome is present in adenovirus 7 transformed hamster cells. This value can be compared to 25-30 viral gene equivalents per cell, measured by the standard DNA reassociation plot and 90-100 equivalent by hybridization with adenovirus 7 cRNA (Green *et al.*, 1970; Green, 1972; Fujinaga and Green, unpublished data). Adenovirus 12 transformed cells analyzed by similarly early kinetic measurements possess approximately 50 copies of 50% of the viral genome (Fujinaga *et al.*, 1974b).

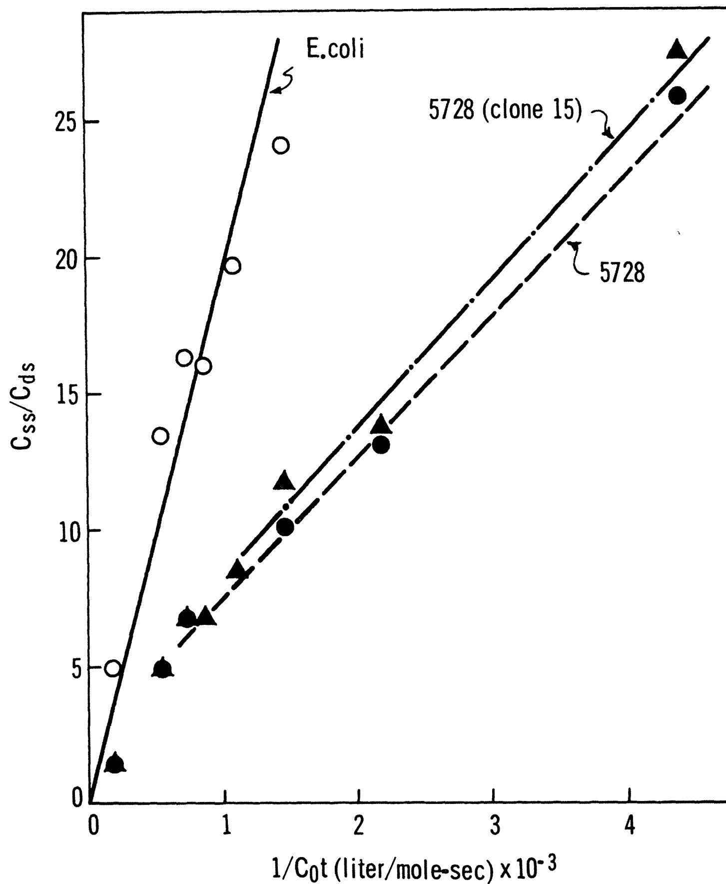

FIG. 7. Reassociation of adeno 7 [^{3}H]DNA in the presence of transformed cell DNA. Adenovirus type 7 [^{3}H]DNA (4.55×10^{-4} OD/ml, 2200 cpm per 100 µl) was sonicated, denatured, and reassociated in the presence of E. coli, 5728 and 5728 clone #15 cell DNA (4.5 OD/ml), at 67°C in 0.40 M phosphate buffer, and the fractions of reassociated DNA fragments were measured by hydroxyapaptite chromatography.

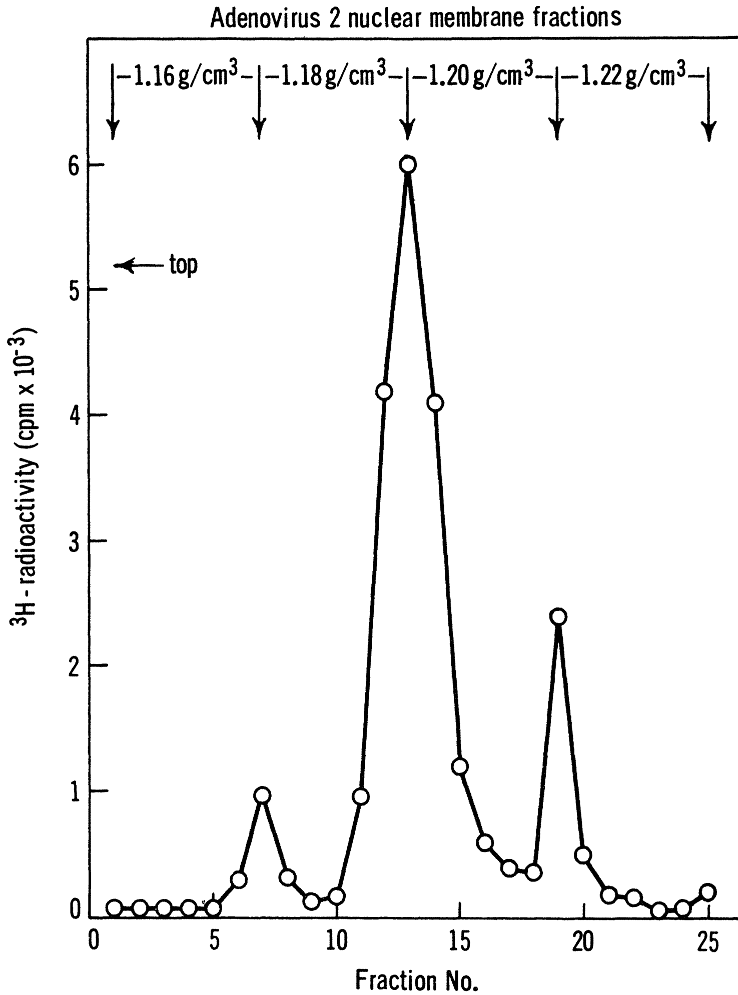

FIG. 8. Association of newly synthesized DNA with nuclear membrane fractions isolated by the discontinuous sucrose gradient procedure. Nuclei prepared from KB cells pulse labeled with ^3H-thymidine for 5 min at 18 h postinfection were sonically disrupted and fractionated by the discontinuous sucrose gradient procedure. After centrifugation for 60 min at 25,000 rpm in a Spinco SW-27 rotor, 1.0 ml fractions were collected and acid-precipitable radioactivity determined.

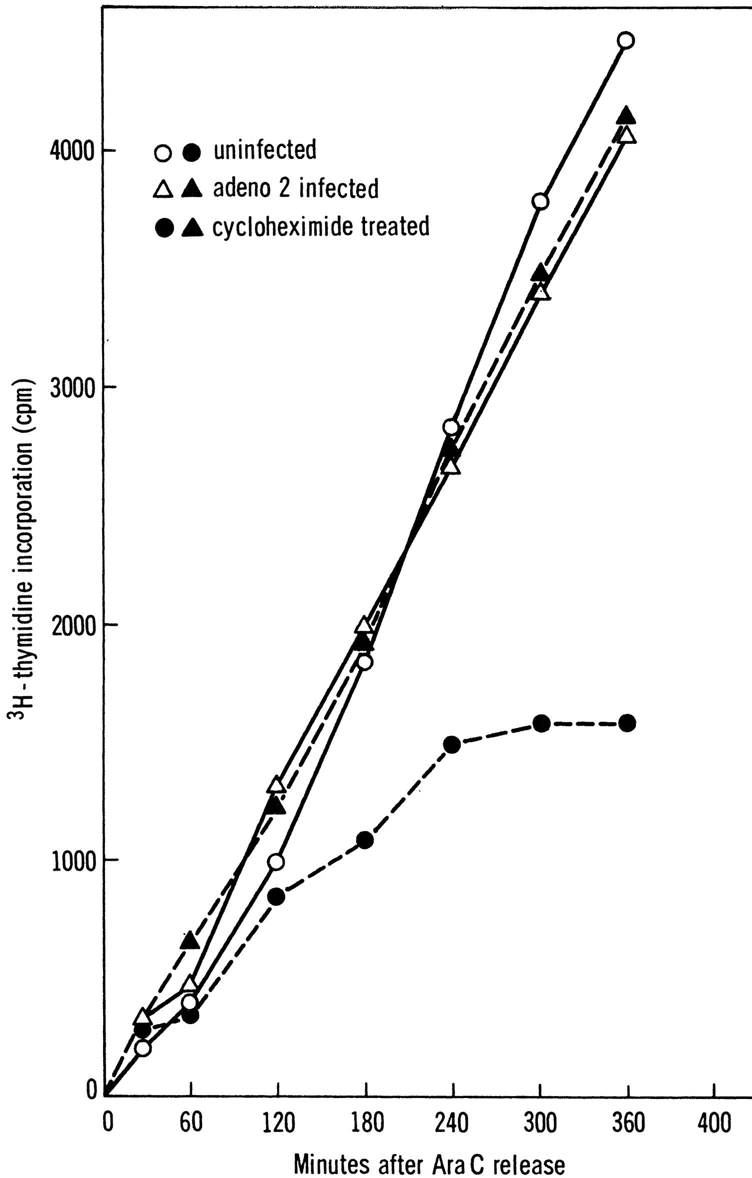

FIG. 9. Effect of inhibition of protein synthesis on DNA synthesis in uninfected and adenovirus 2-infected KB cells after treatment with Ara C. Ara C was added to adenovirus 2-infected and uninfected KB cell cultures at 2 h postinfection. At 24 h postinfection, Ara C was removed and cycloheximide (25 μg/ml), deoxycytidine (50 μg/ml), and ^{3}H-thymidine (2 μCi/ml) were added. Cells were harvested every 60 min and acid-precipitable radioactivity was measured. (O): uninfected cells without cycloheximide; (●): uninfected cells with cycloheximide; (Δ) adenovirus 2-infected cells without cycloheximide; (▲) adenovirus 2-infected cells with cycloheximide.

Thus, we detect large numbers of specific viral DNA fragments in adenovirus 7 and 12 transformed hamster cells. Sharp, Phillipson and Sambrook (personal communication) have found approximately two copies of about 40-50% of the viral genome in adenovirus 2 transformed rat cells. The mechanism of integration could be different for adenovirus 2 transformed cells. It is interesting that adenovirus 7 and 12 belong to the weakly and highly oncogenic groups B and A of human adenoviruses while adenovirus 2 is a member of the group C, which can transform rat embryo cells *in vitro* but does not induce tumors in unconditioned hamsters.

DNA REPLICATION COMPLEX FROM ADENOVIRUS-INFECTED KB CELLS

The well characterized human adenovirus genome is particularly well suited for investigation of the replication of linear duplex DNA molecules in the mammalian cell. The availability of subcellular systems would further facilitate such studies. We have found that a nuclear membrane fraction isolated from adenovirus 2-infected cells late after infection contains newly synthesized viral DNA, two new major proteins, and can synthesize adenovirus DNA sequences *in vitro*. The results of some of these studies are described below.

Adenovirus 2-infected KB cells were pulse labeled with ^{3}H-thymidine for 5 minutes at 18 hours after infection, a time when only viral DNA is synthesized, and the nuclear membrane fraction was prepared by the discontinuous sucrose method of Kashing and Kasper (1969). As shown in Figure 8, the major peak of newly synthesized DNA was associated with the membrane fraction which banded at the interphase between sucrose densities 1.18 and 1.20 g/ml.

Polasa and Green (1965) described experiments suggesting that the synthesis of protein(s) early after infection was required for the replication of adenovirus DNA late after infection. To investigate this requirement further, we carried out experiments with cycloheximide, a specific inhibitor of protein synthesis. To distinguish between early and late viral gene functions, arabinosylcytosine (Ara C) was added to KB cells at 2 hours after infection to block viral DNA synthesis and thus the synthesis of late viral proteins. At 24 hours after infection, Ara C was removed, unlabeled deoxycytidine was added to reverse the effects of Ara C, cycloheximide was added to block further protein synthesis, and ^{3}H-thymidine was added to measure DNA synthesis. As shown in Figure 9, DNA synthesis in adenovirus 2-infected cells continued at the same rate in the presence and absence of cycloheximide. DNA synthesis in cycloheximide-treated uninfected cells was inhibited. These experiments suggest that protein(s) required for adenovirus DNA replication are synthesized early after infection and could represent early viral gene functions. Similar findings were

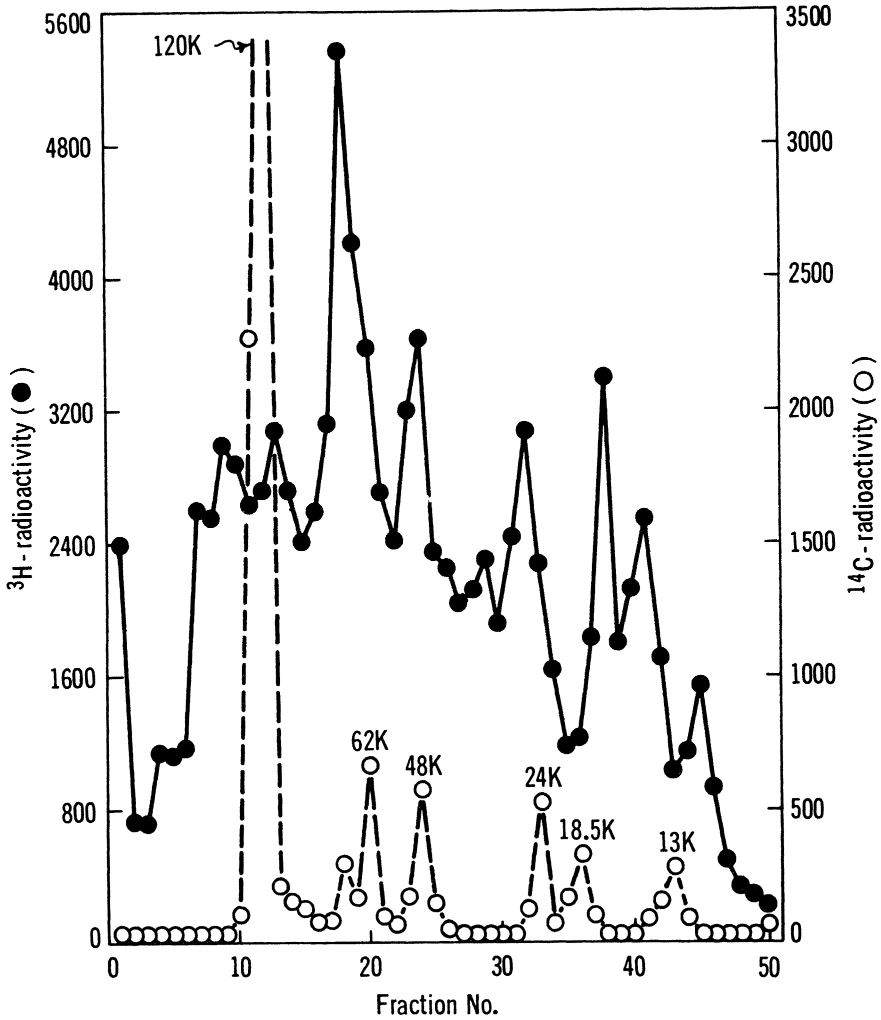

FIG. 10. Coelectrophoresis on Na dodecyl SO_4 gels of ^{3}H-leucine labeled polypeptides of the nuclear membrane isolated by the discontinuous sucrose gradient procedure at the density between 1.18 and 1.20 g/ml together with ^{14}C-leucine labeled adenovirus 2 polypeptides.

reported by Horwitz, Brayton, and Baum (1973) with adenovirus 2-infected HeLa cells.

It was therefore of interest to analyze the nuclear membrane fraction of infected cells for the possible presence of new "virus-specific" proteins. Cells were treated with Ara C at 2 hours after infection and labeled with ^{3}H-leucine from 6-24 hours. Nuclear membranes were isolated, mixed with ^{14}C-leucine labeled adenovirus to provide molecular weight markers, and electrophoresed on Na dodecyl SO_4 gels after dissociation with Na dodecyl SO_4 and β-mercaptoethanol. The gel pattern in Figure 10 revealed a major protein of molecular weight 75,000, a smaller peak at 45,000, and several additional peaks of radioactive polypeptides. The 75,000 and 45,000 dalton polypeptides were not demonstrable in the replication complex from uninfected cells (unpublished data). The 75,000 and 45,000 molecular weight proteins resemble in size the proteins recently isolated from adenovirus 5 abortively infected monkey kidney cells by binding to DNA cellulose columns (A. Levine, personal communication).

We next studied the ability of the nuclear membrane fraction (referred to as the "DNA replication complex") to synthesize DNA *in vitro*. Maximal DNA polymerase activity occurred in the viral complex at pH 8 (Figure 11). KCl and ATP were not essential, but the reaction was enhanced by 50 mM KCl and 2 mM ATP. The optimal Mg^{2+} concentration was 10-20 mM; Mn^{2+} was a poor substitute. The presence of all four deoxyribonucleoside triphosphates provided maximal DNA polymerase activity. Incorporation of ^{3}H-TTP was linear for 20 minutes and reached a plateau after 75 minutes.

The nature of the DNA product synthesized *in vitro* was studied. By hybridization, it was shown that only adenovirus DNA sequences are generated *in vitro*. Thus the specificity that occurs *in vivo* is maintained *in vitro*. The size of *in vitro* synthesized DNA was analyzed by rate-zonal sedimentation in alkaline and neutral sucrose gradients. In alkaline gradients, only small 6-7S Okazaki type DNA fragments were found. In neutral sucrose gradients, these fragments were found hydrogen-bonded to 18S DNA. It is not clear whether 18S DNA represents the template or an endonuclease-cleavage product of template viral DNA molecules. In additional studies, we have found that DNA is synthesized by a semiconservative mechanism and that some RNA species are used as a primer to initiate DNA synthesis *in vivo*. Further analysis of this replication complex should provide an increased understanding of the mechanism of adenovirus DNA replication.

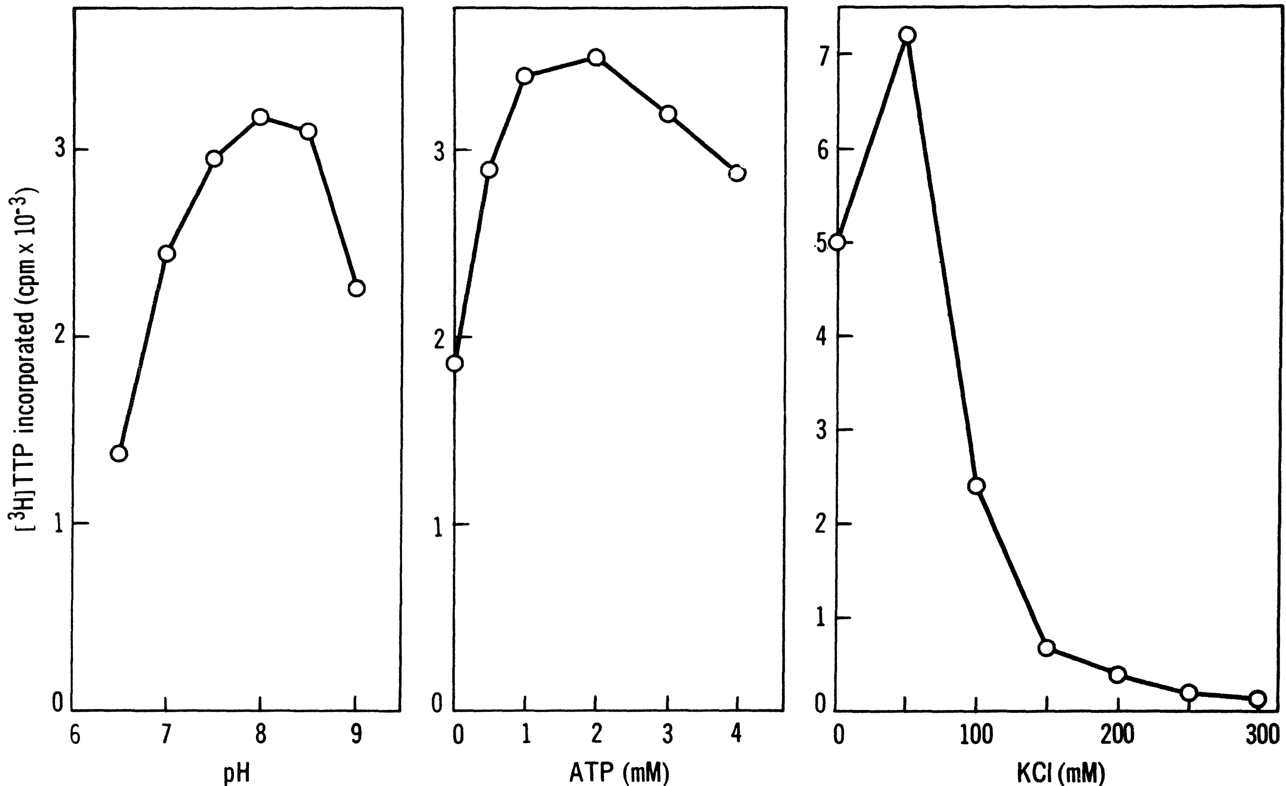

FIG. 11. (a) Effect of pH on DNA polymerase activity of viral DNA replication complex. (b) Effect of ATP concentration on the DNA polymerase activity. (c) Effect of KCl on DNA polymerase activity.

SUMMARY

The transcription of the adenovirus 2 genome early and late during productive infection was analyzed by hybridization of RNA in excess with radioactive viral L and H DNA strands. Early after infection, the equivalent of the entire viral DNA genome is transcribed by cellular enzymes to give two populations of viral RNA: (i) abundant viral RNA transcripts representing 14-16% of the viral genome, and (ii) scarce viral RNA sequences, presumably short-lived precursors to viral RNA. The transcription of the entire viral genome and the conservation of specific mRNA molecules would appear to represent a cellular mechanism for regulating gene expression.

A dramatic change in the pattern of viral gene transcription occurs when late viral genes begin to function. About 80% of viral DNA sequences of the L strand and only 10-20% of the H strand are transcribed at 9-36 hours. These RNA transcripts are of high abundance and represent the viral imposed regulation of gene transcription.

Two major homogeneous early viral mRNA species of molecular weight 0.74 and 0.76 x 10^6 and smaller amounts of 15-18S and 21-26S broad viral RNA peaks have been isolated by hybridization with and elution from L and H DNA strands.

The number of copies and the fraction of the viral genome present in cells transformed by human adenovirus 7 and 12 were estimated by measurements of early kinetics of reassociation of denatured viral DNA in the presence of unlabeled transformed cell DNA. About 300 copies of about 15-20% of the viral genome were found in adenovirus 7 transformed cells and about 50 copies of about 50% of the viral genome in adenovirus 12 transformed cells.

Nuclear membrane fractions containing newly synthesized viral DNA were isolated from adenovirus 2-infected KB cells late after infection. Associated with these fractions are two polypeptides of molecular weight 75,000 and 45,000 that are not detected in similar preparations from uninfected KB cells. A nuclear membrane complex synthesized short viral DNA fragments _in vitro_.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service Grant AI-01725 from the National Institute of Allergy and Infectious Diseases and from Contract N01 CP 70692 from the Virus Cancer Program of the National Cancer Institute.

M. G. is a Research Career Awardee of the National Institute of Health (5K6-AI-4739).

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CHARACTERIZATION AND METABOLISM OF NUCLEAR VIRAL RNA SYNTHESIZED 18 HOURS AFTER INFECTION WITH ADENOVIRUS 2

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SUMMARY

Nuclear viral RNA was studied in cultured human cells (KB) productively infected with adenovirus type 2. The nuclear RNA synthesized beginning 18 hours after infection was at least 50% virus specified. Only 10% of the nuclear RNA bound to poly U filters. More than half of newly synthesized nuclear viral RNA sedimented as greater than 28S, whereas cytoplasmic viral mRNA is of the size range 10S-28S. Nuclear RNA greater than 28S bound to poly U filters 5%, and nuclear RNA in the size range 10S-28S bound nearly 20%.

The metabolic stability of various classes of nuclear viral RNA was analyzed using a "cold chase" method. Total nuclear RNA, nuclear RNA sedimenting as greater than 28S, nuclear RNA sedimenting as 18S-28S and the molecules containing poly A from each of these three classes, all exhibited a slow decay with a half life of 3-5 hours.

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INTRODUCTION

Productive infection of human cells by adenoviruses consists of two distinct phases--the early period which precedes the onset of viral DNA replication, and late events that occur subsequently. Although adenovirus 2 DNA synthesis begins 5-6 hours after infection (Thomas and Green, 1969), most analyses of the late period of replication have been performed 18 hours after infection when viral macromolecular synthesis dominates cellular metabolism (Pina and Green, 1969; White *et al.*, 1969; Raskas and Okubo, 1971). At 18 hours, virus specific RNA is transcribed from most of the genome (Fujinaga and Green, 1970). Newly synthesized viral RNA is found in nuclei as high molecular weight molecules (Parsons, Gardner, and Green, 1971; McGuire, Swart, and Hodge, 1972; Wall, Philipson, and Darnell, 1972). This high molecular weight viral RNA is evidently the precursor of functional RNA, for it contains all the viral RNA sequences present in polysomal viral RNA (Wall *et al.*, 1972). Apparently during conversion of the high molecular weight viral RNA to polysomal RNA (Parsons *et al.*, 1971), some sequences are eliminated; recent experiments have shown that polysomal viral RNA inhibits hybridization of nuclear viral RNA sequences only 70-75% (Wall *et al.*, 1972; Lucas and Ginsberg, 1972).

We have analyzed further the metabolism of nuclear viral RNA. Previous studies had shown that like cellular RNA a very high percentage of polysomal viral RNA contained poly A sequences (Lindberg, Persson, and Philipson, 1972; Bhaduri, Raskas, and Green, 1972; Raskas, Tal, and Brunner, 1973). Poly A sequences were also found in the high molecular weight nuclear viral RNA (Philipson *et al.*, 1971). In the present study we have determined the fraction of the nuclear viral RNA which contains poly A and used a method recently developed by Murphy and Attardi (1973) to examine the metabolic stability of various classes of nuclear viral RNA.

RESULTS

(a) Fraction of Newly Synthesized Nuclear Viral RNA Containing Polyadenylic Acid

To separate molecules containing poly A from those lacking such sequences, we have utilized the observation of Sheldon, Jurale, and Kates (1972) that RNAs containing poly A sequences associate specifically with polyuridylic acid immobilized on fiberglass filters.

Beginning 18 hours after infection, cultures were exposed to labeled RNA precursors for intervals ranging from 30 to 90 minutes.

The nuclear RNAs were purified and assayed for binding to poly U filters. In various preparations the RNA samples bound 8-13% (Table 1). No reproducible difference was observed in the binding of samples labeled for different intervals. Several controls were performed for evaluating the effectiveness of the poly U method. As shown in Experiment II (Table 1), essentially none of the RNA which passed through a poly U filter bound to a second filter. Thus it is likely that all ^{3}H-RNA which can bind to poly U filters does so in the first filtration. In other experiments not shown, commercial ^{3}H-poly A bound to the poly U filters 100% whereas ribosomal RNA bound less than 0.1%. Also, we labeled nuclei with ^{3}H-adenosine and determined that nearly all the nuclear poly A was bound to the poly U filters.

The nuclear RNA synthesized late in infection was separated into three size categories, and the binding of each size class to poly U filters determined. Nuclear RNA labeled for 60 minutes beginning 18 hours after infection was fractionated by sucrose density gradient centrifugation. More than 50% of the ^{3}H-RNA sedimented greater than 28S ribosomal RNA (Figure 2A) with some of the material apparently sedimenting as fast as 80S. The ^{3}H-RNA sedimenting greater than 28S, material sedimenting between 28S and 18S, and the lower molecular weight RNA, less than 18S, were each tested for ability to bind to poly U filters. RNA sedimenting as greater than 28S bound approximately 5%, whereas RNA in the 28S-18S size category bound 20%. Only 4% of the RNA sedimenting as less than 18S bound to poly U filters.

In order to determine the relationship of these different preparations of nuclear RNA to virus specified RNA, hybridizations to adenovirus DNA were performed (Table 2). Total nuclear RNA annealed 45-50% to viral DNA. Likewise, fractionated nuclear RNA, whether binding to poly U filters or not, or sedimenting as greater than 28S or between 28S and 18S, all annealed approximately 50% to viral DNA. Thus, the RNAs containing poly A and those lacking poly A were virus specified to the same extent. Since such a high percent of the nuclear RNA was transcribed from a viral template, we have been able to characterize further the metabolism of nuclear viral RNA without performing hybridization analyses of each RNA preparation.

(b) Conditions for Performing Chase Experiments

To investigate the metabolic stability of the different classes of nuclear viral RNA, chase experiments were performed utilizing the method of Murphy and Attardi (1973). In this procedure cultures are labeled with ^{3}H-uridine, an excess of unlabeled precursors is added, and cultures are then exposed to a cold (4°C) treatment.

TABLE 1. Binding of nuclear RNA to poly U filters

Experiment	^{3}H-RNA sample	Input cpm	cpm bound to poly U filter	cpm bound to blank filter	Percent bound
I	30' label	11,320	1,161	163	8.9
	60' label	11,780	1,328	332	8.5
	90' label	11,360	1,672	210	12.9
II	90' label	278,000	20,759	-	7.5
	Rebinding of unbound RNA	67,000	183	148	0.05

Binding of nuclear RNA preparations was performed as described by Sheldon *et al.* (1972) and Brunner and Raskas (1974). Cultures were labeled with ^{3}H-uridine at 18 hours after infection.

TABLE 2. Hybridization of nuclear RNA to adenovirus 2 DNA

Experiment	RNA sample	DNA/ membrane	Input cpm	Hybridized cpm	Percent hybridized
I	Total nuclear	3	1,731	891	51.5
II	Total nuclear	1.5	1,399	618	44.2
III	>28S eluted	4	1,315	777	59.1
	>28S pass through	4	1,794	937	52.2
	28S-18S eluted	4	1,303	579	44.4
	28S-18S pass through	4	1,960	1,055	53.8

Total nuclear RNA, size fractionated nuclear RNA, and nuclear RNA lacking and containing poly A were assayed for virus specified sequences by hybridization to excess amounts of viral DNA. The three experiments represent three independent preparations each labeled for 60 minutes with ^{3}H-uridine beginning 18 hours after infection.

Since enzymes functioning in nucleotide phosphorylation retain activity at reduced temperatures but macromolecular synthesis is inhibited, this method allows reduction of the specific activity of triphosphate pools in the absence of RNA synthesis and degradation.

When the cold chase method was applied to KB cultures 18 hours after adenovirus 2 infection, the results shown in Figure 1 were obtained. Cultures were exposed to ^{3}H-uridine for 30 minutes, and then transferred to 4°C with the simultaneous addition of 10 mM uridine and 5 mM cytidine. There was no significant increase or decrease in the level of acid-precipitable ^{3}H-uridine during cold treatments as long as three hours. When cultures were returned to 37°C the decay of acid-precipitable ^{3}H-uridine was detectable within one hour, and by 2.5 hours whole cell ^{3}H-RNA had decreased approximately 30%.

Infected cultures held at 4°C still retained the ability to synthesize RNA when the cultures were returned to 37°C. RNA synthesis after cold treatment was compared to synthesis in cultures maintained throughout at 37°C. After 1.5 hours at 4°C, a culture was returned to 37°C, ^{3}H-uridine was added, and the accumulation of acid-insoluble ^{3}H-uridine determined. No lag in RNA synthesis was detected.

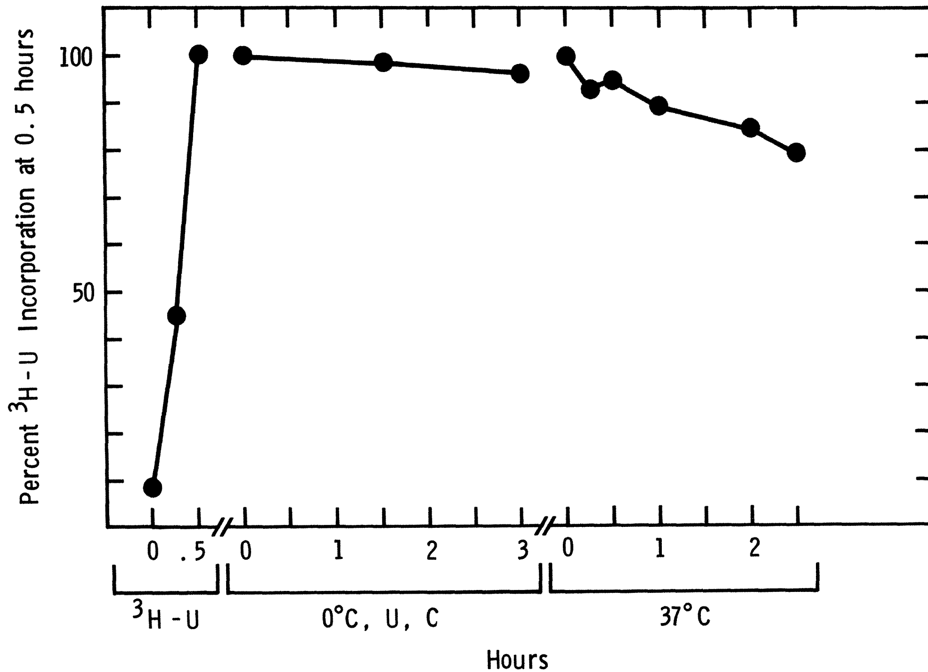

FIG. 1. Decay of whole cell RNA during chase experiments. A culture of KB cells (100 ml) infected with adenovirus 2 was labeled with ^{3}H-uridine for 0.5 hours beginning 18 hours after infection. Unlabeled 10 mM uridine and 5 mM cytidine were then added and the culture placed in an ice bath for 3 hours. The culture was then returned to 37°C. The amount of acid-precipitable ^{3}H-uridine was determined by removing triplicate 1 ml samples into 2 ml of PBS. The samples were collected by centrifugation, washed one time with PBS, and precipitated with 5% trichloroacetic acid. Similar results were obtained when cultures were placed in the ice bath for only 1.5 hours.

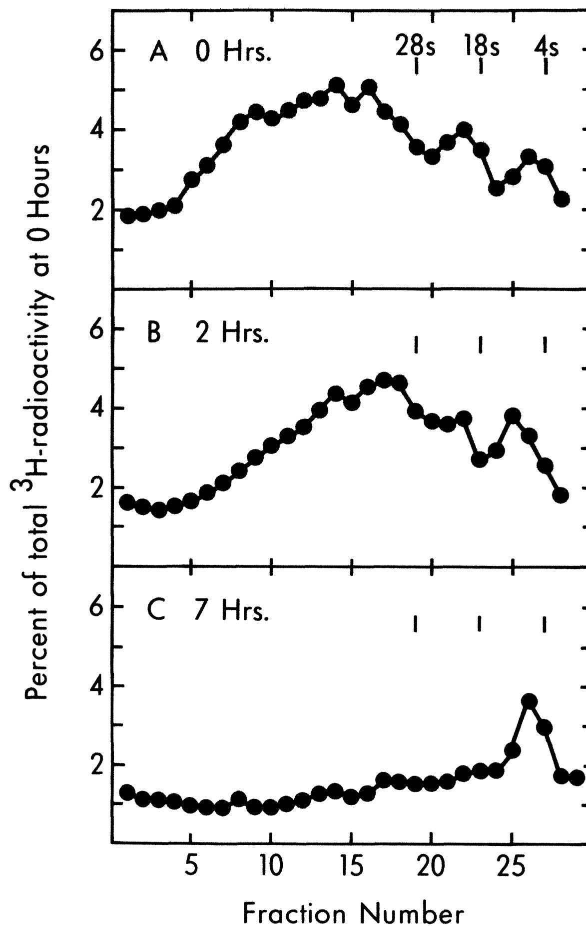

FIG. 2. Size distribution of nuclear RNA extracted at different times during the chase period. One liter of KB cells infected with adenovirus 2 was concentrated threefold and labeled with ^{3}H-uridine for 60 minutes beginning 18 hours after infection. Unlabeled uridine and cytidine were added, the culture was diluted to the original volume with fresh medium and then placed at 4°C for 1.5 hours. The culture was returned to 37°C and 300 ml samples harvested at a) 0 hours, b) 2 hours, and c) 7 hours. Nuclear RNA was purified and analyzed on sucrose gradients containing 0.1 M NaCl, 0.005 M Tris pH 7.4, 0.5% SDS. 0.5 ml fractions were collected, and 10 λ aliquots used to determine the acid-precipitable radioactivity per fraction. The radioactivity for each gradient fraction is presented as the fraction of the radioactivity present at 0 hours, the beginning of the chase period. Thus the sum of values plotted in panel A is 100%, those in panel B sum to 86% and those in panel C to 44%. The total acid-precipitable cpm in the gradient shown in panel A was 72,400.

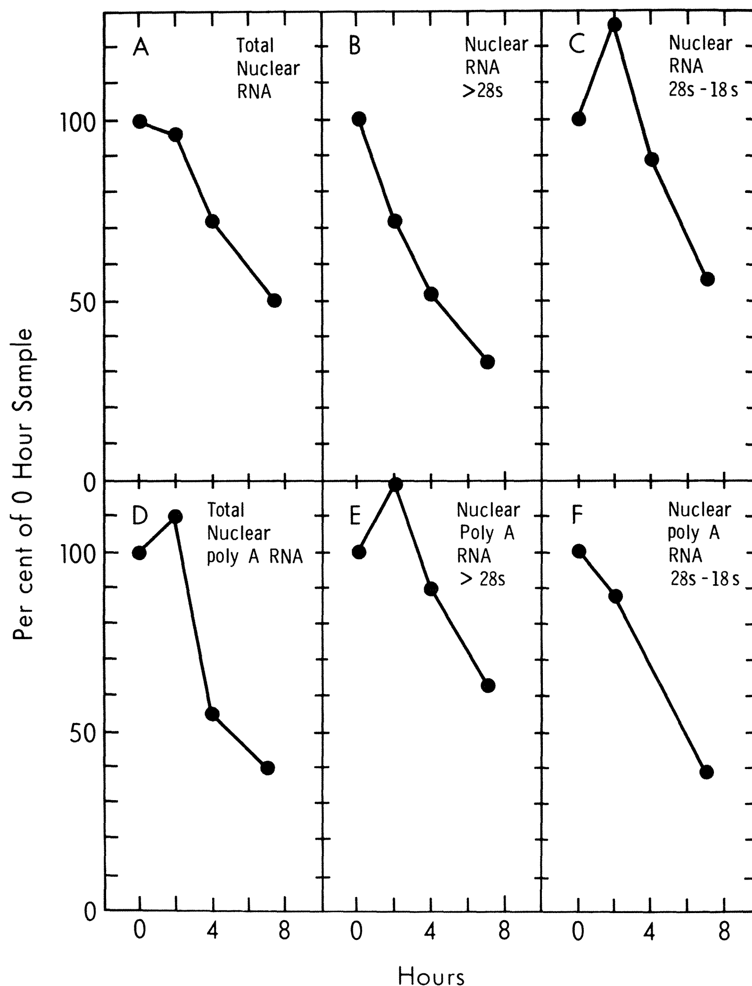

FIG. 3. Decay of total nuclear RNA, size fractionated nuclear RNA, and poly A containing nuclear RNA during chase conditions. Cultures were labeled for 60 minutes and then incubated at 4°C for 1.5 hours. Samples were removed at 0, 2, 4, or 7 hours after returning the culture to 37°C. Nuclear RNA was then purified and a portion fractionated by sucrose density gradient centrifugation as in Figure 2. The ^{3}H-RNA in (a) total nuclear RNA, (b) nuclear RNA sedimenting as greater than 28S and (c) nuclear RNA sedimenting in the range 28S-18S were plotted as a function of hours after returning the cultures to 37°C. Each population of RNA was also analyzed for binding to poly U filters with results as shown in Table 1. Based on such data and the data of panels A-C, the decay of the poly A containing RNAs in each population was determined: (d) total nuclear RNA, (e) nuclear RNA sedimenting as greater than 28S, and (f) nuclear RNA sedimenting as 18S-28S.

(c) Decay of Nuclear RNA During Chase

Approximately 65% of the nuclear RNA labeled during a 60' exposure to ^{3}H-uridine sedimented as greater than 28S. The size distribution of RNA purified from nuclei after incubation at 0°C (Figure 2A) was the same as obtained from nuclei harvested immediately after a 60' labeling period. The sedimentation distribution of RNA extracted from nuclei at 2 hours and 7 hours after returning the cultures to 37° is shown in Figures 2B and 2C. In addition to the decrease in total ^{3}H-RNA at later times in the chase period, there appeared to be a preferential decay of RNA greater than 28S as compared to the 18S-28S molecules.

The decay of various fractions of nuclear RNA was quantitated and is presented in Figure 3. The amount of total ^{3}H-nuclear RNA began to decline two hours after returning the cultures to 37°C. As late as seven hours after beginning the chase, nuclear ^{3}H-RNA was reduced only 50% (Figure 3A). The decay of RNA sedimenting as greater than 28S and RNA in the range 28S-18S was also determined. The high molecular weight ^{3}H-RNA, greater than 28S, was diminished by two hours after beginning the chase period (Figure 3B). By seven hours the ^{3}H-RNA sedimenting as greater than 28S was reduced 65% as compared to zero hours. In contrast the size class 18S-28S contained approximately 25% more ^{3}H-RNA at two hours than at zero hours (Figure 3C). This class of ^{3}H-RNA then decreased; by seven hours the amount remaining was 48% of that present at the onset of the chase period.

The decrease in radioactive nuclear molecules containing poly A was also determined. In these experiments samples were assayed to determine the fraction of the labeled material which bound to poly U filters. Thus Figures 4D-4F were obtained by combining the data used for Figures 4A-4C and assays such as shown in Table 1. After 4 hours of 37°C chase, the total nuclear RNA containing poly A was decreased about 50% (Figure 3D). In contrast, at four hours the amount of high molecular weight RNA containing poly A was 90% of the amount present at zero hours (Figure 3E). Thus there was a preferential decay of total RNA in the high molecular weight category (Figure 4B), but this loss was not found for molecules in this size class which contained poly A. After seven hours of chase, the amount of poly A containing ^{3}H-RNA had been reduced 50-70% for each of the RNA populations examined. Hybridization of the ^{3}H-nuclear RNA obtained after this lengthy chase showed that at least 50% was virus specified.

(d) Continuous Labeling of Nuclear RNA

An alternative approach to determining metabolic stability is

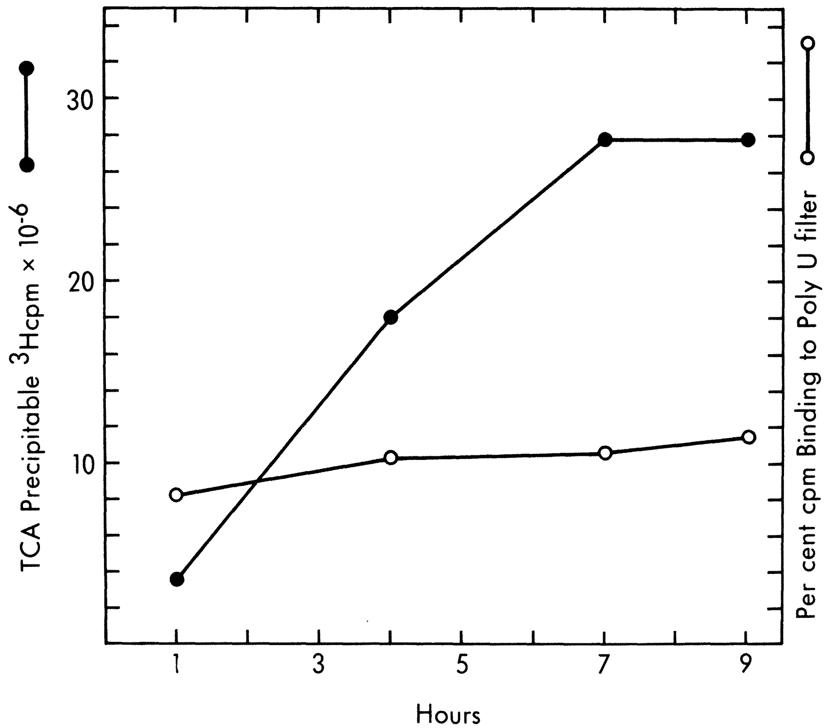

FIG. 4. Continuous labeling of nuclear RNA in cultures infected with adenovirus 2. A culture of KB cells infected with adenovirus 2 was labeled with ^{3}H-uridine (10 μc/ml) beginning 18 hours after infection. At the indicated times 75 ml samples were removed and used as a source of nuclei. Aliquots of nuclei were removed to determine the total acid precipitable nuclear ^{3}H-RNA. Nuclear RNA was purified and assayed for binding to poly U filters.

to measure the time required to label uniformly a given population. Cultures were exposed to ^{3}H-uridine and aliquots harvested at different times. The amount of nuclear ^{3}H-RNA per portion was determined. Labeling of nuclear RNA continued to increase until at least seven hours (Figure 4). Although ^{3}H-RNA accumulation continued for many hours, a constant fraction of the RNA bound to poly U filters (Figure 4) and all samples annealed to viral DNA to the same extent. Thus there was continued accumulation of total adenovirus nuclear RNA as well as viral nuclear RNA containing poly A.

(e) Accumulation of Poly A Containing Molecules in the Cytoplasm During the Chase

Although less than 10% of uniformly labeled nuclear viral RNA contains poly A (Figure 3), most if not all functional viral RNA in the cytoplasm is polyadenylated (Philipson _et al._, 1971; Lindberg _et al._, 1972; Bhaduri _et al._, 1972). These observations suggest that only a fraction of the nuclear viral RNA is transferred to the cytoplasm as functional mRNA. Therefore we quantitated the accumulation of poly A containing molecules in the cytoplasm during the chase period (Table 3). For the experiment shown (Table 3) the nuclei contained 145 x 10^5 cpm of ^{3}H-RNA per sample at the beginning of the chase period. Since approximately 10% of this RNA contained poly A, a maximum of 14.5 x 10^5 cpm might be transferred to the cytoplasm. At the beginning of the chase period the cytoplasm contained 3.4 x 10^5 cpm of RNA which bound to poly U filters. After 4 hours of chase at 37°C, the amount of cytoplasmic RNA binding to poly U filters had increased by 5.1 x 10^5 cpm or 35% of the maximum amount expected after a complete chase. Several factors may reduce the measured amount of cytoplasmic ^{3}H-RNA binding to poly U filters: (i) The nuclear RNA presumably undergoes a processing step which results in the removal of some nucleotides from the high molecular weight nuclear precursors. (ii) The net accumulation of poly A containing RNA in the cytoplasm represents the sum of new RNA molecules leaving the nucleus while at the same time other RNA molecules are being degraded; after seven hours of chase the degradation is evident (Table 3).

DISCUSSION

(a) Nuclear RNA Synthesized 18 Hours After Infection

On the basis of assays with polyuridylic acid filters, only 5-10% of the nuclear RNA synthesized late in infection appears to contain poly A. This value is lower than the 20% reported

for L cell HnRNA (Greenberg and Perry, 1972) and 30-40% for HeLa HnRNA (Jelinek et al., 1972). The various steps in preparation of the nuclear RNA, particularly the DNAse treatment, might allow degradation of some molecules. However, since a high proportion of the purified RNA sediments as greater than 28S it is unlikely that the 90% of the RNA lacking poly A arises from degradation. Moreover the length of the labeling periods as well as the results of the chase experiments which are discussed below, make unlikely the possibility that those molecules lacking poly A are all newly synthesized molecules which are not polyadenylated. An alternative possibility is that all RNA molecules are polyadenylated but that the large precursor molecules are rapidly cleaved. Our finding that only 5% of the nuclear RNA sedimenting as greater than 28S bound to poly U filters argues strongly against this interpretation as do earlier kinetic studies of polyadenylation in HeLa cells (Darnell, Wall, and Tushinski, 1971; Jelinek et al., 1972).

Evidently most nuclear viral RNA lacks poly A. Although 90% of nuclear RNA did not bind to poly U filters, the nuclear RNA containing and lacking poly A annealed to viral DNA to the same extent (Table 2). Since 75% of the viral RNA sequences present in nuclei are also found in polyribosomes (Wall et al., 1972; Lucas and Ginsberg, 1972), the high molecular weight RNAs which lack poly

TABLE 3. Poly A containing molecules found in cytoplasm during chase

Hours of chase	Total nuclear cpm x 10^{-5}	Total cytoplasmic cpm x 10^{-5}	Percent cytoplasmic RNA binding to poly U filters	cpm in cytoplasmic poly A molecules x 10^{-5}
0	145	58	5.8	3.4
2		74	9.9	7.4
4		83	10.3	8.5
7		71	9.4	6.6

KB cultures infected with adenovirus 2 (1200 ml at 3 x 10^5 cells/ml) were concentrated threefold and labeled with ^{3}H-uridine (10 μC/ml) for 60 minutes beginning 16 hours after infection. The culture was then placed at 0°C for 1.5 hours, prior to returning the culture to 37°C. Aliquots of 300 ml were removed for analysis beginning at the time the culture was returned to 37°C (zero hours). Nuclei were prepared by treatment with nonidet P-40 and collected by centrifugation. The cytoplasmic supernatant was further clarified by an additional 15 minutes centrifugation at 18,000 g prior to phenol extraction. The fraction of cytoplasmic RNA containing poly A was assayed by binding to poly U filters. The ^{3}H-RNA is given per 300 ml aliquot.

A most likely include sequences that are also found in polyribosomes. Thus this finding of nonadenylated viral RNAs demonstrates that nuclei can contain RNAs which are not adenylated but which contain sequences that are capable of being functional. Although the low percent of poly A containing molecules in nuclei from infected cultures may reflect a unique metabolic state of infected cells, the results indicate the potential significance of polyadenylation as a regulatory step. In fact, if one assumes an average molecular weight of 5×10^6 for the high molecular weight RNA and 1.2×10^6 daltons for the 18S-28S RNA, and that there are approximately twofold as many ^{3}H-cpm in the high molecular weight size class as compared to the 18S-28S group, then the greater than 28S size class contains one-half as many molecules as the 18S-28S population. Thus the ratio of polyadenylated molecules in the 18S-28S class as compared to the high molecular weight population would then be 8:1.

(b) Decay of Nuclear Viral RNA During Chase

The nucleotide triphosphate pools in eukaryotic cells make chase experiments difficult to perform. However the finding that actinomycin D inhibits protein synthesis in addition to inhibiting RNA transcription (Singer and Penman, 1972) has encouraged the search for approaches which might allow studies of mRNA metabolism without addition of drugs.

In our studies we have used the cold chase method (Murphy and Attardi, 1973). This approach is valid if the cold treatment allows reduction of the specific activity of triphosphate pools without altering macromolecular metabolism. Murphy and Attardi (1973) found that after 1.5 hours of cold treatment in the presence of 10 mM uridine and 5 mM cytidine the UTP and CTP pools were reduced to 15% and 40% of their original values. By 4 hours the CTP specific activity is further reduced to 15%. The reduction in UTP specific activity is the result of an increase in the pool size by a factor of 2.5 and a loss of acid-soluble radioactivity from the cells. Others have also reported a two- to threefold expansion of the UTP pool when excess uridine is added to the growth medium of various types of cells (Plaggemann, 1972; Kramer, Wiegers, and Hilz, 1973). We have found that the radioactivity in the UTP pool in infected cultures was reduced 50% after 1.5 hours of cold chase. Taking into account the increased pool size, this amounts to an 80% reduction in specific activity of the UTP pool. Since the CTP and UTP pools equilibrate (Plaggemann, 1972; Kramer _et al._, 1973) and the specific activity of the CTP pool appears to be reduced slower than that of the UTP pool (Murphy and Attardi, 1973), our measurements of the half-lives of nuclear RNAs may be somewhat overestimated.

Our studies of the decay of various classes of nuclear viral RNA during chase lead to two major conclusions: First, the half-lives of the various nuclear RNAs are 3.5 hours, considerably longer than those reported for other nuclear RNAs by other methods. Second, molecules containing poly A disappear from nuclei with the same kinetics as molecules lacking poly A. In previous studies the half-lives of nuclear cellular RNAs were determined either from continuous labeling experiments (Soeiro, Birnboim, and Darnell, 1968) or by utilizing actinomycin D (Scherrer et al., 1966; Attardi et al., 1966). More recently, Jelinek et al. (1973) utilized cordycepin (3'-deoxyadenosine) to determine the decay of HeLa nuclear poly A. Following treatment with cordycepin, poly A synthesis was inhibited and poly A disappeared from nuclei with a half-life of approximately 50 minutes, a more rapid decay than we have found for viral molecules containing poly A.

There are obvious limitations to the quantitation that can be achieved with the methods we have used. The possibilities for variation in chase effectiveness are considerable, and the fraction of molecules that bind to poly U varies from one preparation to another. For this reason it is difficult at this point to draw firm conclusions about possible differences in decay kinetics for different nuclear RNAs. However, if the data obtained by the cold chase method are a reasonable indication of RNA half-lives, then our results do predict that a lengthy period would be required to label uniformly total nuclear RNA. Such was the result obtained (Figure 4).

The chase experiments did allow a measurement of the flow of viral RNA into the cytoplasm. If only 10% of nuclear RNA contained poly A at the conclusion of labeling but a substantial portion of the RNA was subsequently adenylated, then during the chase period considerably more than 10% of the nuclear RNA should accumulate in the cytoplasm. Rather, the poly A containing RNA that reached the cytoplasm was less than 10% of the nuclear RNA at the time the chase began. Thus, it seems likely that a significant fraction of nuclear viral RNA is never polyadenylated and never functions as mRNA. The fate of these RNA molecules remains to be determined.

ACKNOWLEDGEMENTS

We thank Mark Telle and Joe Kelley for technical assistance. This research was supported by grants from the American Cancer Society (VC-94) and the National Cancer Institute (CA 16007).

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ONCORNAVIRUS,
ONCOGENIC RNA VIRUSES

THE PROVIRUS OF ROUS SARCOMA VIRUS: SYNTHESIS, INTEGRATION AND TRANSCRIPTION*

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ABSTRACT

RNA-directed DNA synthesis by detergent-disrupted virions of Rous sarcoma virus initiates by the covalent attachment of pdA to the 3'-terminal rA of a 4S RNA hydrogen-bonded to the 70S RNA template. This 4S "primer" has structural features of tRNA and can be aminoacylated with methionine. Synthesis and integration of provirus DNA can be monitored in both permissive (duck) and non-permissive (mouse) cells acutely infected with Rous sarcoma virus. Synthesis of provirus is a prerequisite for both viral replication and cellular transformation by the virus. However, neither integration of viral genes into the host chromosome nor transcription from these genes is necessarily followed by transformation.

INTRODUCTION

Infection of cells by RNA tumor viruses requires the synthesis of virus-specific DNA, hereafter called "provirus" or "proviral DNA" (Temin, 1971). This communication summarizes the current status of our efforts to elucidate the details of provirus synthesis in the

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*A portion of these data will also appear in the Proceedings of the VIth International Symposium on Comparative Leukemia Research.

case of Rous sarcoma virus (RSV). We assume that proviral DNA is transcribed from viral RNA by the RNA-directed DNA polymerase ("reverse transcriptase") of the virus (Temin and Baltimore, 1972), and we will describe and compare certain features of that transcription as it occurs _in vitro_ and _in vivo_.

INITIATION OF DNA SYNTHESIS

The natural template for RSV reverse transcriptase is the 70S RNA of the viral genome, although several low molecular weight RNAs of the virus are also transcribed _in vitro_ at a low frequency (Garapin _et al_., 1973). Transcription of DNA from 70S RNA initiates by the covalent attachment of a deoxynucleotide to the 3' terminus of an RNA primer (Leis and Hurwitz, 1972; Verma _et al_., 1971). The resulting RNA-DNA "joint" has been identified for a variety of RNA tumor viruses, and has generally proven to be rA-dA (Flügel and Wells, 1972; Gilden _et al_., 1972; Taylor _et al_., 1973; Verma, Meuth, and Baltimore, 1972). Flügel and Wells found both rA-dA and rU-dC joints in the case of AMV when the enzymatic reaction was elicited by disrupting virus with ether rather than with one of the more generally used non-ionic detergents (Flügel and Wells, 1972). We find only rA-dA with a variety of avian RNA tumor viruses irrespective of the manner in which virions are disrupted (Table 1). By contrast, DNA synthesis with visna virus (kindly provided by A. Haase) initiates by the formation of rA-dG (Table 1).

TABLE 1. RNA-DNA linkages formed by initiation of DNA synthesis

Virus	Polymerase reaction* Endogenous		Purified enzyme
	NP-40	Ether	
SR-RSV (A)	rA-dA	rA-dA	rA-dA
B77-RSV (C)	rA-dA		
PR-RSV (C)	rA-dA		
RAV-2	rA-dA		
Visna	rA-dG		

*The techniques for DNA synthesis with disrupted virions and purified DNA polymerase of RSV, and the procedure for identification of the RNA-DNA joints have been described (Taylor _et al_., 1973). Abbreviations: RSV, Rous sarcoma virus; SR, Schmidt-Ruppin strain; B77, Bratislava 77 strain; PR, Prague strain; RAV, Rous associated virus.

TABLE 2. The primer of RSV 70S RNA as tRNA*

1) 75 nucleotides (4S).
2) 5' pGp.
3) 3' pG-U-C-A-C-C-A_{OH}.
4) [ΨΨC]G oligonucleotide.
5) Ten distinct minor nucleosides, but rT is absent.
6) Accepts amino acid (methionine).

*The structural data referred to in this table will be published elsewhere (Bishop *et al*., 1973; Faras *et al*., manuscript in preparation).

IDENTIFICATION AND CHARACTERIZATION OF RNA PRIMER

We have identified and purified an RNA molecule which serves as a primer for DNA synthesis *in vitro* with RSV 70S RNA as template (Faras *et al*., 1973). This primer is bound to the viral genome by non-covalent forces, and has both structural and functional properties of tRNA (Table 2). In particular, the RNA is structurally homogeneous (Bishop *et al*., 1973), has 3' and 5' termini characteristic of tRNA, contains a variety of methylated and rare nucleosides (Table 3), and can be acylated only with methionine (Table 4). This primer is thus a distinct and homogeneous fraction derived from the total population of 4S RNAs associated with the viral genome (Erikson and Erikson, 1971; Faras *et al*., 1973a). These are structurally heterogeneous (Erikson and Erikson, 1971; Faras *et al*., 1973a), and accept a variety of amino acids in the case of both AMV (Rosenthal and Zamecnik, 1973) and RSV (Table 4).

TABLE 3. Minor nucleosides found in primer 4S RNA*

Pseudo-uracil
Dihydro-uracil
2'-O-methylguanine
1-methylguanine
7-methylguanine
Methyl cytosine
Two additional, presently unidentified cytosine derivatives
1-methyl adenine
Unidentified derivative of guanine

*The purification of primer RNA and its compositional analysis have been described elsewhere (Bishop *et al*., 1973; Faras *et al*., 1973b).

TABLE 4. Acylation of RSV 4S RNAs with amino acids*

Amino acid	Acceptance by 70S-a 4S RNAs (pmole amino acid/pmole RNA) Primer-free fraction (63°) PR-RSV(C)	"Primer" (80°) PR-RSV(C)
Alanine	.002	--
Arginine	.006	--
Asparagine	.005	--
Aspartic	--	--
Cystine	--	--
Glutamic	.001	--
Glutamine	--	--
Glycine	.002	--
Histidine	.019	--
Hydroxyproline	--	--
Isoleucine	.005	--
Leucine	.004	--
Lysine	.035	--
Methionine	.091	.226
Phenylalanine	.003	--
Proline	.002	--
Serine	.027	--
Threonine	.007	--
Tryptophane	--	--
Tyrosine	--	--
Valine	.040	--
TOTAL	.249	.226

*Primer was separated from the remainder of 70S-a 4S RNA by sequential denaturation at 63°C and 80°C (Faras et al., 1973a). This produces two fractions of 4S RNA: primer-free (released at 63°) and purified primer (released at 80°). Purity of the primer was documented by analysis of the oligonucleotides released by hydrolysis with T_1 RNase (Faras et al., 1973a). Acylation with [^{3}H]amino acids was carried out according to conventional techniques using aminoacyl tRNA synthetases prepared from chicken liver (a gift of E. Penhoet).

PROVIRUS IN INFECTED CELLS

Some of the genes of RSV are homologous to genes contained in normal chicken cells, and there is a corresponding homology between the genome RNA of RSV and nucleotide sequences in chicken DNA (Table

TABLE 5. Host cells for RSV and consequences of infection

Cell	Endogenous RSV genes	Reaction of RSV 70S RNA with DNA*	Transformation	Virus production
Chick	+**	50% 75% (RAV-0)	+	+
Duck	0	0	+	+
Mouse	0	0	+	0
Rat	0	0	+	0

*[^{32}P]70S RNA was incubated with a vast excess (1×10^6) of denatured cell DNA to a $C_0t = 10^4$, then tested for resistance to hydrolysis by RNase. The procedure is described in more detail elsewhere (Bishop et al., 1973).

**+ = ostensibly normal cells release RAV spontaneously, after infection, or after chemical or physical induction.

5). These facts complicate efforts to monitor the synthesis and fate of RSV provirus in its natural host. However, we have identified both permissive (duck) and non-permissive (mammalian) cells which are devoid of RSV-specific nucleic acids in the uninfected state (Varmus, Vogt, and Bishop, 1973), and we have measured the accretion of RSV provirus in these cells following acute infection (Tables 6 and 7). In both duck (permissive) and mouse (non-permissive) cells RSV proviral DNA is detectable within 3-12 hours following infection. Integration of provirus into host chromosomal DNA occurs between 10 and 36 hours. Prior to integration, provirus is restricted to cytoplasmic fractions from infected cells. However, we consider this observation provisional and in need of further validation.

TABLE 6. Acute infection of duck cells, synthesis of RSV DNA

Virus	Time (hours)	Viral DNA (copies/cell) Cytoplasm		Nucleus	
RSV-C	0	0	0	0	0
	3	0.6	0.43 (-)	0	0
	6	0.1	0.43 (-)	0.18	0.05 (-)
	9.5	0	0	0.8	0.21 (+)
	24	0	0	1.5	0.65 (+)

(-) = sequences not integrated
(+) = sequences integrated

TABLE 7. Synthesis and transcription of RSV provirus in mouse cells (BALB/c 3T3)

Time	Passage*	Viral DNA	Integration	Viral RNA (copies/cell)
12 hr	0	+	0	nt
24 hr	0	+	+	nt
48 hr	0	+	+	nt
66 hr	0			200
66 hr	RSV(A)**	0	0	2
6 days	1-2	+	+	nt
14 days	2			< 0.2
20 days	3			nt
30 days	5			< 0.02
Cloned	3T3(RSV)	+	+	2
Normal	3T3	0	0	-

*Mass cultures of BALB-3T3 mouse cells were infected at 0 hr with RSV(C).

**This is a control infection with a subgroup of RSV which does not transform mouse cells.

The synthesis of provirus is considered a prerequisite for the initiation of viral infection (Temin, 1971). We have substantiated this view by carrying out experiments with two conditional mutants of Prague-RSV(C), ts335 and ts337. These mutants, isolated in the laboratory of Peter Vogt (Wyke and Linial, 1973), possess temperature-sensitive RNA-directed DNA polymerase (Linial and Mason, 1973; see also Baltimore _et al_., this volume). Infection of permissive cells by either of these mutants at the permissive temperature (35°C) leads to the synthesis of virus-specific DNA (Table 8), followed by virus replication and cellular transformation. By contrast, infection at the restrictive temperature (41°C) precludes normal levels of proviral DNA synthesis (Table 8), and there is a corresponding reduction in both viral replication and cellular transformation (Linial and Mason, 1973). These observations document the central role of the viral polymerase in both replication of and neoplastic transformation by RNA tumor viruses, and provide further evidence in support of Temin's "provirus hypothesis" (Temin, 1971).

Cells chronically infected with and transformed by RSV all contain two or more copies of RSV provirus per cell (Table 9). In every instance examined to date the provirus is covalently integrated into chromosomal DNA of the host. Sub-clones of hamster cells originally transformed by RSV but now phenotypically reverted (DNA provided by I. Macpherson) (Macpherson, 1965) retain RSV provirus

TABLE 8. Proviral DNA synthesis by RSV ts mutants*

Virus	DNA copies/cell		Activity ratio (41°/35°)
	35°C	41°C	
Wild type	2.8	2.8	1.0
ts335	2.95	0.5	0.18
ts337	2.25	0.65	0.29

*Duck cells were infected with wild type and mutant Prague-C RSV. After 36 hours at the indicated temperatures, cellular DNA was extracted and tested for the presence and amount of RSV-specific nucleotide sequences. These experiments were carried out in collaboration with Drs. W. Mason and P. Vogt. A full report will appear elsewhere (Varmus et al., manuscript in preparation).

through numerous passages subsequent to their reversion. We have yet to determine whether the provirus in these cells is integrated, but its stability and constancy of amount during passage suggest that it is.

It is technically difficult to assess physically whether only part or all of the RSV genome is represented in the DNA of various cells. The data given in Table 9 for chicken, mouse and rat cells, all infected with RSV, are provisional minimum estimates. We expect that the entire RSV genome is present as provirus in those cells which can yield virus either spontaneously or after induction by any of several means, but we have no definitive physicochemical evidence in this regard.

TRANSCRIPTION OF RNA FROM INTEGRATED PROVIRUS IN NON-PERMISSIVE CELLS

RSV-transformed mouse, rat and hamster cells contain small amounts of RSV RNA (Table 9). At least one line of BALB/c 3T3(B77) mouse cells may contain the entire viral genome as RNA despite the failure of these cells to produce either virus or measurable viral antigen.

Mouse cells acutely infected with RSV contain relatively large amounts of viral RNA (Table 7). This RNA is readily detectable 66 hours after infection, and small amounts are still present after several passages of the cells. The gradual dissipation of viral RNA may reflect institution of transcriptional controls in the face of persisting integrated provirus DNA.

Hamster cells reverted from a transformed to a normal phenotype retain RSV proviral DNA as noted above, and continue to transcribe

TABLE 9. RSV-specific nucleic acids in normal and infected cells

Cell	Virus	Viral DNA (copies/cell)	Fraction of RSV genome	Integration	Viral RNA (copies/cell)	Fraction of RSV genome
Chick (gs^--chf^-)	0	ca. 10	> 40%	nt	< 0.05	-
Chick (gs^+ chf^+)	0	ca. 10	> 40%	nt	ca. 5	nt
Chick	RSV(C)	ca. 10	> 75%	nt	5000	100%
Duck	0	0				
Duck	RSV(A)	0				
Duck	RSV(C)	1-4	nt	+	5000	nt
Mouse (BALB-3T3)	0	0				
Mouse (BALB-3T3)	RSV(C)	1-2	> 30%	+	ca. 5	> 75%
Rat (NRK)	0	0				
Rat (NRK)	RSV(C)	1-2	nt		10-50	> 50%
Rat (XC)	RSV(C)	20	> 75%	+		> 50%
Hamster	0	0				
Hamster	RSV(D)	1-2	nt	nt	1-5	> 50%
Hamster (reverted)	RSV(D)	1-2	nt	nt	ca. 1	> 50%

the provirus into RNA (Table 9). We have not measured what fraction of the viral genome is represented in that RNA.

FEATURES OF PROVIRUS SYNTHESIS AND TRANSCRIPTION IN NON-PERMISSIVE CELLS

Provirus is physically detectable in the acutely infected mouse cell only if the infecting virus has been passed previously through the same host (Varmus et al., 1973). Thus, RSV grown strictly on chicken cells does not give rise to appreciable provirus in mouse cells (unpublished observation). (Provirus synthesis must occur under these circumstances, but at a level too low for detection by current physical techniques.) This observation conforms to the report of Altaner and Temin (1970) that "passage" of RSV in mammalian cells increases the efficiency of plating (i.e., transforming efficiency) of RSV on the same cells.

The number of non-permissive cells which are infected and contain integrated provirus from which RNA is transcribed must be far greater than the number which are ultimately transformed (ca. 10^{-3} -10^{-5}). Therefore, neither integration of viral genes into the host chromosome nor transcription of RNA from these genes is necessarily sufficient cause for transformation. We draw the same tentative conclusion from the fact that RSV provirus persists and is transcribed in hamster cells which have reverted from a transformed to a phenotypically normal state.

CONCLUSION: TRANSCRIPTION OF 70S RNA *IN VITRO* AND *IN VIVO*

There are presently only limited parallels apparent between transcription of 70S RNA *in vitro* and provirus synthesis *in vivo* (Table 10). The same polymerase is almost certainly involved, but still unidentified host factors may contribute to the events *in vivo*. A primer for DNA synthesis *in vitro* has been identified, but we have no evidence that transcription of viral genome *in vivo* initiates at this same site(s). The DNA synthesized *in vitro* by reverse transcriptase with 70S RNA as template consists of relatively short polynucleotides (Temin and Baltimore, 1972), and under most circumstances represents only a limited portion of the total template (Garapin et al., 1973). By contrast, we have preliminary evidence that prior to integration, the RSV provirus consists of DNA chains approximately 7500 nucleotides long (i.e., the length of high molecular weight subunits of the viral genome) and that these chains contain nucleotide sequences representing most or all of the viral genome (unpublished observations). The products of transcription from RSV 70S RNA include RNA-DNA hybrids, single-stranded DNA, and double-stranded DNA, both *in vitro* (Temin and Baltimore, 1972) and

TABLE 10. Transcription of RSV 70S RNA *in vitro* and *in vivo**

	In Vitro	In Vivo
Template	70S RNA	70S RNA
Enzyme	RSV DNA polymerase	RSV DNA polymerase
Primer(s)	4S RNA	?
Products	H	(H)
	ssDNA	(ssDNA)
	dsDNA	dsDNA
Chain length (nucleotides)	100-1000	(ca. 7500)
Fraction of template transcribed	10-100%	(100%)

*Items in parentheses represent provisional conclusions. Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; H, DNA-RNA hybrid.

in vivo (preliminary observations). However, we have yet to determine which of these products is the active intermediate in integration. In summary, there is considerable evidence that viral reverse transcriptase is the enzymatic vehicle for provirus synthesis, but the details of that synthesis and the mechanism of provirus integration remain to be elucidated.

ACKNOWLEDGEMENTS

We thank P. K. Vogt, I. Macpherson, K. Smith, S. Heasley, E. Penhoet, A. Haase, and B. Evans for valuable assistance. Our work is supported by grants from the American Cancer Society VC-70, the USPHS AI 08864, CA 12380, CA 12705, CA 14026, AI 06862, AI 00299, and Contract No. N01 CP 33293 within the Virus Cancer Program of the National Cancer Institute. J. M. T. acknowledges the support by Senior Dernham Fellowship (D-201) of the American Cancer Society, California Division. H. E. V. is a recipient of Research Career Development Award (CA 70193) from the National Cancer Institute.

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TEMPERATURE-SENSITIVE DNA POLYMERASE FROM ROUS SARCOMA VIRUS MUTANTS

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The DNA polymerase which can be isolated from the virions of RNA tumor viruses (the "reverse transcriptase") has many properties which suggest that its role is to synthesize a DNA copy of the viral RNA genome (Temin and Baltimore, 1972). Only genetic experiments, however, are able to definitely establish whether it does act to copy the viral genome. Recently, Linial and Mason (1973) and Wyke (1973) have isolated and characterized temperature-sensitive mutants of Rous sarcoma virus which show temperature-sensitive synthesis of DNA *in vitro*. These are mutants in a function necessary only very early in the viral growth cycle. It therefore seems likely that these mutants represent temperature-sensitive DNA polymerase mutants and could be utilized to delineate the function of the DNA polymerase found in the virions of RNA tumor viruses.

In order to characterize the properties of the DNA polymerase isolated from the virions of the temperature-sensitive mutants, we have purified the enzymes from two mutants (called ts335 and ts337) and from the parental wild type Prague C Rous sarcoma virus. The enzymes were purified by the standard methodology utilized in this laboratory (Verma and Baltimore, 1973) and behaved normally throughout the purification scheme. They did appear, however, to be more labile than wild type enzymes and preparations of the enzymes tended to lose activity rapidly even when frozen. Analysis of the subunit

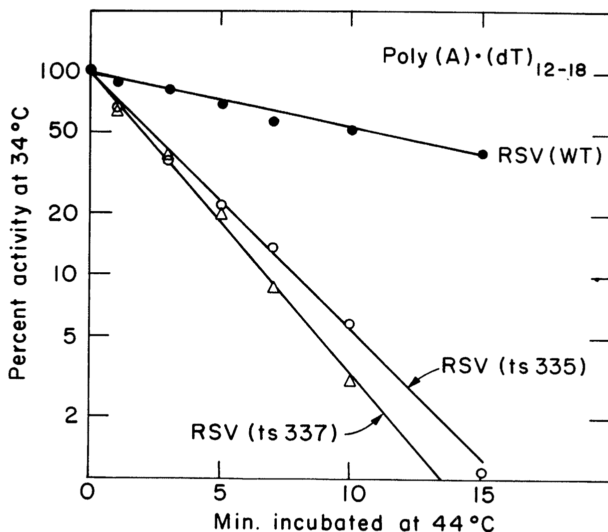

FIG. 1. Inactivation rate of ts335, ts 337 and wild-type Rous sarcoma virus DNA polymerase using poly(A)·oligo(dT) as a template·primer. Synthesis of poly(dT) using (^{3}H)-TTP as a radioactive substrate was assayed at 34° after various times of incubation at 44°. Assays were performed as described previously (Baltimore and Smoler, 1971). Incubation at 44° was carried out in a 2-fold concentrated reaction mixture lacking template, primer and substrate.

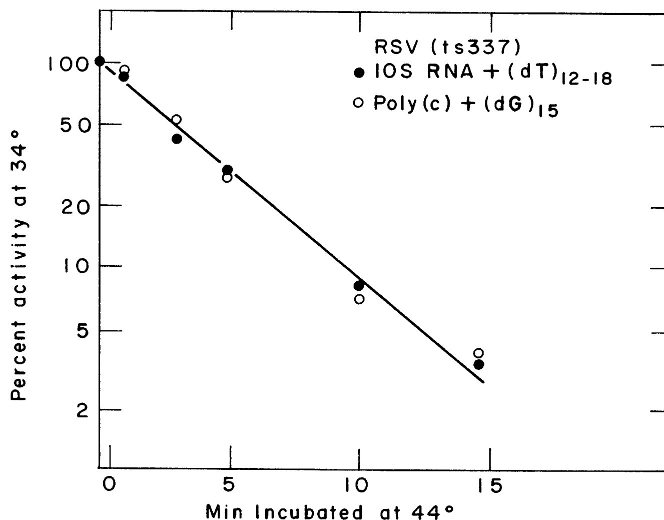

FIG. 2. Inactivation of ts337 and wild-type Rous sarcoma virus DNA polymerase using poly(C)·oligo(dG) and 10S globin mRNA·oligo(dT) as template·primers.

structure of the virion DNA polymerases by electrophoresis through polyacrylamide gels showed two subunits having the same size as the two subunits isolated from the avian myeloblastosis virus DNA polymerase (Kacian et al., 1971).

The heat lability of the DNA polymerases was assayed by incubating the enzymes for various lengths of time at 44° and then assaying their ability to synthesize DNA at 34°. In all cases comparisons were made between the mutant enzymes and enzymes isolated from the parental wild type virus. Using a template·primer combination of poly(A)·oligo(dT) to analyze the RNA-dependent DNA polymerase activity of the purified proteins (Baltimore and Smoler, 1971), we found that both mutant enzymes were inactivated about 3 times more rapidly at 44° than was the wild type enzyme (Figure 1). A similar rate of inactivation was found with poly(C)·oligo(dG) as was found with poly(A)·oligo(dT). A reproducible small difference between the rates of inactivation of the two mutants was routinely observed, with ts337 being more heat-sensitive than ts335.

The virion DNA polymerase is able to copy cellular messenger RNAs if oligo(dT) is used as a primer to initiate DNA synthesis (Verma et al., 1972; Kacian et al., 1971; Ross et al., 1972). The oligo(dT) appears to bind to poly(A) sequences at the 3'-end of the messenger RNAs. Figure 2 shows that the heat inactivation rate of ts337 DNA polymerase was the same utilizing 10S globin messenger RNA primed with oligo(dT) as it was with poly(C)·oligo(dG). The mutant enzymes are therefore as heat-labile using RNA heteropolymer templates as they are with RNA homopolymers.

The virion DNA polymerase is able to copy both RNA and DNA (Temin and Baltimore, 1972). In order to determine the heat inactivation kinetics of the enzyme using a DNA template·primer, poly(dC)·oligo(dG) was utilized. Again, the mutant enzymes were much more heat-sensitive than the wild type enzyme, and the ts335 enzyme was found to be slightly less thermosensitive than the ts337 enzyme (Figure 3). Utilizing nuclease-treated calf thymus DNA, data indistinguishable from that in Figure 3 was obtained with both of the mutant enzymes. The rate of inactivation using a DNA template was slightly less than the rate found with RNA templates.

The virion DNA polymerase isolated from avian viruses is not only a DNA polymerase but is also a ribonuclease H (Molling et al., 1971; Baltimore and Smoler, 1972). Such a nuclease is able to remove selectively the RNA moiety from a RNA-DNA hybrid (Hausen and Stein, 1970). Figure 4 shows that the activity of ribonuclease H in the ts337 purified enzyme was much more heat-labile than the wild type activity at 47°. Assays of heat inactivation at 44° showed that the ribonuclease H activity was more stable than the DNA polymerase activity although a difference between the mutant and the

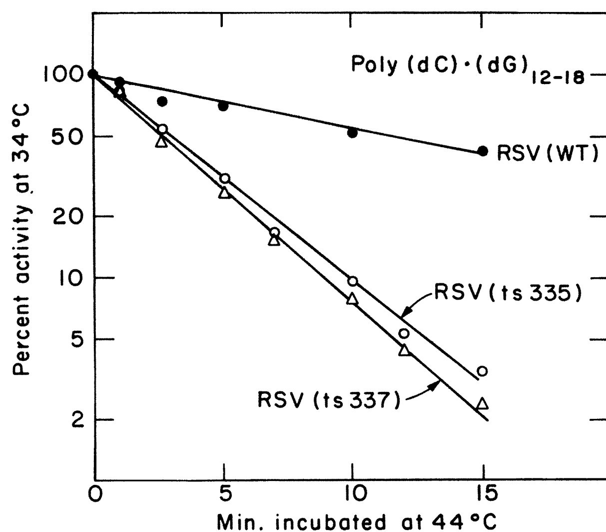

FIG. 3. Inactivation rate of ts335, ts337 and wild-type Rous sarcoma virus DNA polymerase using poly(dC)·oligo(dG).

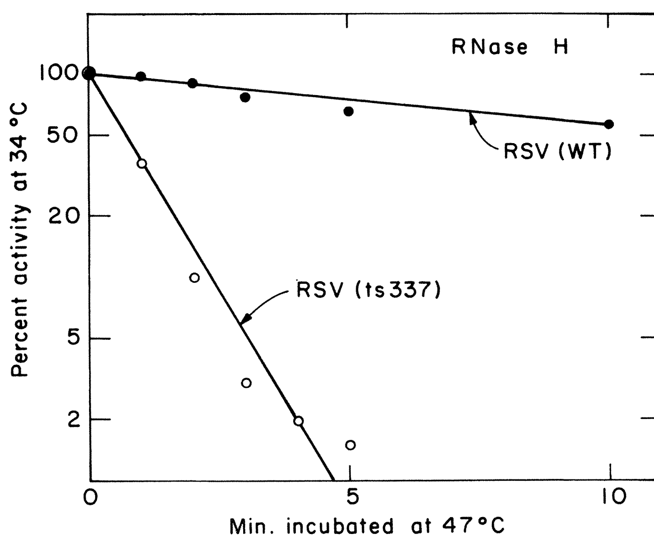

FIG. 4. Inactivation rate of ts337 and wild-type Rous sarcoma virus ribonuclease H using (^{3}H) poly(A)·poly(dT) as substrate.

wild type enzymes was evident at that temperature also (data not shown).

One way to determine whether the temperature-sensitive in vivo properties of the viruses are due to the temperature sensitivity of the DNA polymerase is to select recombinants between the mutants and wild type virus. If the temperature-sensitive growth and transformation properties of the virus are due to the heat-lability of the DNA polymerase, the two characteristics should segregate together. Such recombinants were selected by infection of cells with the temperature-sensitive Rous sarcoma virus and a non-transforming group B leukosis virus. Two temperature-sensitive recombinants and two wild type recombinants were selected and their DNA polymerase activities were assayed. The temperature-sensitive viruses had heat-labile polymerases, while the wild type viruses had normal polymerases.

These data demonstrate that the DNA polymerase found in the virions of RNA tumor viruses is encoded by a gene of the virus. Previous evidence had been in agreement with this conclusion but the genetic proof is much stronger than the biochemical evidence. Furthermore the studies on recombinants, and studies on revertants which are in progress, strongly support the idea that the DNA polymerase is responsible for an early step in virus production. Studies from H. Varmus and J. M. Bishop's laboratory indicate that less viral DNA is produced by the mutants at non-permissive temperatures than is produced by wild type virus (unpublished results). Taken together these results indicate that a virus-encoded DNA polymerase is responsible for the synthesis of a DNA copy of the viral genome shortly after infection of cells by RNA tumor viruses.

ACKNOWLEDGEMENTS

The work was supported by Contract #N01 CP 33348 from the Virus Cancer Program and grant #CA 14051 from the National Cancer Institute.

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MECHANISM OF INHIBITION OF RNA TUMOR VIRUS REVERSE TRANSCRIPTASE BY RIFAMYCIN SV DERIVATIVES

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ABSTRACT

The rifamycin SV derivatives AF/ABDMP (2,5-dimethyl-4N-benzyl demethyl rifampicin), AF/013 (O-n-octyloxime of 3-formyl rifamycin SV) and C-27 [3-(dicyclohexyl-alkyl piperidyl)-rifamycin SV] have been tested against a variety of purified enzymes, including the α and αβ forms of the reverse transcriptase purified from avian myeloblastosis virus (AMV). These derivatives appear to be specific for nucleic acid polymerizing enzymes; they were found inactive when tested against non-polymerizing enzymes such as alkaline phosphatase, glutamateoxaloacetate transaminase, RNase A, DNase I, even at very high ratios of drug to enzyme molecule. The derivatives were very active against the DNA polymerase and RNase H activities of AMV. The mechanism by which the derivative AF/ABDMP inhibits the reverse transcriptase activities of AMV and murine leukemia-sarcoma virus, Moloney strain [MSV-MLV(M)] has been studied. The drug interacts with the enzyme, not with templates, and the inhibition is completely reversible. Lineweaver-Burke analysis demonstrated that the inhibition is of the non-competitive type with regard to the binding of templates and triphosphates. Inhibition occurs at an early step(s) of transcription and is the result of the cooperative binding of drug molecules to a region distinct from the active site. The kinetic data of polymerization obtained with the two different forms of the AMV enzyme, α and αβ were analyzed according to Hill in order to calculate the number of drug molecules that bind and inhibit the viral enzyme. A straight line with a slope of 6.5 was obtained with the α enzyme, which has one subunit. A

biphasic plot was obtained with the αβ enzyme which has two subunits; the values obtained for the two slopes were 3 and 7, with the change in slope occurring at a drug concentration that gives approximately 65% inhibition. A slope of 1 was obtained when the inhibition of E. coli RNA polymerase by AF/ABDMP was studied in an analogous way. The evidence discussed in this paper suggests that the drug binds to a hydrophobic site on the enzyme which exerts a regulatory function on the initiation of DNA synthesis.

INTRODUCTION

Rifampicin is a semisynthetic derivative of rifamycin B, a natural product of fermentation of S. mediterranei. The antibacterial effect of the drug is the result of the inhibition of RNA synthesis, for the drug specifically interacts with the E. coli DNA dependent RNA polymerase. The antipolymerase activity of rifampicin is limited to bacteria and their bacteriophages (see Riva and Silvestri, 1972, for a review). However, modification of the side chain of this compound provided derivatives which were effective inhibitors of the endogenous polymerase activity of several RNA tumor viruses (Gurgo et al., 1971; Green et al., 1971). Subsequent screening uncovered more powerful inhibitors of the reverse transcriptase (Gurgo, Ray, and Green, 1972; Green et al., 1972a; Green, Bragdon, and Rankin, 1972b; Yang et al., 1972). These compounds were also found to be active, to different extents, against purified mammalian DNA and RNA polymerases (Green et al., 1972a, 1972b; Yang et al., 1972). The ability of some rifamycin derivatives to inhibit the reverse transcriptase of RNA tumor viruses and the possibility that these viruses could be involved in human neoplasia stimulated several laboratories to investigate the effect of these inhibitors in vivo. Calvin et al. (1971) reported that AF/ABDMP inhibited both Moloney murine sarcoma virus (M-MSV) induced cell transformation and MSV(MLV) replication at drug levels slightly below those toxic to cells. The same derivative was reported to delay the onset of benzanthracene induced tumors in rats (Joss, Hughes, and Calvin, 1973) and to inhibit murine leukemia virus (MLV) induced cell transformation without inhibiting MLV replication (Hackett and Sylvester, 1972). A correlation between the ability of rifamycin SV derivatives to inhibit DNA polymerases of RNA tumor viruses and to inhibit cell transformation induced by M-MSV (Green et al., 1972b) and by MSV(MLV) (Ting, Yang, and Gallo, 1972) has been reported. An analogous correlation was observed in the ability of several rifamycin derivatives to inhibit leukemia induced in rats by Rauscher leukemia virus (RLV) (Wu, Ting, and Gallo, 1973). However, as it is known that the

rifamycin SV derivatives tested thus far have a low selectivity for different polymerases, it is difficult to give an unambiguous interpretation of the results obtained in vivo.

The possibility that the rifamycin derivatives have as a general property the ability to inhibit enzymes in a non-specific way has been ruled out, by experiments in which potent inhibitors of the reverse transcriptase were found inactive when tested against a variety of non-polymerizing enzymes. In this article we discuss our recent studies (Gerard et al., 1973) on the specificity of several rifamycin derivatives for polymerizing enzymes and the mechanism of inhibition by AF/ABDMP of the reverse transcriptase of two oncornaviruses (Gurgo et al., 1974). These studies demonstrate that the drug interacts with the enzyme at a site other than the active site. The mechanism of interaction differs from that found for the inhibition of the bacterial RNA polymerase by rifampicin (RNA Polymerase and Transcription, I Lepetit Colloguim, 1970).

SPECIFIC INHIBITION OF POLYMERIZING ENZYMES BY RIFAMYCIN SV DERIVATIVES

To substantiate the suggested specificity of certain rifamycin derivatives for polymerizing enzymes, three compounds were selected from among inhibitors of the reverse transcriptase and tested against a variety of enzymes. These derivatives, AF/ABDMP, C-27, and AF/013 (Figure 1) belong to different subgroups of rifamycin SV derivatives, with major differences in the structure of the side chain. All three derivatives were found active against the polymerase and RNase H activities of the α and αβ forms of the AMV enzyme (Grandgenett, Gerard, and Green, 1973) at 20 μg/ml, which corresponds to a ratio of 500-1000 drug molecules per enzyme molecule. In Table 1 the results obtained with the α polymerase are presented; similar results were obtained with the αβ enzyme. Partially purified preparations of DNA-directed DNA polymerase I and II and reverse transcriptase from 3T6 cells transformed by Harvey [MSV(MLV)] were also completely inhibited by the three derivatives (results not shown).

The same derivatives were tested against non-polymerizing enzymes such as pancreatic DNase I, pancreatic RNase A, alkaline phosphatase, and glutamateoxaloacetate transaminase; all drugs were found inactive at ratios of drug to enzyme of 700,000 to 1, 30,000 to 1, 50,000 to 1, and 35,000 to 1, respectively. These results are illustrated in Tables 2 and 3.

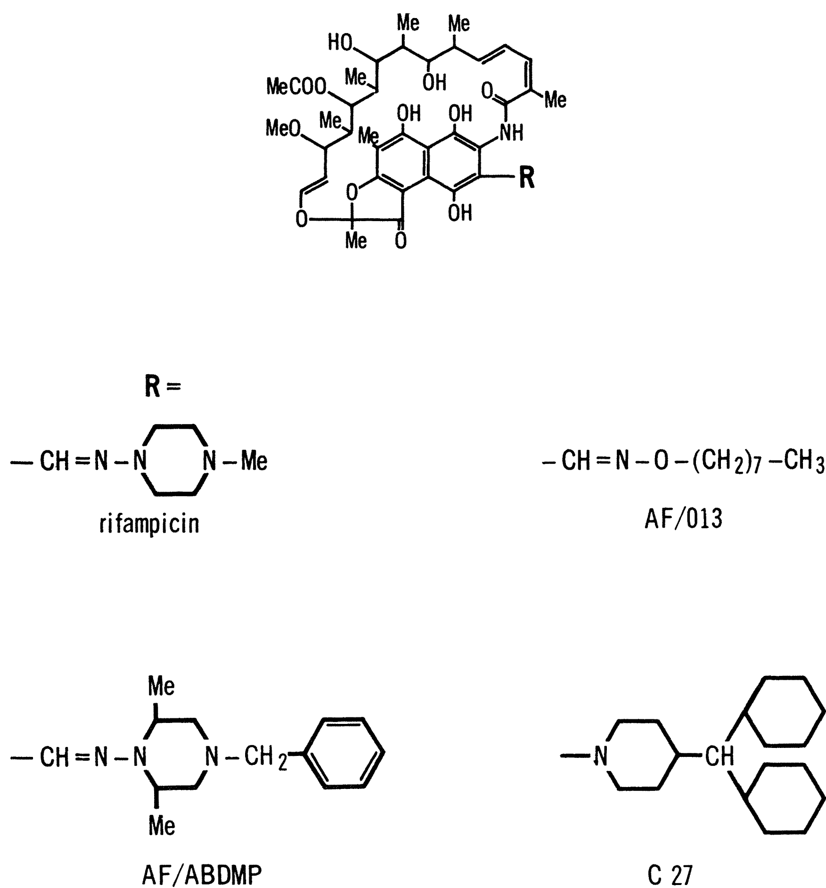

FIG. 1. Structure of rifamycin derivatives.

MECHANISM OF INHIBITION OF AMV AND MSV(MLV)(M) DNA POLYMERASES BY AF/ABDMP

Reversible Interaction of Drug with the Enzyme Molecule at a Site Different from the Active Site

AF/ABDMP inhibits the activity of the AMV and MSV(MLV)(M) polymerases by interacting with the enzyme molecule, not with the

TABLE 1. The effect of rifamycin SV derivatives on the activities of α AMV DNA polymerase

Derivative	Derivative concentration μg/ml	^{3}H-TTP incorp. directed by poly(A)-oligo(dT) cpm/15 min	^{3}H-TTP incorp. directed by poly(dA-dT) cpm/30 min	^{3}H-poly(A)-poly(dT) solubilized cpm/60 min
None	0	32,190 (100)*	24,320 (100)	1,050 (100)
Rifampicin	20	37,940 (118)	33,100 (136)	1,030 (98)
	40	31,890 (99)	19,300 (79)	975 (93)
	100	37,640 (117)	26,190 (170)	1,040 (99)
AF/ABDMP	20	12,320 (38)	2,230 (9)	83 (8)
	40	870 (3)	0 (0)	30 (3)
	60	430 (1)	0 (0)	31 (3)
C-27	20	7,620 (24)	0 (0)	9 (0.9)
	40	6,640 (21)	0 (0)	4 (0.4)
	60	6,730 (21)	0 (0)	4 (0.4)
AF/013	20	720 (2)	0 (0)	90 (9)
	40	65 (0.2)	0 (0)	38 (4)
	60	0 (0)	0 (0)	0 (0)

*The numbers in parentheses indicate percent of control activity.

The DNA polymerase from AMV was purified through the glycerol gradient step (Grandgenett *et al.*, 1973). The reaction mixture (0.1 ml) for the assay of RNA and DNA directed DNA polymerases and for RNase H activity (Grandgenett, Gerard, and Green, 1972) contained: 50 mM Tris-HCl (pH 8), 5 mM dithiothreitol, 50 mM NaCl, 10 mM $MgCl_2$, 3 μg/ml of enzyme, and one of the following (i) 10 mM ^{3}H-TTP (3000 cpm/pmole) and 50 mM poly(A) plus 10 mM oligo dT (12-18), (ii) 10 μM ^{3}H-TTP (3000 cpm/pmole), 0.1 μM ATP, and 40 μM poly(dA-dT), or (iii) a hybrid of ^{3}H-poly(A) (3 μM, 40 cpm/pmole) and poly dT (13 μM). The amount of DNA product formed and of ^{3}H-poly(A) solubilized was determined as previously described (Grandgenett *et al.*, 1972 and 1973).

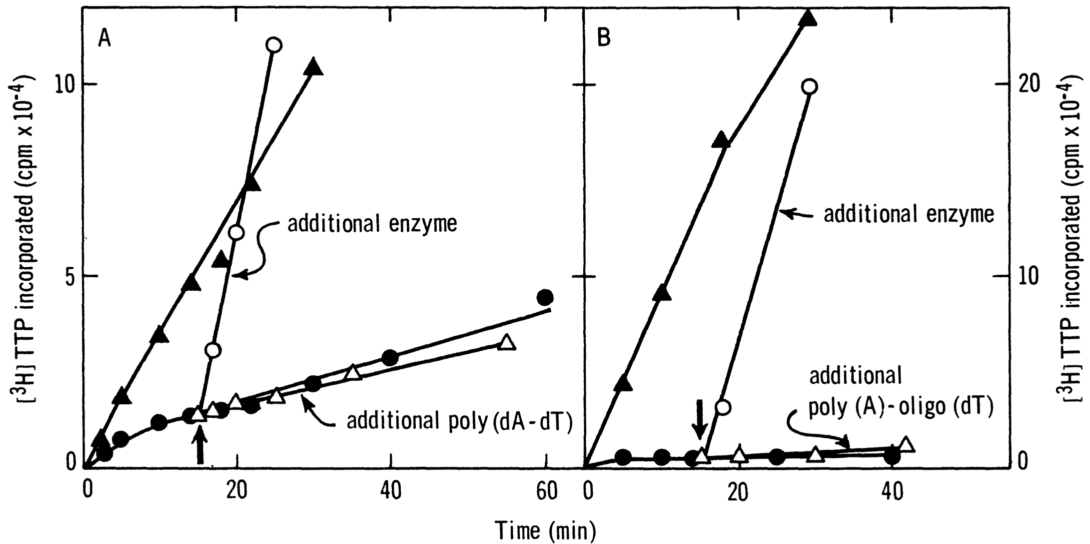

FIG. 2. The effect of additional DNA polymerase or template on AMV polymerase activity. The standard reaction mixture for the assay of the AMV DNA polymerase contained 45 mM Tris-HCl (pH 8.3); 30 mM NaCl; 0.08 mM each of d-ATP, d-CTP, d-GTP; 0.1 mM ^{3}H-TTP (150 μCi/ml); and poly(dA-dT) or poly(A)-oligo(dT) as indicated. (A) A 2-ml reaction mixture containing poly(dA-dT) (2 μg/ml) and AF/ABDMP (1.08 x 10^{-5} M) was started by the addition of AMV polymerase (3 μg/ml) (phosphocellulose purified, Grandgenett et al., 1973). After 12 minutes of incubation the reaction mixture was divided into three equal fractions. At 15 minutes, additional enzyme (O) or template (Δ) were added to separate fractions at 7.5 and 100 μg/ml respectively, with one fraction remaining as drug control (●). At the indicated times, 90 μl samples were removed and assayed (Blatti et al., 1970). The control curve (▲) was obtained with Me_2SO present instead of AF/ABDMP. (B) An identical experiment was performed as above except that the reaction mixture contained poly(A)-oligo(dT) (20:1 μg/ml) and AF/ABDMP (2.6 x 10^{-5} M). The reaction was initiated by the addition of AMV polymerase (0.6 μg/ml). Additional enzyme (2.5 μg/ml) (O) and poly(A)-oligo(dT) (Δ) (140 and 7 μg/ml, respectively) were added as previously described. The drug control (●) and Me_2SO (▲) have been described.

templates. The evidence supporting this is presented in Figure 2. An amount of drug was added to the assay mixture to allow a limited reaction of polymerization. At a given time after the reaction was initiated, more enzyme was added. A rapid increase in the rate of polymerization was observed. When an excess of template was added to a parallel assay, the effect of the drug was not overcome. Dilution of the enzyme-drug complex promptly restored the enzymatic activity, indicating that the binding is completely reversible (Table 4).

When the kinetic data were analyzed according to Lineweaver-Burke, the drug behaved as a non-competitive inhibitor with respect to the binding of templates and triphosphates. The double reciprocal plot of the initial velocity of polymerization as a function of

TABLE 2. The effect of rifamycin SV derivatives on the activity of pancreatic DNase I and RNase A

Derivative	Derivative concentration μg/ml	^{3}H-poly(dA-dT) solubilized cpm/20 min	^{3}H-poly(U) solubilized cpm/5 min
None	0	4,600 (100)*	1,985 (100)*
Rifampicin	40	5,390 (118)	-
	100	5,120 (112)	-
	200	-	2,920 (147)
AF/ABDMP	40	4,870 (106)	-
	100	4,810 (105)	-
	200	-	3,433 (173)
C-27	40	7,800 (170)	-
	100	3,510 (75)	-
	200	-	1,800 (91)
AF/013	40	4,660 (102)	-
	100	4,940 (108)	-
	200	-	1,695 (85)

*The numbers in parentheses indicate percent of control activity.

Pancreatic DNase I was added (0.01 μg/ml) to a reaction mixture (0.1 ml) containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 20 μM ^{3}H-poly(dA-dT) (14 cpm/pmole). Pancreatic RNase A was added (0.2 μg/ml) to a reaction mixture (0.1 ml) containing 200 mM ammonium acetate (pH 6.5) and 200 μM ^{3}H-poly(U) (250 cpm/nmole). The amount of ^{3}H-poly(dA-dT) or ^{3}H-poly(U) solubilized was determined as described for the RNase H assay, legend to Table 1. Enzymes were from Worthington Biochemicals.

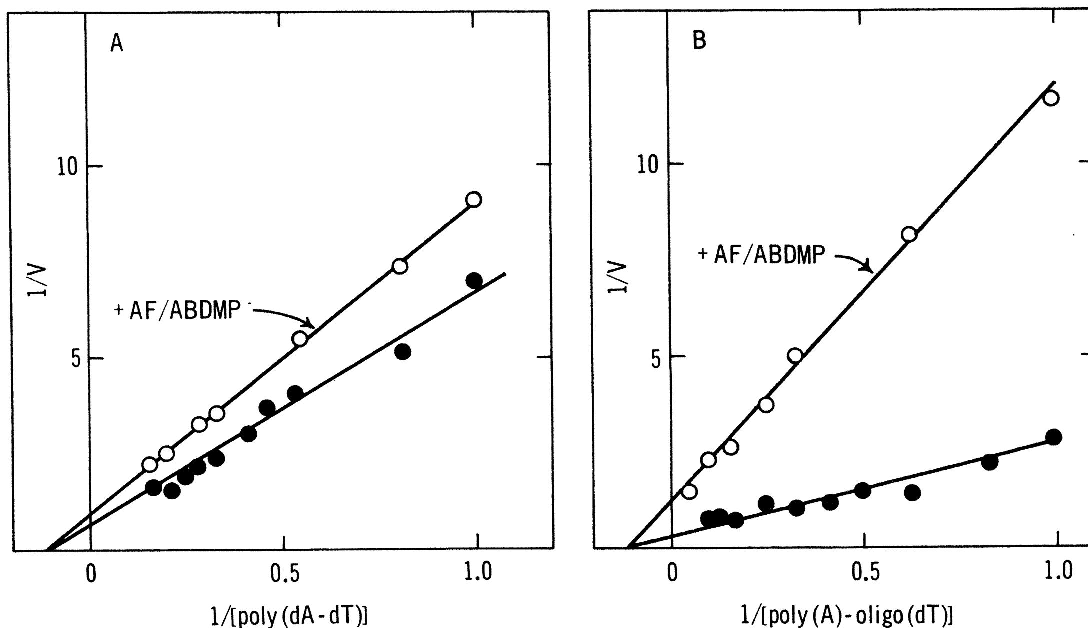

FIG. 3. Lineweaver-Burke plot of AMV DNA polymerase activity as a function of template concentration in the presence or absence of AF/ABDMP. (A) A standard reaction mixture (see legend to Figure 2) containing AF/ABDMP (O) (1.08×10^{-5} M) was distributed in a series of tubes containing poly(dA-dT) at a final concentration which ranged from 0.4 to 12 μg/ml. A parallel experiment using Me_2SO (●) instead of AF/ABDMP was used as a control. The reaction was started by the addition of AMV polymerase (7.5 μg/ml). Aliquots were taken after 3, 6, and 9 min, and initial velocities were determined. (B) An additional experiment was performed as described above except poly(A) ranged in concentration from 1 to 80 μg/ml with the amount oligo(dT) maintained at a ratio of 1:20 with respect to the amount of poly(A). The reaction was started by the addition of enzyme (0.6 μg/ml). Me_2SO (●) was present in the control reaction mixture.

TABLE 3. The effect of rifamycin SV derivatives on the activity of E. coli alkaline phosphatase and glutamate-oxaloacetate transaminase

Derivative	Derivative concentration μg/ml	Alkaline phosphatase ΔA_{410}/min	Glutamate-Oxaloacetate transaminase ΔA_{366}/min
None	0	0.79 (100)*	1.55 (100)
Rifampicin	50	-	1.45 (94)
	100	0.77 (97)	1.45 (94)
	200	0.84 (106)	-
AF/ABDMP	50	-	-**
	100	0.79 (100)	-**
	200	0.80 (101)	-
C-27	50	-	1.61 (104)
	100	0.83 (105)	1.65 (106)
	200	0.94 (119)	-
AF/013	50	-	1.57 (101)
	100	0.85 (108)	1.67 (108)
	200	0.92 (116)	-

*The numbers in parentheses indicate percent of control activity.

**The high extinction coefficient of AF/ABDMP at 366 nm prevented determination of the ΔA_{366}.

Escherichia coli alkaline phosphatase was added (0.7 μg/ml) to a reaction mixture containing 150 mM Tris-HCl (pH 8.0) and 1 mM p-nitrophenylphosphate. The initial velocity of the reaction was determined spectrophotometrically, by monitoring the change in absorbance at 410 nm with time. Glutamate-oxaloacetate transaminase activity was analyzed spectrophotometrically in a coupled reaction with malate dehydrogenase in the presence of NADH. "Statzyme GOT" from Worthington Biochemicals was used and the change in absorbance with time at 366 nm catalyzed by 0.7 μg/ml of glutamate-oxaloacetate transaminase was monitored.

poly A·oligo dT or poly d(AT) is presented in Figure 3. The data indicated that the maximal velocity of polymerization decreases in the presence of the drug while the apparent K_m remains unaffected. The same type of inhibition was observed when the effect of the drug was analyzed at various triphosphate concentrations in an analogous way. The results therefore indicate that the drug binds reversibly to a site which is different from the catalytic site.

TABLE 4. Reversal of AF/ABDMP inhibition by dilution

	^{3}H TTP incorporated before dilution	^{3}H TTP incorporated after dilution	% Recovery of activity after dilution
Control 2% Me_2SO	60	74	123
AF/ABDMP, 2 x 10^{-5} M	5.9	58	98
AF/ABDMP, 2.6 x 10^{-5} M	1.24	59	99
AF/ABDMP, 3.2 x 10^{-5} M	0.77	66	110

AMV polymerase at a concentration of 3 μg/ml was added to four assay mixtures (see legend to Figure 2) containing poly(A)-oligo(dT) (20 μg/ml) and Me_2SO or AF/ABDMP at the above molar concentrations. After 1 min incubation at 37°, 50 μl was taken from each tube and diluted ten times with a complete assay mixture containing bovine serum albumin (200 μg/ml) but lacking Me_2SO or drug. Aliquots were taken from these diluted samples and the undiluted controls after 0, 5, and 10 min of incubation and assayed according to Blatti _et al._, 1970. Incorporation is expressed as cpm x 10^4/10 min incubation.

As the inhibitory effect of AF/ABDMP is dependent on the presence of a strongly lipophilic side chain, it is reasonable to postulate that the binding occurs at a hydrophobic region. The presence of a region which exerts a regulatory function on the activity of the enzyme is also suggested by the finding that low concentrations of detergents activate the enzyme (Thompson _et al._, 1972).

Inhibition of an Early Step(s) of Transcription

Evidence that AF/ABDMP inhibits an early step(s) of transcription is based on the following observations:

1) Preincubation of the enzyme with template partially protects the enzymatic activity against inhibition by the drug. That is, enzyme and template are able to form a complex which can subsequently initiate in the presence of the drug (Table 5).

TABLE 5. Protection of DNA polymerase activity by preincubation with template

Polymerase	Preincubation with template	Control ^{3}H TTP incorporated (cpm x 10^4)	^{3}H TTP incorporated in the presence of AF/ABDMP (cpm x 10^4)
AMV	-	16.9	4.4 (26)
	+	16.7	9.5 (57)
M-MSV(MLV)	-	5.3	0.8 (14)
	+	6.2	3.3 (53)

AMV polymerase (1.35 µg) was preincubated (2 min total) in AMV diluting buffer (90 µl) with (+) or without (-) poly(A)-oligo(dT) at 25°C. The diluting buffer contained 0.2 M potassium phosphate (pH 8.0), 2 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol. After a 1 min preincubation, Me_2SO (control) or AF/ABDMP was added. The reaction was initiated by adding the components of the standard reaction mixture (see legend to Figure 2) with or without added template and aliquots were taken after 5, 15, and 30 min incubations at 37°C. The final concentrations of polymerase and drug were 4.5 µg/ml and 2.4 x 10^{-5} M, respectively. The same experiment was repeated with MSV(MLV)(M) polymerase (1 µg) in 60 µl of diluting buffer with or without poly(dA-dT) except the total preincubation time was 5 min. Me_2SO or AF/ABDMP were added after 2.5 min preincubation. The final polymerase and drug concentrations were 3.2 µg/ml and 2.2 x 10^{-5} M, respectively. The numbers in parentheses are expressed as percentage of control activity.

2) The order of addition of enzyme, drug, and template is important for the inhibition of polymerase activity. When the drug and enzyme are mixed in a standard assay prior to the addition of template, complete inhibition is observed. In the presence of limited amounts of drug, the rate of the partially inhibited reaction proceeds linearly as does the uninhibited control. When the drug is added during polymerization, inhibition is never observed immediately after the addition. This is illustrated in Figure 4. An amount of drug which completely inhibits the enzyme in the absence of the template was added to a complete reaction mixture during polymerization. The reaction proceeded unabated for at least two minutes, progressively decreasing to reach complete inhibition after 30 minutes. When higher amounts of drug were used, the reaction proceeded unaffected for at least 1 minute, and a plateau was reached after 5 minutes. A further increase of drug concentration did not produce additional inhibition. The data suggest that only reinitiation is inhibited when the drug is added during the reaction of polymerization.

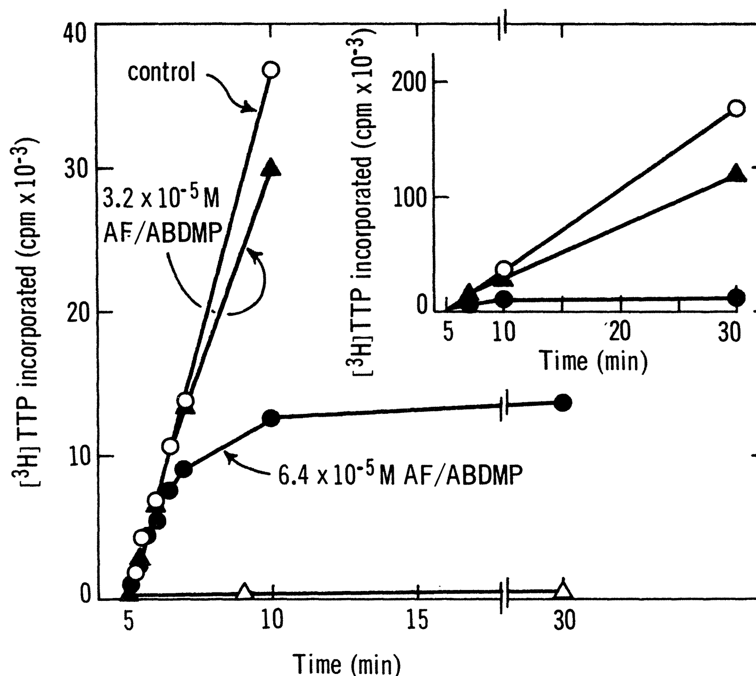

FIG. 4. Protection by template of AMV DNA polymerase activity against inhibition by AF/ABDMP. AMV polymerase (5.8 μg/ml) was added to a standard reaction mixture containing poly(A)-oligo(dT) (20 μg/ml) except that cold TTP (7×10^{-6} M) was present instead of ^{3}H-TTP. After 5 min incubation ^{3}H-TTP (3×10^{-6} M) and AF/ABDMP at 3.2 (▲) or 6.4 (●) x 10^{-5} M were added together and aliquots taken as indicated. Only ^{3}H-TTP and Me_2SO were added to a control tube (O). In a separate experiment, the above enzyme was preincubated with AF/ABDMP (3.2×10^{-5} M) at 4°C for 1 min with no template present in the above reaction mixture which had ^{3}H-TTP (3×10^{-6} M). Template was added at 0 min (Δ). A control without AF/ABDMP present (data not shown) demonstrated that the polymerase activity remained stable during preincubation. The insert presents data from the same experiment on an expanded scale.

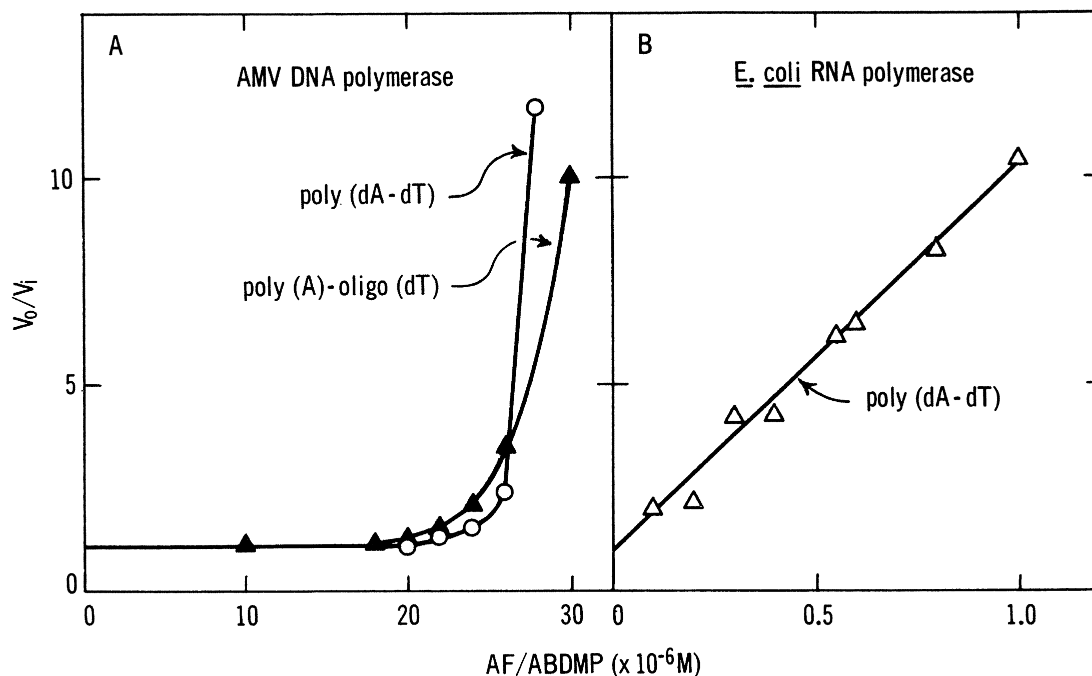

FIG. 5. Cooperative interaction of AV/ABDMP molecules with AMV DNA polymerase. (A) Reaction mixtures containing poly(dA-dT) (O) and poly(A)-oligo(dT) (▲) were distributed into a separate series of tubes containing AF/ABDMP at the concentrations indicated. The reaction was initiated by AMV polymerase (1.2 µg/ml) and aliquots were taken from each tube at 1, 2, and 3 minutes of incubation. Using initial velocities, the ratio (V_o-V_i) (V_o = ^{3}H-TTP incorporated in the absence of drug; V_i = ^{3}H-TTP incorporated in presence of drug) was plotted as indicated. (B) E. coli RNA polymerase (12.5 µg), purified by glycerol gradient centrifugation according to Burgess (1969), was preincubated 2 minutes at 4°C in a series of tubes containing 50 mM Tris-HCl (pH 8), 8 mM $MgCl_2$, 1 mM dithiothreitol, and different amounts of AF/ABDMP, in 0.2 ml. Tubes were then prewarmed for 20 seconds at 37°C and the reaction was initiated by adding 0.2 ml of substrate mixture (prewarmed to 37°C) which contained Tris-HCl (pH 8) and $MgCl_2$ at the above concentration plus 6 x 10^{-4} M ATP, 6 x 10^{-5} M ^{3}H-TTP (20 µCi) and poly(dA-dT) (25 µg/ml). After 30, 60, and 90 seconds of incubation, aliquots were taken and initial velocities were used to calculate (V_o/V_i).

Cooperative Interaction

Studies concerning the effect of drug concentration on the initial velocity of polymerization yielded a result not expected for a simple non-competitive inhibitor. The drug was found active within a very small range of drug concentrations, with inhibition increasing exponentially as though the binding of some molecules facilitated the binding of other molecules. When plotted as a function of drug concentration, the ratio of the uninhibited to inhibited activity increased exponentially, while a linear relationship was observed for the inhibition of the bacterial RNA polymerase studied in an analogous way (Figure 5). The effect of AF/ABDMP on the bacterial enzyme was studied as a control, for the mechanism by which rifamycins inhibit the bacterial enzyme is well documented (Di Mauro _et al._, 1969).

In an attempt to calculate the number of drug molecules that bind to the viral enzyme in order to give complete inhibition, the method of Hill was used (Koshland, 1970). As the drug binds to the enzyme in a reversible way, free and drug-bound enzyme will be in equilibrium at any drug concentration:

$$\frac{E\text{-}AF/ABDMP_{\eta}}{E[AF/ABDMP]_{\eta}} = K \qquad (1)$$

Equation (1) can be linearized in the following way:

$$\log\left(\frac{E^{o} - E}{E}\right) = \log K = \eta \log [AF/ABDMP] \qquad (2)$$

where

$E^{o} - E = E\text{-}AF/ABDMP_{\eta}$

E^{o} = concentration of enzyme in the absence of drug

E = concentration of drug free enzyme in presence of drug

$E\text{-}AF/ABDMP_{\eta}$ = concentration of enzyme-drug complex

K = association constant

Assuming that _n_ molecules of drug bind to the enzyme to give complete inhibition, and that the reaction of polymerization in the presence of drug is carried on by the drug-free enzyme, under conditions of steady state equilibrium, the initial velocity of polymerization can be used as a measure of enzyme concentration and substituted in equation (2) where Vo and Vi are the initial velocity of polymerization in the absence and presence of the drug.

$$\log\left(\frac{Vo - Vi}{Vi}\right) = \log K + \eta \log [AF/ABDMP] \qquad (3)$$

Equation 3 resembles the general form of the Hill equation used in studying cooperative binding of ligands to allosteric enzyme.

When kinetic data are plotted according to the Hill equation, a straight line is observed and the slope of this line is an index

of the number of molecules that bind and affect the enzymatic activity with a cooperative interaction. The value of the slope depends also on the degree of interaction among the binding sites and gives, therefore, an approximate number of binding sites (Koshland, 1970).

When the inhibition of the *E. coli* DNA-dependent RNA polymerase was studied in this way, a slope of 1 was observed (Figure 6); this is consistent with the direct determination of the binding of rifampicin to the bacterial enzyme. A slope of 6.5 was obtained with the α form of the AMV DNA polymerase, which is a single polypeptide (Grandgenett *et al.*, 1973). This suggested that a minimum of six to seven molecules bind to α in order to give complete inhibition. A biphasic plot was observed with the αβ form of AMV DNA polymerase, which has two subunits. The values obtained for the two slopes were 3 and 7, with the change in slope occurring at approximately 65% inhibition. We interpret these results in terms of subunit dissociation. If α is the only subunit with polymerase activity, then the binding of β to α might mask some of the binding sites for the drug on α, or reduce the affinity of some of these binding sites, so that only 3 molecules bind to α in αβ, and 7 after dissociation of αβ into α and β subunits has occured. Alternatively, binding of drug to αβ could cause dissociation into two subunits, both having polymerase activity, with sequential inhibition of β and then α, since the value for *n* for the second slope is close to the value observed for the α subunit alone. Although the difference between the effect of AF/ABDMP on αβ and α is intriguing, it is difficult to interpret this effect on the basis of kinetic data alone.

DISCUSSION

Several rifamycin derivatives have been found which inhibit the reverse transcriptase of RNA tumor viruses. A bulky lipophilic side chain is a common feature of all these derivatives. The macrocyclic structure (or part of it) seems to provide important steric factors necessary for binding and inhibition, because the side chain components themselves do not have inhibitory activity (Gurgo *et al.*, 1971). Our recent studies, presented in this article, show that the mechanism of inhibition of the reverse transcriptase by AF/ABDMP is different from the mechanism of inhibition of the bacterial RNA polymerase by rifampicin because: 1) the presence of a suitable side chain component is a requirement for the inhibition of the viral, but not the bacterial, polymerase; 2) several molecules bind, with a cooperative interaction, to a hydrophobic region of the viral enzyme, while only one molecule of rifampicin or AF/ABDMP binds to the *E. coli* enzyme. This argues against the possibility that polymerases have a common feature which is the target for different rifamycin derivatives. Moreover, it is not

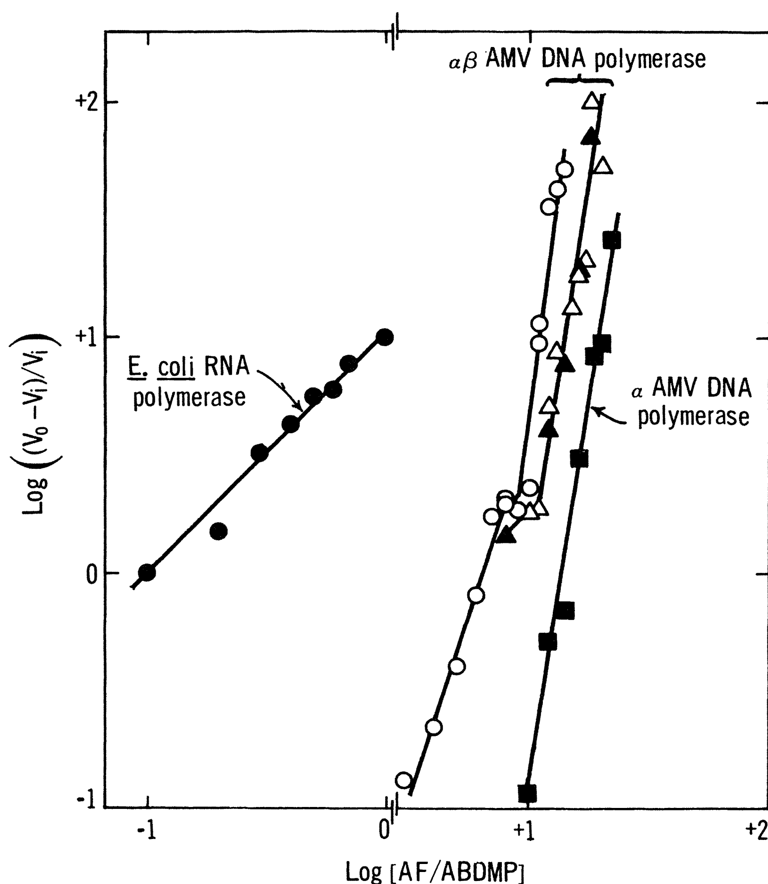

FIG. 6. Plots of log (V_o - V_i/V_i) vs. log AF/ABDMP to determine Hill coefficients. V_o is the initial velocity without AF/ABDMP and V_i is the initial velocity in the presence of AF/ABDMP. The Hill coefficient, n, is the slope of each line. Glycerol gradient purified αβ AMV polymerase at 3.5 μg/ml was preincubated for 15 sec at 37°C in 360 μl of assay mixture lacking template and primer, but containing 10 μCi (O) or 30 μCi (▲, Δ) of ^{3}H-TTP (10^{-4} M) and AF/ABDMP at the concentrations (μg/ml) indicated in the figure. The reaction was started by the addition of poly(A)-oligo(dT). After 2 min, 58,400 (Δ) and 59,000 cpm (▲) were incorporated in untreated controls, and in a third experiment, after 8 min, 90,000 cpm (O) were incorporated. Phosphocellulose purified α AMV polymerase at 15 μg/ml was assayed as described above (■); 15 μCi of ^{3}H-TTP (10^{-4} M) per assay were used. After 2 min, 40,000 cpm were incorporated in the untreated control. The data for E. coli RNA polymerase (●) included in this figure were obtained from the experiment illustrated in Figure 4. The molecular weight of the drug is 927.

possible to generalize that all the rifamycin derivatives inhibit the reverse transcriptase by the same mechanism, for major differences in the structure of the side chain may impart different modes of action with different degrees of specificity, as it has been shown in the case of T_7 RNA polymerase (Chamberlin and Ring, 1972) and RNA polymerase of human leukemic lymphocytes (Tsai and Saunders, 1973).

The attention given by several laboratories to rifamycin derivatives as potential specific inhibitors of viral reverse transcriptase is justified by the possible involvement of RNA tumor viruses in human cancer. Evidence for a viral etiology is suggested by the experimental data thus far available (for a review, see Green and Gerard, 1974).

Provided that the RNA tumor virus is proven to be an etiological agent in human cancer, it is difficult to see how specific inhibitors of the reverse transcriptase could be useful after viral information becomes stably integrated into the host chromosome. Specific inhibitors of the viral reverse transcriptase could, however, be useful in cancer chemotherapy if: 1) the enzyme is necessary for the maintenance of the transformed state; or 2) if viral information is transferred horizontally. That such a spread of viral information can occur has been recently documented by Hardy et al. (1973) who reported contact infections, among cats, of feline leukemia virus, with subsequent development of lymphosarcoma. Additional support for the idea that transformation may be the consequence of the spread of information from one cell to another has been provided by Fialkow et al. (1971) who related the case of a female who had recurrent leukemia after irradiation and bone marrow transplantation from her brother; cytogenetic studies indicated that the leukemia cells were of the male karyotype only.

Because of the antipolymerase activity observed with several rifamycin SV derivatives and the great number of chemical modifications that it is possible to introduce into the basic structure, one should be able with suitable compounds to discriminate among polymerases from various sources which differ in structure and template specificity. Whether to be directed against the viral reverse transcriptase or other polymerases involved in the transcription of DNA of viral origin, specific or selective inhibitors will be useful in establishing the role of such enzymes in human neoplasia.

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CELL SURFACE ANTIGENS IN ONCORNAVIRUS TRANSFORMED CELLS

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Of the various physiological changes detectable after conversion of cells to malignancy, the expression of new antigens on the cell surface could be a key phenomenon, the study of which might contribute essentially to answering basic questions of cancer research.

The appearance of new cellular antigens which are foreign to the tumor-bearing host and therefore capable of provoking an immunological defense response implies the possibility to prevent or cure cancer by immunological means. This could be achieved either by 1) unspecific stimulation and enhancement of the natural immune response of the organism, 2) injecting tumor antigen-specific immune sera or lymphocytes, or 3) finally by vaccination with the respective tumor antigens. The expression of new cell surface antigens, on the other hand, might reflect molecular changes that influence growth control mechanisms. The investigation of the release from growth control might lead to an understanding of the mechanism of tumor development.

Thus, the investigation of the immunology, biochemistry and biosynthesis of tumor specific cell surface antigens (TSSA) could possibly help to solve the <u>academic</u> problem of how cancer develops as well as the <u>medical</u> problem of how cancer can be cured.

For several reasons we have chosen the avian RNA tumor virus (ATV) system in order to study TSSA. A variety of well-defined virus strains is available which are closely related to each other with respect to structure and replication mechanism, but which are different in immunological and biological properties. Progress has been made in studying the genetics of these viruses and series of

temperature-sensitive mutants have been obtained which are defective either for replication or for transformation or for both. It is also possible to induce virus-producing as well as non-virus-producing tumors, and some viruses (leukosis virus) can replicate in tissue culture without transformation. Thus, a variety of virus-cell interactions facilitates the investigation of the question as to which cellular modifications occurring after oncornavirus infection are related to the transformation process.

As will be demonstrated, the results obtained indicate the existence of TSSA as a stable marker of transformed cells. TSSA expressed on different target cells such as leukemia and sarcoma cells, and also on cells of different species transformed by ATV are identical or related. So far, TSSA could not be identified with virion or embryonic antigens (Gelderblom, Bauer, and Graf, 1972; Gelderblom and Bauer, 1973; Kurth and Bauer, 1972a, 1972b, and 1973a).

MATERIALS AND METHODS

For convenience, the results will be essentially described as obtained with viruses of subgroup A and D with the exception of one experiment in which the R77 strain as ASV-C and the avian myeloblastosis virus (AMV) strain as ALV-B were used. Viruses belonging to the same subgroup cross-react in neutralization tests, i.e. have related virus envelope (Ve) antigens, which also determine the virus host range (Vogt, Ishizaki, and Duff, 1966). Sarcoma virus (ASV) and leukosis virus (ALV) members of subgroup A and D were used. ASV transform chicken embryo cells (CEC) *in vitro*, whereas ALV do not, although ALV are oncogenic *in vivo*. The virus strains used were the Schmidt-Ruppin (SR) ASV strains of both subgroups A and D, RAV-1, an ALV of subgroup A (ALV-A) and NC-SRV-D, an ALV of subgroup D (ALV-D). The NC-SRV-D is a derivative of SR-ASV-D (Graf *et al.*, 1971) and has been shown to behave *in vivo* like leukosis viruses (Biggs *et al.*, 1973).

Cells from 11-day-old chicken embryos were taken either as normal control, or productively infected by ALV, or transformed by ASV. ALV-B transformed myeloblasts were obtained from diseased chickens. Mouse embryo cells from inbred STU mice served as control when mouse tumors were investigated. The mouse tumor cells were *in vitro* cell cultures from ASV-D-induced STU mouse tumors (Bauer *et al.*, 1969).

A variety of methods has been used for assaying TSSA, embryonic antigens (EA), and virion antigens and was described in detail previously. Immune sera prepared in chickens were used in the immuno-ferritin technique (Gelderblom *et al.*, 1972; Gelderblom and

Bauer, 1973), the immunofluorescence method (Kurth and Bauer, 1972b) and the radioimmunoassay (Kurth and Bauer, 1973a). Immune lymphocytes from chicken and mice as well as mouse immune sera were used in cytotoxic assays (Kurth and Bauer, 1972a, 1972b, and 1973b).

RESULTS

Demonstration of Type-and Group-Specific Cell Surface Antigens

DNA tumor viruses were first described to induce new cell surface antigens (Habel, 1961; Sjögren, Hellström, and Klein, 1961). These antigens could not be identified with virion constituents. In contrast to papovaviruses, oncornaviruses do replicate under natural conditions while transforming natural host cells. For this reason and as an interpretation of experimental data, it was a general assumption for years that the Ve antigen exposed at the cell surface during the maturation process of oncornaviruses was the main or even the only cell surface antigen-specific for these tumor viruses.

This was investigated by our laboratory in a direct approach by testing CEC that were infected or transformed by ALV and ASV, respectively, of subgroups A and D. Cell surface antigens were tested by chicken immune sera with the immunoferritin method (Gelderblom et al., 1972), and by immune lymphocytes (IL) in cytotoxic assays where the destruction of target cells was evaluated microscopically or by trypan blue staining (Kurth and Bauer, 1972a).

The results with both sera and IL were essentially similar, as summarized in Tables 1 and 2. ALV-infected cells react only with immune serum or lymphocytes prepared against viruses of the same subgroup, i.e. via related Ve antigens.

The immunoferritin staining of Ve antigen occurred at the budding site of particles as well as at non-budding areas of the cell surface.

ASV-transformed cells are additionally stained by antiserum against ASV of a different subgroup and killed by immune lymphocytes prepared against ASV as well as ALV of different subgroups.

Several conclusions can be drawn from these results:

A virus type-or subgroup-specific cell surface antigen is detectable on transformed as well as untransformed but productively infected CEC. From the fact that the respective antisera stain the

TABLE 1

Cell Surface Staining by Hybrid Antibody Technique of Transformed and/or Infected Chicken Cells by Chicken Immune Sera

Chicken Immune Sera* against	Transforming and/or infecting viruses			
	ALV-A	ASV-A	ALV-D	ASV-D
ALV-A	+[§]	+	ϕ	ϕ
ASV-A	+	++	ϕ	+
ALV-D	ϕ	ϕ	+	+
ASV-D	ϕ	+	+	++

*Taken after a single virus injection

§Degree of staining

TABLE 2

Cytotoxic Effect of Immune Lymphocytes Against Transformed and/or Infected Cells of Chicken

Immune lymphocytes against*	Transforming and/or infecting viruses			
	ALV-A	ASV-A	ALV-D	ASV-D
ALV-A	+	++	ϕ	+
ASV-A	+	++	ϕ	+
ALV-D	ϕ	+	+	++
ASV-D	ϕ	+	+	++

*Taken after repeated virus injections

virus envelope antigen which is known as the only virus strain-specific antigen, it is concluded that the cell surface antigen in that subgroup-specific reaction represents Ve antigen (Gelderblom et al., 1972).

A group-specific antigen is found only on the surface of transformed cells but not on the virion. The failure of ALV-antisera to detect the group-specific antigen is probably due to the immunization procedure: immune sera were obtained after a single and lymphocytes after several virus injections (Kurth and Bauer, 1972a).

Both kinds of antigen act as transplantation antigens in the sense that they stimulate specifically cytotoxic lymphocytes.

The finding that IL obtained after repeated immunization with ALV also reacted with a group-specific TSSA suggested the expression of antigenically related TSSA on both leukemia and sarcoma cells. This was proved more directly by an *in vitro* approach in which AMV-transformed chicken myeloblast cells were investigated for the presence of TSSA. It turned out that radioactively labeled chicken IgG sensitized against TSSA were specifically absorbed by myeloblast cells in comparison with normal white blood cells, normal and ASV-transformed chicken embryo cells (Kurth and Bauer, in preparation).

TSSA Common to ATV-Induced Tumors of Different Species

Cross-reacting TSSA in papovavirus-induced tumors of different species have been well documented. *In vivo* experiments devoted to that question in the ATV systems have given equivocal results, probably due to an inadequate sensitivity of transplantation assay (Jonsson, 1966). We reinvestigated this by the more refined *in vitro* techniques as mentioned above.

Mammalian (mouse, hamster) and chicken cells transformed by ATV were investigated with immune sera and lymphocytes of heterologous origin in different immunological tests. Cross-reactions were observed in both directions (Kurth and Bauer, 1972b; Gelderblom and Bauer, 1973). No virus type or subgroup-specific antigen could be detected on mammalian cells, but instead group-specific TSSA. According to staining with ferritin-labeled or fluorescent chicken IgG, it seemed to be expressed even in quantitatively larger amounts than in infected cells of the natural host. The mouse immune sera and lymphocytes, on the other hand, had a weaker cytotoxic effect on transformed CEC when compared to mouse tumor cells. Whether this was due to a weaker expression of TSSA on chicken cells or indicated a qualitative difference, i.e. partial cross-reactivity between ATV-induced TSSA of different species could not be decided with certainty by those experiments.

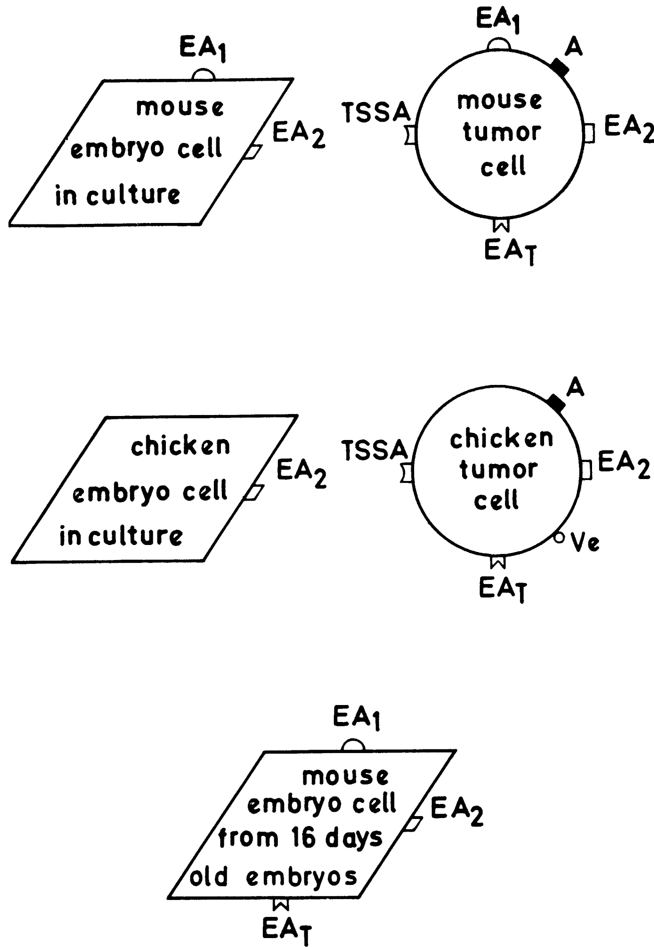

Fig. 1. Schematic representation of cell surface antigens.

Demonstration of Embryonic Antigens

The latter results could also indicate the presence of several antigenically different tumor cell surface antigens, some of which could be species-specific. Because of the recent detection of embryonic antigens in certain tumor cells it was also considered that such tumor-associated antigens might be of embryonic origin, being re-expressed after cell transformation. To study that possibility, both mice and chickens were repeatedly injected with the respective syngeneic minced embryonic tissue. Immune sera and lymphocytes were found to react to different extents with homologous and heterologous embryonic and tumor cells. By extensive series of absorption experiments in which TSSA-specific sera were included, several antigens could be distinguished on ATV-transformed cells (Kurth and Bauer, 1973c) (Figure 1).

1) one antigen of embryonic origin which is interspecies-specific for mouse and chicken tumor cells and tentatively called EAT,
2) one mouse-specific embryonic antigen found an transformed as well as embryonic mouse cells (EA_1),
3) one antigen of embryonic origin that was demonstrable on both normal and transformed mouse and chicken cells (EA_2),
4) one antigen that could not be identified as of embryonic origin and which will henceforth tentatively be called TSSA.

Consistency of TSSA and EA

Any biological phenomenon characteristic of the malignant cell could be either correlated directly with the malignant growth behavior, or reflect a certain step in a series of reactions that lead to that final state of malignancy. This distinction may be tested after reversion of a cell from the malignant to a normal growth behavior. A reversion might be achieved either by genetical manipulation such as using temperature-sensitive viruses or cells, or by influencing certain metabolic steps of the tumor cells. The latter is possible by increasing the intracellular level of cyclic adenosine 3':5' monophosphate (cAMP) by addition of dibutyrylic (db) cAMP and theophylline (Th) to the medium of cultured tumor cells. This has been reported to normalize the growth behavior of tumor cells (Kurth and Bauer, 1973c).

This technique of normalization of tumor cell growth was used to investigate the expression of TSSA and EA as compared to normal xenogeneic cell surface antigens under different growth conditions of the tumor cell (Kurth and Bauer, 1973c). A non-virus-producing mouse tumor cell line and the $chromium^{51}$ release technique as previously described was used (Kurth and Bauer, 1972b).

In accordance with the results described in other systems by several investigators, the morphology, the growth properties, and the agglutinability of tumor cells reverted nearly to normal when the intracellular cAMP level was raised. This effect was best achieved by cell treatment with 10^{-5} M dbcAMP plus 10^{-3} M (Th). Theophylline has been reported to potentiate the effect of dbcAMP. Likewise, the cytotoxic effect directed against normal cell surface antigens decreased considerably. Surprisingly, the cytotoxic effect exerted by TSSA and EA-antiserum was not reduced but instead significantly increased under the same conditions.

CONCLUSION

As a model for oncornavirus-directed cell surface changes, the ATV system has provided an answer to a variety of basic questions. From the results obtained, it is quite clear that not only one but several new antigens are expressed on the surface of transformed cells. Those include virion antigens, embryonic antigens, and TSSA. The latter are apparently neither constituents of the virion nor of embryonic origin.

The type-specific virus envelope glycoprotein is only found in virus-producing cells of the natural host. It is remarkable that this antigen is located at non-budding areas and mediates the cytotoxicity of immuno lymphocytes. From this observation one can conclude that it acts as a transplantation antigen.

One antigen reacting with immune sera and lymphocytes prepared against embryonic tissue is specifically expressed after transformation only and is not found in productively infected but untransformed cells. It cross-reacts even between chicken and mouse cells and is considered as re-expressed embryonic antigen. Further embryonic antigens detectable in transformed chicken and mouse cells are also formed on embryonic cells in tissue culture. Since the differentiation state and growth properties of embryonic cells are to some degree comparable to those of tumor cells, the embryonic antigens might be linked with the growth regulation. Though only to a weak extent, embryonic antigens also induce cytotoxic reactions of the host and therefore might also be considered as useful in immunotherapy or prophylaxis of cancer cells.

TSSA represent the strongest antigens in cytotoxic *in vitro* tests and are assumed to be the major correlate to the antigens involved in *in vivo* transplantation experiments as described years ago by several groups for that system. TSSA are not only group-specific for avian sarcoma virus strains of different subgroups, but also for leukosis and sarcoma viruses as has now directly been proven by comparing leukemia and sarcoma cells *in vitro* (Kurth and

Bauer, in preparation). This is in accordance with earlier _in vivo_ experiments in mice (Bauer _et al_., 1969) and chickens (Meyers, Sigel, and Holden, 1972) where the immunization with leukosis viruses has led to an immunity against sarcoma viruses of unrelated Ve antigen.

The interspecies specificity of TSSA seems to favor the idea that these antigens are coded by the virus genome. One has to be aware, however, that the experiments so far done do not exclude with certainty an embryonic origin of TSSA and that interspecies-specific embryonic antigens seem to exist. TSSA could well consist of several antigens, some of which could be of cellular and others of viral origin.

The cAMP experiments have clearly distinguished between TSSA and EA on the one hand and agglutinin receptors (A in Figure 1) as a different marker of the tumor cell surface on the other. Whether the failure of correlated expression of these markers reflects a difference in biological functions is an interesting question. A more thorough study of isolated cell surface antigens will be undertaken to approach this and other problems.

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STABILITY OF AVIAN ONCORNAVIRUS PRECURSOR PROTEIN IN A LINE OF RSV-TRANSFORMED HAMSTER CELLS

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ABSTRACT

Antibody to disrupted avian myeloblastosis virus was used to precipitate rous sarcoma virus-specific proteins from extracts of RSV-transformed hamster cells. These cells and heterologous transformed cells in general are known not to produce virus particles. When analyzed by dodecyl sulfate gel electrophoresis, the immune precipitates were shown to contain a polypeptide with exactly the same mobility as the AMV polypeptide that has been demonstrated previously to be a precursor to AMV structural proteins in infected chick cells. Tryptic fingerprints indicated that the hamster cell-RSV-polypeptide and the AMV-precursor are closely related. Unlike the AMV-precursor in chick cells, however, the hamster cell-RSV-polypeptide is not cleaved proteolytically to yield virion proteins. It is suggested that the block to virus production in mammalian cells transformed by avian oncornaviruses may be due to the inability of such cells to process the viral precursor polypeptide.

INTRODUCTION

Avian oncornaviruses can productively infect as well as transform susceptible avian cells. In addition, several strains of these viruses are capable of transforming mammalian cells, both in tissue culture and in animals, without production of virus (Zilber, 1965; Altaner and Temin, 1970). For example, mammalian cell lines infected by the Prague, B77, and Schmidt-Rupin strains of rous sarcoma virus (RSV) have been judged to be transformed by the criteria of tumorgenicity, focus formation, and morphology

(Svoboda, 1960). That these transformed heterologous cells do not produce RSV has been determined by the lack of detectable radioactive particles with density 1.16 g/cm^3 in the supernatant of ^{3}H-uridine labeled cells, the absence of RNA dependent DNA polymerase activity in the supernatant, and by the negative results of electron microscopic examination for virus particles (Coffin and Temin, 1971; Kotler, 1971; Fleissner, 1970). Nevertheless, at least some viral genes are expressed in these RSV transformed cell lines, since both intracellular viral specific RNA (Coffin and Temin, 1972) and viral antigen (Bauer and Janda, 1967; Huebner *et al.*, 1964) can be detected.

Although not produced by heterologous transformed cells alone, infectious virus can be "rescued" by fusion or co-cultivation of the cells with uninfected or infected chick cells (Hanafusa and Hanafusa, 1966; Sarma, Vass, and Huebner, 1966; Vigier, 1967). Neither the nature of the block to virus production nor of the rescue of virus has yet been elucidated.

Using an antiserum against avian myeloblastosis virus (AMV) structural proteins as a probe for intracellular viral proteins, we recently demonstrated that in productively infected chick cells, the main structural proteins of AMV and RSV are synthesized as a large polypeptide precursor of 76,000 MW (Vogt and Eisenman, 1973). The precursor molecule is proteolytically cleaved to yield at least the three major viral group specific proteins (Vogt and Eisenman, 1973, and unpublished results). This paper extends the investigation of intracellular oncornaviral proteins to an RSV transformed cell line (H-RSV(BH), ref. 11) derived from a tumor in hamsters infected with the Bryan High Titer strain of RSV. We show that in these cells the 76,000 MW precursor polypeptide is also present, but that it is completely stable and that no virion proteins are formed.

METHODS AND MATERIALS

Cells and Virus

H-RSV(BH) cells, a line of hamster cells transformed *in vivo* by the Brian High Titer strain of RSV (Sarma, Vass, and Huebner, 1966) were obtained from Erwin Fleissner and grown as monolayers in Dulbecco's modified Eagle's medium with 7.5% tryptose broth and 7.5% fetal calf serum. Chick embryo fibroblast cells and labeled AMV were prepared as described previously (Vogt and Eisenman, 1973).

Labeling and Lysis of Cells and Immune Precipitation

The procedure for preparing labeled precursor protein has been described previously (Vogt and Eisenman, 1973). Briefly, cells were washed with phosphate buffered saline and pulsed for 10 minutes with ^{35}S methionine (25 μC or 250 μC) in 1.5 ml Earle's saline at 37°C. Cells were lysed in the cold by adding 1 ml of 0.02 M Tris-HCl (pH 7.5), 0.05 M NaCl, 0.5% NP40, 0.5% sodium deoxycholate. The lysed cells were scraped off the plate, vortexed for 30 seconds and centrifuged for 10 minutes at 10,000 x g. To the pulse labeled supernatant were added 3 μg of unlabeled AMV and 0.1 ml of anti-AMV rabbit serum. The antiserum was prepared by repeated bimonthly injections of 0.5 mg of NP40-disrupted AMV mixed with complete Freund's adjuvant. The antibody treated cell cytoplasm was incubated for 16 hours at 4°C and the immune precipitate washed in lysis buffer, dissolved in electrophoresis buffer containing 5% SDS, and applied to a 15% polyacrylamide slab gel.

RESULTS

In order to determine whether H-RSV(BH) cells synthesize a high molecular weight precursor protein similar to that found in RSV and AMV infected chick cells, we pulse labeled the transformed hamster cells with ^{35}S methionine. After lysis of cells with an NP40-deoxycholate detergent mixture, the cytoplasm was incubated with antiserum against virion proteins. The resulting precipitate was dissolved in dodecylsulfate buffer and electrophoresed on a polyacrylamide-dodecyl sulfate slab gel. Figure 1 shows the autoradiograph of such a gel containing immune precipitates from H-RSV(BH) cells, AMV infected chick cells, and uninfected hamster cells (BHK). Column G in the figure is the characteristic pattern from pulse labeled AMV infected chick cells. The band marked Pr 76 is the 76,000 MW precursor reported previously (Vogt and Eisenman, 1973). Pr 66 and Pr 55 are intermediate cleavage products of Pr 76, as indicated by kinetic and fingerprinting analysis (unpublished observations). H 45 corresponds to a protein also precipitated from non-infected chick cells (Vogt and Eisenman, 1973). The polypeptides of purified ^{35}S-methionine labeled AMV are depicted for comparison in Figure 1F. At least those of molecular weight 24,000, 19,000, and 11,000 are derived from the 76,000 MW precursor (Vogt and Eisenman, 1973, and unpublished results).

The pattern of proteins from an extract of the pulse labeled transformed hamster cells is shown in Figure 1B. In addition to a polypeptide migrating at the same position as the infected chick cell protein marked H-45, the transformed hamster cells contain an immune precipitable polypeptide with exactly the same mobility as the AMV precursor protein. As illustrated in Figure 1A, no

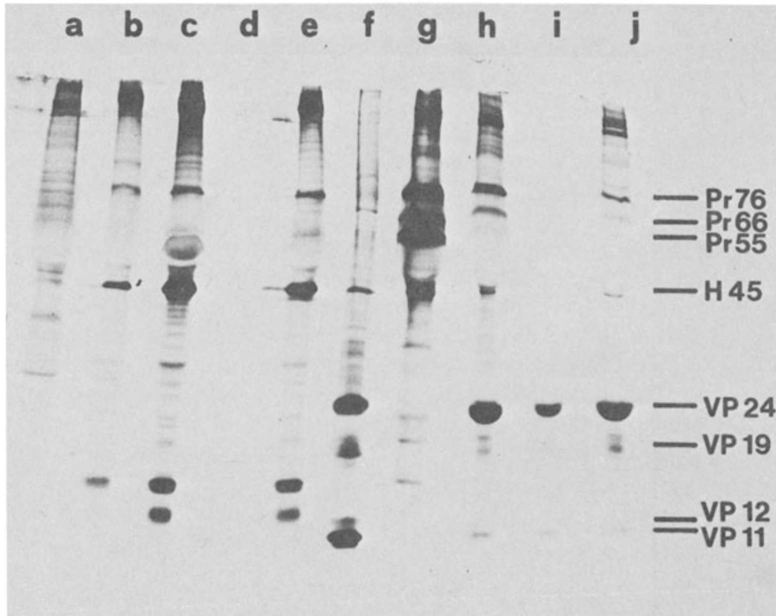

FIG. 1. Autoradiograph of SDS gel electrophoresis of immune precipitates. Plates of AMV-infected chick cells and untransformed (BHK) and RSV transformed (H-RSV(BH)) hamster cells were pulse-labeled for 10 minutes with 25 μC, 250 μC, and 250 μC ^{35}S-methionine, respectively. The cells were either lysed immediately as described in Methods, or incubated in complete medium for another one or three hours and then lysed. Immune precipitates from the cytoplasmic fractions of these cells were dissolved in dodecyl sulfate buffer and then electrophoresed on a 15% polyacrylamide slab gel. The medium from the three hour chase was centrifuged at 80,000 x g for 30 minutes, and the resulting pellet redissolved in lysis buffer and then immune precipitated in parallel with the cytoplasmic fractions.

A. BHK cells, pulse.
B. H-RSV(BH) cells, pulse.
C. H-RSV(BH) cells, pulse and 1 hour chase.
D. Particulate fraction from medium of H-RSV(BH) cells, 3 hour chase.
E. H-RSV(BH) cells, pulse and 3 hour chase.
F. Purified ^{35}S-methionine AMV (not antibody precipitated).
G. AMV-infected chick cells, pulse.
H. AMV-infected chick cells, pulse and 1 hour chase.
I. Particulate fraction from medium of chick cells, 3 hour chase.
J. AMV-infected chick cells, pulse and 3 hour chase.

labeled protein of this molecular weight can be detected in immune precipitates of extracts of BHK, a hamster cell line not infected with RSV, suggesting that this is the precursor protein for RSV.

To demonstrate its viral origin more directly, we recovered the ^{35}S-methionine labeled 76,000 MW hamster cell protein from a gel and digested it with trypsin. The resulting peptides were separated by cation exchange chromatography (Chromo Beads P, Technicon) together with a mixture of tryptic digests of the four ^{3}H-methionine labeled purified AMV virion proteins of molecular weights 24,000, 19,000, 12,000 and 11,000. All major methionine containing tryptic peptides from the four proteins are resolved by this procedure. The elution profiles of ^{35}S and ^{3}H from this column showed striking similarity, with 5-7 of the ten identifiable ^{3}H-peaks coinciding with ^{35}S-peaks (data not shown), thus indicating that the precursor and AMV proteins are related. However, there were several differences which are not seen in similar tryptic chromatographs of the ^{35}S-methionine AMV precursor and ^{3}H-methionine AMV peptides. The differences may result from a non-identity of AMV and RSV structural proteins. Although the two viruses share the determinants of their group specific antigens (Bauer and Janda, 1967; Armstrong, 1969; Roth and Dougherty, 1969), it is not known whether these proteins differ in amino acid sequence from one virus to another. We are currently comparing fingerprints of the H-RSV(BH) precursor with those of RSV structural proteins.

Hamster cells contain a much smaller quantity of immune precipitable pulse label in the precursor polypeptide than do AMV infected chick cells. Although the H-RSV(BH) cells incorporated 7 times as much ^{35}S-methionine as the chick cells, comparison of columns B and G in Figure 1 indicates that there is still less radioactivity in the hamster than the chick precursor band. To quantitate this difference, the proteins in these bands from a parallel gel were eluted, counted for radioactivity, and calculated as percentage of total labeled protein. While the recovered AMV precursor represented 0.2% of total pulse labeled protein, the H-RSV(BH) precursor represented only 0.01-0.02%.

Figure 1 also shows that the bands at 66,000 MW and 55,000 MW found in the pulse labeled AMV infected chick cells (G) are absent from the H-RSV(BH) cells (B). These proteins are intermediate cleavage products derived from the large precursor (unpublished results). The fact that they are not found in the hamster cells suggested a lack of cleavage of the precursor protein into virion protein.

To test this idea we pulse-labeled H-RSV(BH) cells as before and then continued to incubate the cells with unlabeled methionine-

containing medium for 1 hour or 3 hours before lysis and antibody precipitation. The results of such an experiment, along with a similar pulse-chase of AMV infected cells, are shown in Figure 1, panels C, E, H, and J. While after 1 hour the majority, and after 3 hours almost all of the radioactivity in the AMV precursor is chased into virion polypeptides (H and J, as shown by comparison with a parallel AMV marker in F), the H-RSV(BH) 76,000 MW polypeptide remains stable even after 3 hours incubation (C, E). Antibody precipitation of a particulate fraction from the medium verified that the chased chick cells released labeled virions (I), but that the hamster cells did not (D). Furthermore, no intracellular hamster cell polypeptides the size of virion proteins appear in the immune precipitates. Three low molecular weight bands do increase in the chase, but these do not correspond to RSV proteins, which coelectrophorese with those of AMV. Thus it appears that the RSV precursor polypeptide in H-RSV(BH) cells is not processed intracellularly to give virion proteins. The labeling techniques used would not exclude cleavage of a small fraction of the precursor, however.

Additional evidence for the stability of the H-RSV(BH) precursor stems from Coomasie Blue staining of the slab gels before autoradiography. Although it is highly labeled, the AMV-infected chick cell precursor is never observed as a stained protein, presumably because it is rapidly cleaved. The precursor from the hamster cells, on the other hand, shows a clearly discernible stained band superimposable over the labeled band seen in autoradiography.

The origins of the several highly labeled proteins (MV > 100,000, 45,000, 16,000, and 14,000) other than the viral precursor visible in Figure 1, panels B, C, and E are unclear. We have considered the possibilities that the smaller of these are aberrant degradation products of the 76,000 MW precursor and that this latter itself is derived from the >100,000 MW protein. However, preliminary evidence from tryptic fingerprinting of methionine labeled peptides on cation exchange chromatography indicates that neither the >100,000 nor the 16,000 MW protein shares significant sequence homology with any of the four major virion proteins. The hamster cell bands could be host proteins that bind to the immune precipitate non-specifically, or they could be viral specific proteins present in only minor amounts in virions.

Taken together, these results indicate that cells of this RSV transformed line are capable of synthesizing the polypeptide which is precursor to RSV structural proteins, but are incapable of generating these proteins by subsequent proteolytic cleavage.

DISCUSSION

Although heterologous cells transformed by RSV generally do not produce virus, they do synthesize small quantities of viral specific proteins (Bauer and Janda, 1967; Huebner *et al.*, 1964). The hamster cell line used in this study, H-RSV(BH), is reported to contain viral antigen as measured both by fluorescent antibody staining and complement fixation (Fleissner, 1970). Identical results are obtained with either antisera from hamsters bearing RSV-induced tumors, or rabbit antisera to disrupted AMV (Fleissner, 1970). We have demonstrated here that the viral specific antigen is not present in virion proteins as expected, but rather in the form of a polypeptide precursor to these proteins having the same antigenic determinants as the virion proteins. Unlike the AMV and RSV precursor in infected permissive (chick) cells (Vogt and Eisenman, 1973), which has a half life of about one half hour (unpublished results), the precursor in this line of hamster cells is stable. The lack of cleavage of the precursor raises the possibility that it is the inability of the cells to process this polypeptide that constitutes the block to virus production.

At least two kinds of models explain the stability of the precursor in H-RSV(BH) cells. The cells might simply lack the specific proteolytic activity that in chick cells generates virion polypeptides from the higher molecular weight protein. Alternatively, in hamster cells the configuration or location of the precursor might make cleavage impossible. The latter model is plausible since in AMV infected chick cells the precursor polypeptide as well as the proteolytic activity necessary to cleave it appear to be bound to a membrane fraction (unpublished results). It has also been demonstrated that in H-RSV(BH) cells the viral specific antigen is membrane associated (Fleissner, 1970). Either model is consistent with the finding that in virus rescue after fusion of heterologous RSV transformed cells with chick cells, it is the latter that donate a function essential for virus production (Coffin, 1972).

It remains to be seen whether the inability to process avian oncornavirus precursor polypeptide is characteristic of heterologous transformed cells in general. If it is, this would lend support to the notion that the block to virus production is due to the failure of this post-translational modification step.

ACKNOWLEDGEMENTS

We are grateful to Tom MacPherson for technical assistance, to Erwin Fleissner for his gift of the H-RSV(BH) cell line, and to J. W. Beard and The American National Cancer Institute for leukemic

chicken plasma. We thank Heidi Diggelmann and Bernhard Hirt for encouragement and criticism. This work was supported by a post-doctoral fellowship from the European Molecular Biology Organization (R.E.) and by Grant No 3.826.72 from the Fonds National Suisse.

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COMPARATIVE STUDIES ON THE MOLECULAR BASIS FOR A VIRAL INVOLVEMENT IN MURINE AND HUMAN MAMMARY ADENOCARCINOMA

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The etiologic role of RNA tumor viruses in a variety of cancers in animals is well established. In many instances proof of causation has been achieved by the inoculation of purified cell-free virus preparations in experiments that satisfy Koch's postulates. Indirect evidence has been accumulating during the last decade that RNA tumor viruses similar to the mouse mammary tumor virus (MMTV) may be involved in the etiology of human breast cancer.

Particles morphologically similar to known RNA tumor viruses have been observed with the electron microscope in samples of human milk (Gross, Gessler, and McCarty, 1950; Feller and Chopra, 1969; Moore *et al.*, 1971) and in tissue from biopsies of human breast tumors (Dmochowski *et al.*, 1968).

Particles from human milk contain the biochemical features of the known RNA tumor viruses, i.e.: 1) These particles have a buoyant density in sucrose of 1.16-1.19 g/ml (Schlom, Spiegelman, and Moore, 1971). 2) They contain a single-stranded 70S RNA (Schlom, Spiegelman, and Moore, 1972b). 3) This 70S RNA contains a poly rA sequence of about 200 nucleotides long (Schlom et al., 1972a). 4) These particles possess a RNA directed DNA polymerase (reverse transcriptase) (Schlom et al., 1972b). 5) These particles appear to infect and replicate in human embryo fibroblast cells (Keydar et al., 1973). Human mammary adenocarcinomas have also been shown to contain particles with characteristics of the known RNA tumor viruses, i.e., these particles have a buoyant density of 1.16-1.19 g/ml, contain a single-stranded 70S RNA and a RNA directed DNA polymerase (Axel, Gulati, and Spiegelman, 1972). The RNA extracted from human mammary adenocarcinoma hybridizes specifically to a radioactively labeled DNA complementary to the 70S RNA of MMTV (Axel, Schlom, and Spiegelman, 1973).

A homology between the radioactively labeled DNA synthesized from the RNA of human milk particles and RNA from 3 out of 9 human mammary adenocarcinomas has been reported (Das et al., 1972). The observation of related particles found in milk of normal women and in malignant breast tumors is in striking similarity to that of the murine model (Spiegelman, Axel, and Schlom, 1972; Schlom and Spiegelman, 1973).

We describe here an additional characterization of the virus-like particles from human milk by the isolation of the cores from these particles. We also confirm and extend the observation of homology in nucleic acid sequences between the virus-like particles from human milk and from human mammary adenocarcinoma. In both cases we describe the analogous experiments performed on the mouse model systems: the isolation of cores from MMTV from mouse milk and the nucleic acid homology between virus particles from mouse milk and mouse mammary tumors.

MATERIALS AND METHODS

Simultaneous Detection Test

Concentrated "viral" or "core" preparations, resuspended in 0.2% NP 40 and 0.1 M dithiothreitol (60 μl volume), are kept at 4°C for 10 minutes. Actinomycin D is added to a final concentration of 100 μg/ml and oligo $(dT)_{12-18}$, functioning as a primer, is added to a final concentration of 0.32 mg/ml. This suspension is then added to a standard simultaneous detection (Schlom and Spiegelman, 1971) reaction mixture (125 μl final volume) containing

6.25 μmol of Tris HCl (pH 8.3), 1 μmol of $MgCl_2$, 1.25 μmol of NaCl, 0.2 μmol each of unlabeled deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and 0.02 μmol of (^3H) deoxythymidine triphosphate (New England Nuclear Corp.) to a final specific activity of 24,000 cpm per picamole. After a 15 minute incubation at 37°C, the reaction is terminated by the addition of NaCl and sodium dodecylsulfate to final concentrations of 0.2 M and 1% respectively. After addition of an equal volume of chloroform phenol-cresol (8:7:1) mixture containing 8-hydroxyquinoline (0.185 g per 100 ml of mixture), the mixture is shaken at 25°C for 15 seconds (Vortex) and centrifuged at 3000 X g for 5 min at 25°. The aqueous phase is then layered over a linear glycerol gradient (10-30% in TNE) and centrifuged at 40,000 rpm for 3 hours at 4°C (Spinco SW-41 rotor). 28S and 18S (^3H) RNA from NC-37 cells are used as external size markers. Fractions are collected from below, and portions of each fraction were assayed for acid precipitable radioactivity (Spiegelman et al., 1970).

Synthesis of a Tritium Labeled DNA Probe

Mouse mammary tumor virus tritiated (^3H) DNA probe was synthesized via the simultaneous detection technique (Schlom and Spiegelman, 1971) as described in legends of Figure 1 with omission of the primer oligo dT_{12-18}. The 60-70S region of the glycerol gradient was pooled, precipitated by the addition of 0.4 M NaCl and yeast RNA carrier (15 μg/ml final concentration). After storage at -20°C overnight, and centrifugation at 16,000 x g for 20 min at -10°C, the resulting pellet was resuspended in 100 μl of 0.002 M EDTA and 10 μl of 4 N NaOH. After incubation at 37°C for 16 hours, 10 μl each of 4 N HCl and 1 M Tris buffer, pH 7.5 was added. The ^3H-DNA product, freed of protein and RNA, was examined by equilibrium centrifugation in Cs_2SO_4. The ^3H-DNA probe solely banded in the DNA region of this gradient.

Preparation of Polysomal RNA

For the preparation of polysomal RNA, approximately 2 grams of tissue were first finely minced with scissors and then disrupted with a Silverson homogenizer at 4°C in 25 ml of TNM buffer (0.01 M Tris, pH 7.4, 0.15 M NaCl, 0.002 M $MgCl_2$) containing 5% sucrose. The suspension was centrifuged at 12,000 rpm (Sorvall RC-2B) for 40 min at 0°C to remove nuclei and heavy membranes. The resulting supernatant fluid was layered over 12 ml of 25% sucrose in TNM and centrifuged at 45,000 rpm for 3 hours at 4°C (Spinco 60 Ti rotor). The resulting pellet was resuspended in 1 ml TNE containing 1% SDS, and the RNA was extracted with an equal volume of phenol cresol, pH 8.3. After centrifugation at 8,000 rpm for 10 minutes

at room temperature, the aqueous phase was re-extracted with phenol-cresol and re-centrifuged. The RNA in the final aqueous phase was precipitated by the addition of 0.4 M LiCl and 2 volumes of ethanol. After storage at -20°C overnight and centrifugation at 10,000 rpm at -10°C for 30 min, the resulting pellet was resuspended in 50 µl of FE mix (50% formamide, 50% 0.003 M EDTA, pH 8.3).

Hybridization Reactions

1,200 cpm of ^{3}H-MMTV-DNA in 1.5 µl, 8 µl of formamide, and 10 µl of E. coli DNA (10 µg/ml in FE mix) were mixed and heated at 80°C for 10 minutes to denature the ^{3}H-MMTV-DNA and then placed at 4°C. 200 µg of RNA in FE mix were added to give a final reaction volume of 80 µl containing 50% formamide and 0.4 M NaCl. Tubes were sealed and incubated at 37°C for 16 hours, after which 11 ml of Cs_2SO_4 at 50% saturation in 0.003 M EDTA (density 1.52 g/ml) were added; the resulting solution was centrifuged at 44,000 rpm for 60 hr at 15°C in a 50 Ti rotor (Spinco). Fractions (0.4 ml) were collected through a needle inserted in the bottom of the tube and assayed for acid precipitable radioactivity. These hybridizations were performed to a C_0t value (i.e., molar phosphate X sec X liter^{-1}) of 400.

Analysis of Hybridization Reaction

Hybridization reactions of tritiated MMTV-DNA to cellular RNA were scored as "positive" or "negative" by the amounts of tritiated DNA hybridized to the larger cellular RNA molecules and thus found in the RNA region after Cs_2SO_4 equilibrium gradient analysis. To achieve the accuracy desired, 10 minute counts (cp10m) of gradient fractions were taken after correction for background counts. The sum of the tritium counts in the RNA density region (1.63-1.68 g/ml) were used to measure the amount of DNA complexed to RNA. An operational mean background and its standard deviation were empirically determined by the total cp10m count of 3 tubes in the negative regions (tubes 2, 3, 4) of each of 50 gradients. All specimens showing cp10m of less than 3 standard deviations in the RNA density region were considered negative. This procedure ensures that all samples scored as "positive," i.e., with greater than 3 sd in the RNA region of the gradient, provide a 99.9% confidence and better that they are statistically significant.

RESULTS AND DISCUSSION

The Isolation of Cores from the Mouse Mammary Tumor Virus

Cores from avian tumor viruses (Stromberg, 1972; Bader, Brown, and Bader, 1972; Bolognesi *et al.*, 1972; Coffin and Temin, 1971), murine leukemia (O'Connor *et al.*, 1966; Gerwin *et al.*, 1970; Fink *et al.*, 1969; Lang *et al.*, 1973), and murine mammary tumor viruses (MTV) (Sarkar, Nowinski, and Moore, 1972; Hageman, Calafat, and Daams, 1973; Calafat and Hageman, 1969) have been produced by the use of surfactants, ether, and phospholipase. These cores contain the viral 60-70S RNA (Bolognesi *et al.*, 1972) and a DNA polymerase activity (Stromberg, 1972; Bader *et al.*, 1972; Bolognesi *et al.*, 1972; Coffin and Temin, 1971; Gerwin *et al.*, 1970). Cores were isolated from MMTV (Feldman, Schlom, and Spiegelman, 1973) using phospholipase C (EC 3.14.3).

Mouse mammary tumor virus from 10 ml RIII mouse milk was treated with phospholipase C-ether as described in the legend of Figure 1. The preparation was then layered on a 25-50% linear sucrose gradient and centrifuged at 98,000 X g for 16 hr at 4°. Each fraction was assayed for endogenous DNA polymerase activity in the presence of actinomycin D (100 μg/ml).

As can be seen in Figure 1, the DNA polymerase activity after MMTV core isolation is located in the fraction with a density of 1.23 g/ml. No activity could be observed in the "viral" fraction of the gradient with a density of 1.16-1.19 g/ml. Figure 2 shows the application of the simultaneous detection test to mouse mammary tumor virus cores with a buoyant density of 1.23 g/ml. A clear distinct peak of TCA precipitable ^{3}H-TTP associated with 70S RNA was obtained.

The Isolation of Cores from Human Milk

Ten to fifteen individual samples of human milk (10-100 ml each) were pooled and mixed with one quarter volume of 0.5 M EDTA (pH 8.3) and centrifuged at 2500 X g for 10 min at 4°. The clear milk-plasma zone was removed, mixed with glycerol to a final concentration of 20%, and centrifuged at 30,000 rpm for two hours at 4° Spinco Ti 15-batch rotor. The resulting supernatant fluid was aspirated and discarded, and the outer wall of the rotor was gently scraped with a spatula and washed with 0.01 M Tris HCl (pH 8.3) to remove pelleted particles. This resuspended pellet, in 0.01 M Tris HCl pH 8.3, was layered over a preformed 20-70% sucrose gradient in TNE and subjected to equilibrium centrifugation. Half of each of the resulting fractions was assayed for the presence of

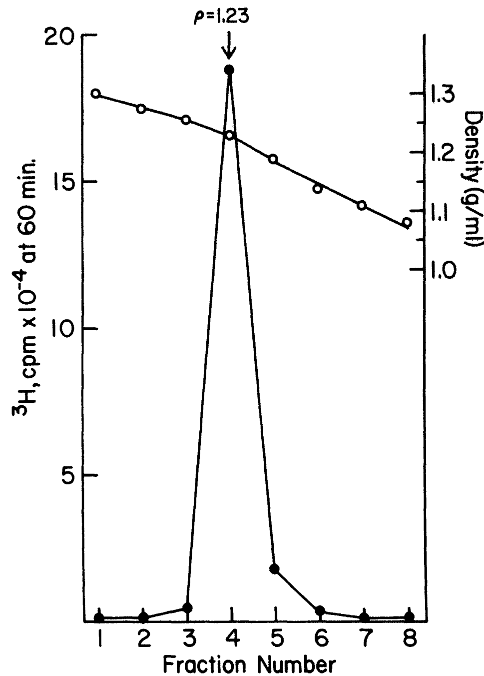

FIG. 1. Production of cores from mouse mammary tumor virus.

In concentrating viruses from milk it is desirable first to remove the casein and lipid components, a step readily accomplished by adding an equal volume of 0.15 M EDTA followed by centrifugation at 8000 g for 10 minutes at 4°C. The middle clear "milk-plasma" layer is then separated from the top lipid layer and the precipitated casein. The virions are then concentrated by centrifugation at 98,000 g for 60 min at 4°C through a column of 20% glycerol in TNE (.01 M Tris, HCl pH 8.3- 15 M NaCl -0.002 M EDTA, ethylenediethyltetraacetate) on a cushion of 100% glycerol. The viral material on top of the 100% glycerol layer is resuspended in 0.01 M Tris HCl pH 7.3. 250 μl of 1 M dithiothreitol is mixed with an equal volume of polyvinyl sulfate (1 mg/ml) in a 15 ml of Corex tube at 4°C. 1 ml of purified murine milk concentrate is then added to the tube followed immediately by 2 μl of phospholipase C (Sigma Chemicals Type 1; 10 mg/ml in 1% bovine serum albumin), giving a final concentration of 0.01 mg/ml. Phospholipase C was stored as a dry powder at 4°. The mixture is placed in a 37° water bath, swirled gently for 30 seconds, kept at 25° for 10 min with gentle swirling for 10 seconds every 2 minutes, and then placed in an ice bath. 1 ml of cold ether (Fisher, Anhydrous) is added, and the tube is swirled gently for 10-20 seconds to allow mixing. The resulting emulsion is then centrifuged at 1500 X g for 10 minutes at 4°C. A lower aqueous phase, an upper ether phase, and an interphase that contained some gelatinous material resulted. The

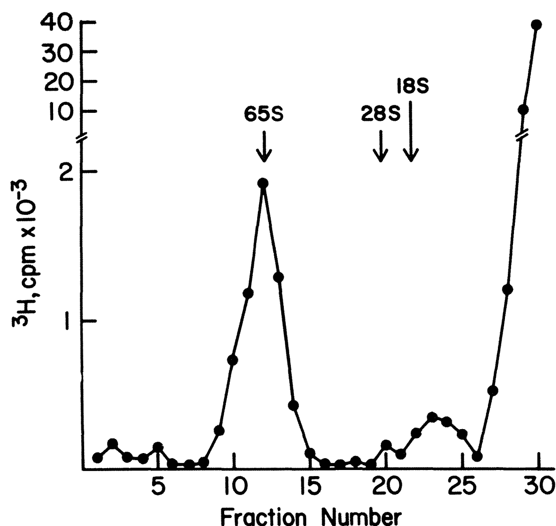

FIG. 2. Application of simultaneous detection test to mouse mammary tumor virus cores. Mouse mammary tumor virus cores were prepared as described in the legend of Figure 1. Particles banding at a density of 1.23-1.24 g/ml were assayed by the simultaneous detection test for (^{3}H) DNA 60-70S RNA complexes.

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lower aqueous phase is removed with a Pasteur pipette and transferred to a 20 ml Corex tube at 4°. A stream of nitrogen is blown over the surface for 1-2 min to aid removal of ether. The resulting solution is layered on a 25-65% linear sucrose gradient (in TNE buffer) and centrifuged for 16 hr at 40,000 rpm at 4°C (Spinco SW-41 rotor). All sucrose solutions are prepared on a weight/weight basis. 10 fractions of equal volume are collected from below, and the density of each fraction was determined (Zeiss refractometer). Each fraction is then diluted in 0.01 M Tris - HCl (pH 9.3), pelleted at 98,000 X g for 30 minutes, and resuspended in 60 μl of 0.01 M Tris HCl (pH 8.3) containing 0.2% NP-40 (a Shell nonionic detergent) and 0.1 M dithiothreitol for simultaneous detection analysis or endogenous DNA polymerase activity. All reagents are dissolved in 0.01 M Tris HCl (pH 8.3) unless otherwise specified.

a 70S RNA associated DNA polymerase (Schlom and Spiegelman, 1971). As can be seen in Figure 3C, particles with a 70S RNA associated DNA polymerase activity can be observed in the fraction with a density of 1.14-1.20 g/ml. The other half of this fraction was treated with phospholipase C as described above and then subjected to equilibrium centrifugation in a preformed 20-70% sucrose gradient in TNE. The assay for a particle with a 70S RNA associated DNA polymerase in the resulting fractions of this equilibrium gradient reveals (Figure 3E) these particles in a fraction with a density of 1.26-1.27 g/ml. No DNA polymerase activity is now found, moreover, in the "viral" region of the gradient with a density of 1.14-1.20 g/ml.

If the core preparation of human milk is pretreated with RNase A (Sigma) or if one of the ribodeoxynucleotide triphosphates is omitted in the reaction mixture of the simultaneous detection test, no TCA precipitable ^{3}H-TTP could be found associated with 70S RNA (Table 1). This assures the 70S RNA associated DNA polymerase found in the core particles to be a true reverse transcriptase. Thus, using phospholipase C, cores with a density of 1.26-1.27 g/ml can be isolated from human milk particles. These cores contain a reverse transcriptase and a 70S RNA.

TABLE 1. Properties of the RNA-directed polymerase of cores from human milk particles

	(^{3}H) cpm in 60-70S Region	Percent of Complete Reaction
Standard reaction mixture	872	100
plus RNase (25 μg/ml)	155	18
minus dATP	125	14

Cores from 80 ml of human milk were purified and concentrated as described. The core pellet was resuspended in three 60 μl aliquots containing 0.01 M Tris HCl, 0.2% NP-40, and 0.1 M dithiothreitol. The first aliquot was tested by the standard simultaneous detection test as described (Figure 1). The second aliquot was pretreated with RNase A (sigma) at a concentration of 25 μl/ml. Deoxyadenosine triphosphate was omitted from the reaction mixture of the third aliquot. (^{3}H) cpm in the 60-70S region of the resulting glycerol gradients is given.

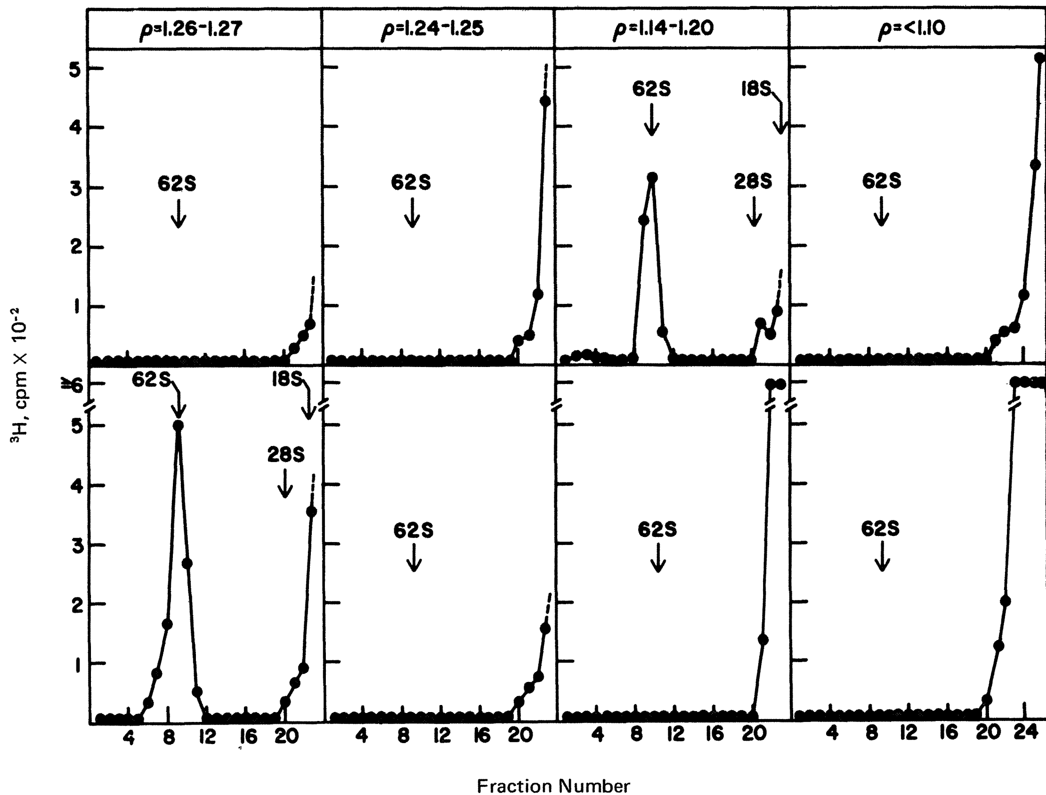

FIG. 3. Conversion of simultaneous detection activity in human milk from "viral" to "core" density region. Several human milk samples were pooled, and particles were concentrated as described. 2 ml of milk pool concentrate, representing 160 ml of starting milk, were layered over a 25-65% linear sucrose gradient and centrifuged for 4 hours at 98,000 X g at 4°C (Spinco SW-27). Fractions were collected from below, and the density of each fraction was taken. One-half of each of four density regions--1.26-1.27 (A), 1.24-1.25 (B), 1.14-1.20 (C), and 1.10 g/ml (D)--were diluted in 0.01 M Tris HCl, pelleted at 98,000 X g for 30 min at 4°, and assayed by the simultaneous detection test. The other half of the 1.14-1.20 g/ml density region (C) was diluted 0.01 M Tris HCl, layered over 6 ml of 100% glycerol (Spinco SW-27), and centrifuged at 97,000 X g for 30 min at 4°. The material on top of the 100% glycerol cushion was treated with phospholipase C-ether as described. The resulting aqueous phase was layered over a 25-65% linear sucrose gradient and centrifuged at 98,000 X g for 16 hr at 4° (Spinco SW-41). The 1.26-1.27 (E), 1.24-1.25 (F), 1.14-1.20 (G), and 1.10 g/ml (H) density regions were then diluted in 0.01 M Tris HCl, pelleted at 98,000 X g for 30 min at 4°C and assayed by the simultaneous detection test.

MOLECULAR BASIS OF MURINE MAMMARY CARCINOMA

Murine mammary neoplasia has been shown to result from the interaction of hormones, a suitable genetic constitution, and a variety of agents belonging to the type B RNA tumor viruses. All these viruses have the same morphology but may differ in antigenicity, virulence, mode of transmission, pathology of induced tumors, or host range (Muhlbock and Bentvelzen, 1969; Bentvelzen and Daams, 1969; Bentvelzen, Daams, and Hageman, n.d.). By means of molecular hybridization, mouse mammary tumor virus-specific RNA can be detected in virus producing mammary tumors, nonvirus producing mammary tumors, and in different organs of mice of strains with a high and a low incidence of mammary tumors (Schlom _et al._, 1973).

Specificity of the Hybridization Reaction

A radioactive MMTV probe (MMTV ^{3}H-DNA) was synthesized from the 60-70S RNA of MMTV by means of the simultaneous detection test (Schlom and Spiegelman, 1971). The specificity of the hybridizations with MMTV ^{3}H-DNA is clearly demonstrated by the results shown in Figure 4. An evident shift of TCA precipitable tritium labeled DNA from the DNA region (1.43 g/ml) to RNA region (1.65 g/ml) of the Cs_2SO_4 gradient is observed if MMTV ^{3}H-DNA is annealed to RIII mammary tumor RNA, but not if this probe is annealed to NIH Suisse liver RNA or poly rA. This shows that the MMTV ^{3}H-DNA probe does not contain poly dT stretches copied from the poly rA stretches known to be present in RNA tumor viruses (Gillespie, Marshall, and Gallo, 1972) and in messenger RNA (Darnell _et al._, 1971).

With this single DNA probe, MMTV specific RNA is found in breast tumors of all mouse strains examined (Table 2), even of the low producer or nonproducer Balb/c and C57BL tumors. The further detection of viral-specific RNA in certain tissues of mice (Table 3) with no evidence of virus particles or malignancy may lead to an understanding of the pathogenesis and ultimately to a means of detection of incipient breast cancer in humans. MMTV-specific RNA can be detected in normal lactating breast tissue of the GR high mammary tumor strain and occasionally in the Balb/c strain (Table 2). This is of interest in view of the analogous observation that the milk of some normal women contains particles that have all the biochemical properties of RNA tumor viruses.

In mouse strains with a high incidence of mammary tumors as GR, MMTV-specific RNA is detectable in organs other than the mammary gland (Table 3). Previous biological studies (Hageman _et al._, 1973; Bentvelzen _et al._, n.d.; Smith, 1966; Parks _et al._, 1972) showed the presence of mouse mammary virus in these "normal" organs. This indicates that although MMTV-specific RNA can be detected in other

TABLE 2. MMTV-specific RNA in mouse mammary tumors and normal breast tissues

Organs	Strain	Result	SD*
Mammary Tumor	RIII	+	14.0
	GR	+	11.5
	C_3H	+	4.7
	C_3Hf C_{57}	+	7.2
	Balb/c	+	5.5
	$C_{57}BL$	+	3.2
Normal Breast Tissue	$C_{57}BL$	-	1.5
	Balb/c, lactating	1+/5-	6.8/1.4-2.0
	GR	+	12.6

*Standard deviation value in the RNA region of the Cs_2SO_4 gradient.

The hybridization is scored as positive if this value is greater than 3.0 (see Figure 4).

organs, the mouse mammary tumor virus transforms only the mammary gland tissue.

In mouse strains with a low incidence of mammary tumors as Balb/c, MMTV-specific RNA can be detected in the spleens and kidneys of mice 12-16 months old (Table 3), but not at the age of 3-6 months. This correlates with the appearance of mammary tumors late in life and indicates an influence of aging on the expression of mouse mammary tumor virus in this strain. In the other mouse strains with a low incidence of mammary tumors, C57BL, MMTV-specific RNA was conspicuously detected in the epididymis (Table 3).

Nucleic Acid Sequence Homology Between Virus Particles from Human Milk and Human Mammary Adenocarcinoma

Analogous to the molecular basis of murine mammary carcinoma, in which a homology in nucleic acid sequence between the RNA in virus particles from mouse milk and RNA from the mouse mammary tumor can be found (Figure 4), a homology can be found in nucleic acid sequences between the virus particles in the human milk and RNA from the human mammary adenocarcinoma (Das et al., 1972; Michalides, Schlom, and Spiegelman, no date). The tritium labeled DNA probe complementary to the RNA of the human milk particles is synthesized via an endogenous reaction using cores from the human milk particles (Feldman et al., 1973).

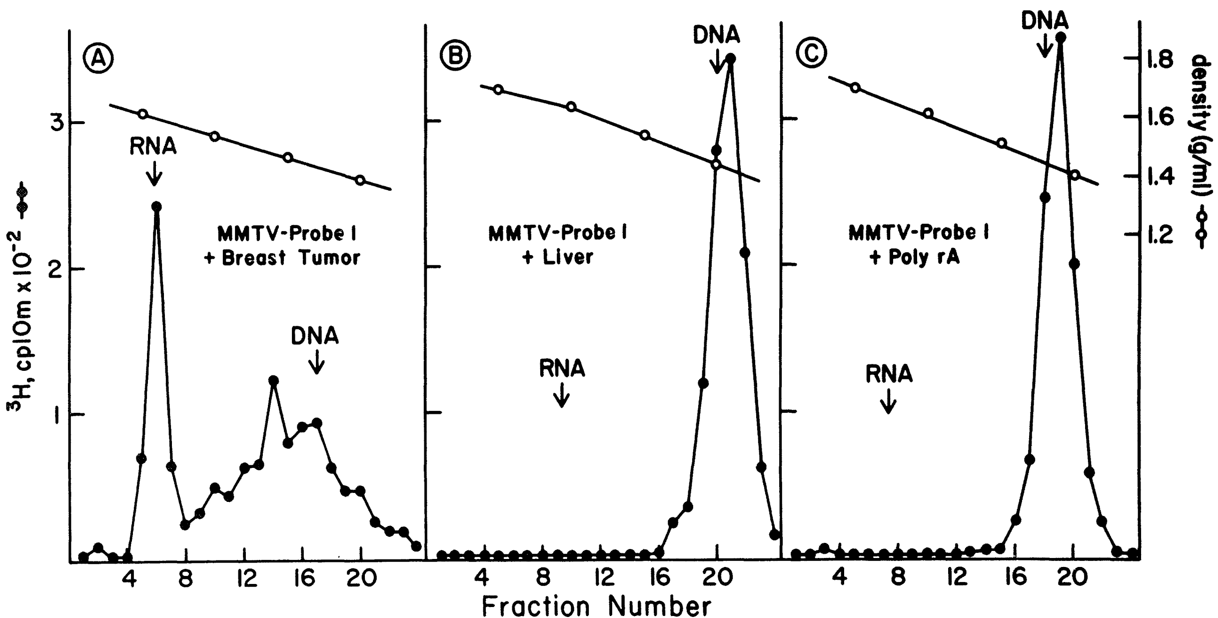

FIG. 4. Cs_2SO_4 equilibrium density gradient centrifugation of MMTV ^3H-DNA after annealing to (A) 200 μg RNA from RIII breast tumor, (B) 200 μg RNA from NIH swiss liver, (C) 5 μg polyadenylic acid.

TABLE 3. MMTV-specific RNA in normal mouse tissue

Strain	Organ	Age of mouse 3-6 months	12-16 months
Balb/c	Spleen	-(1.0)**	+(6.8)
	Kidney	-(0.0)	+(3.4)
	Epididymis	-(0.0)	NT
	Brain	-(0.8)	-(0.3)
	Liver	-(0.3)	-(1.0)
	Thymus	-(0.5)	-(1.9)
	Embryo (17 day)	-(0.7)	
$C_{57}BL$	Spleen	-(0.1)	-(0.7)*
	Kidney	-(0.9)	-(0.0)
	Epididymis	+(6.3)	NT
	Brain	-(0.0)	-(0.1)
	Liver	-(2.8)	-(0.6)
	Thymus	NT	-(0.6)
	Embryo (17 day)	-(0.7)	
GR	Spleen	+(3.9)	
	Kidney	+(6.5)	
	Epididymis	+(3.4)	

NT = Not tested

*One of seven $C_{57}BL$ spleen samples was positive (3.8)

**Standard deviation value in the RNA region of the Cs_2SO_4 gradient in parenthesis. The hybridization is scored positive if this value is greater than 3.0 (see Figure 4).

The ^{3}H-DNA product, freed of protein and RNA by phenol extraction and alkali treatment, was examined by equilibrium centrifugation in Cs_2SO_4 gradient. After this probe is annealed to poly rA and the hybridization is analyzed in a Cs_2SO_4 gradient, no TCA precipitable radioactivity is found in the RNA region.

If 1000 cpm of this probe were hybridized to 350 μg polysomal RNA of human mammary adenocarcinoma to a Cot value of 2500, a positive result was obtained (Figure 5) while annealing the same probe to polysomal RNA from human benign breast tumor revealed a negative result. In the case of human benign breast tumors, pools of 4 tumors were used for the RNA extraction.

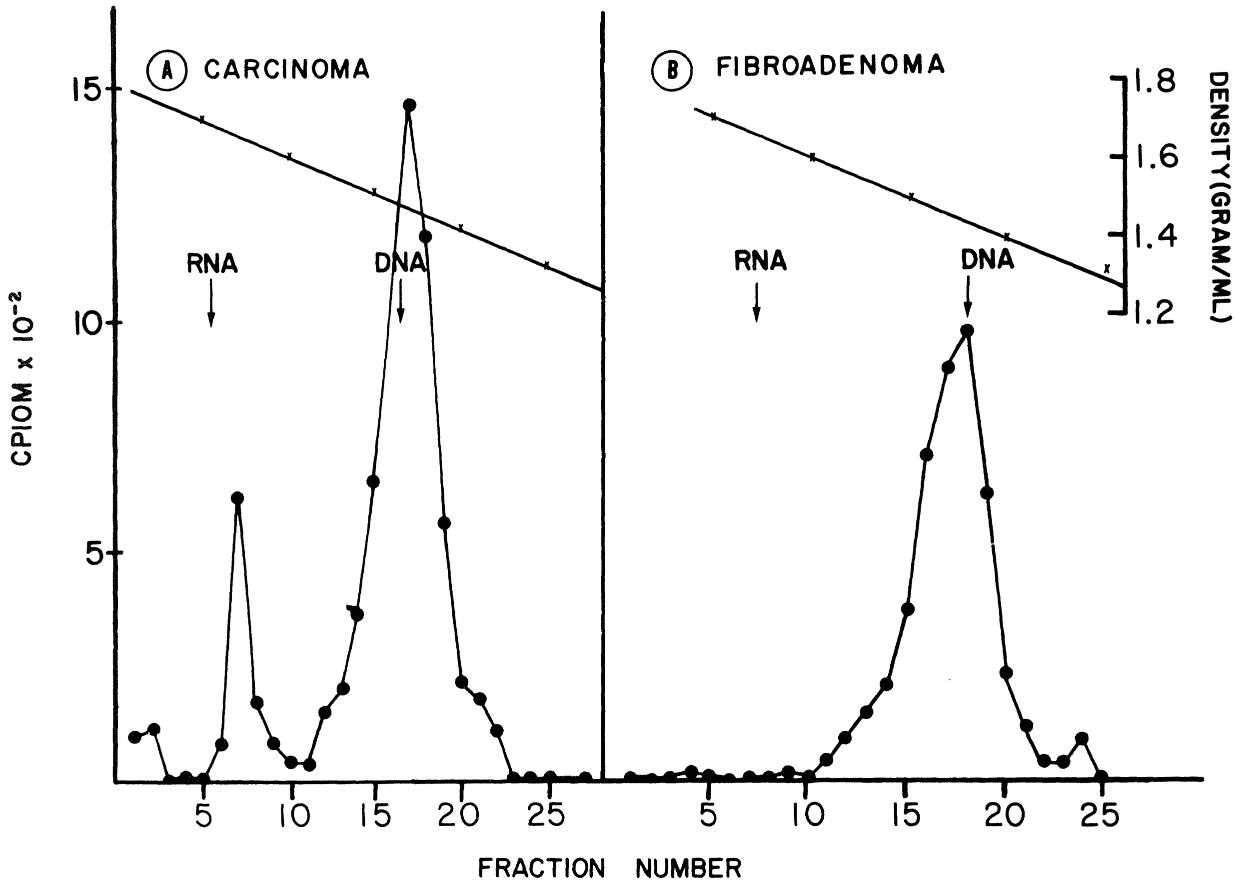

FIG. 5. Cs_2SO_4 equilibrium density gradient centrifugation of human milk ^{3}H-DNA after annealing to 350 μg polysomal RNA of (A) human mammary adenocarcinoma, (B) human mammary fibroadenoma.

The polysomal RNA of 7 out of 14 malignant human breast tumors hybridized significantly with the ^{3}H-DNA probe synthesized from the RNA of human milk cores with standard deviation values ranging from 3.0-11.0. No significant hybridization could be detected if the same probe was hybridized to the polysomal RNA of human benign breast tumors, human normal breast tissue, human sarcoma, and leukemic tumors and of "normal" human spleen.

Reasons why not all of the fourteen human malignant breast tumors tested were positive in the hybridization with a DNA probe synthesized from the RNA of human milk cores may be: (a) Different histologic types of human malignant breast tumors were used. (b) Only a portion of the tumor sample used for RNA extraction could actually be tumor cells. (c) Condition of the tumor, i.e., hemorrhagic, etc. (d) The low sensitivity of the test; the hybridizations were performed to a Cot value of 2500. (e) Only a certain percentage of breast tumors contain RNA related to these particles from human milk.

In a search for increasing the sensitivity of the hybridization the human milk core ^{3}H-DNA probe was hybridized to the RNA of different fractions of the human malignant breast tumor to a Cot value of 7000.

For that purpose human breast tumor tissue (25 g) was finely minced in ice. After adding 5% sucrose in TNE (2 ml per gram tissue) the tumor material was homogenized for 2 minutes using a Silverson homogenizer. The suspension was then centrifuged for 10 minutes at 3000 g at 4°C for 1 hour. The pellet was dissolved in 2 ml TNE and RNA was extracted as described above for polysomal RNA. This RNA preparation contained possible viral RNA and membrane associated RNA. This pellet sample was layered over a preformed 20-60% sucrose gradient in TNE in a SW 27 tube and spun at equilibrium for 6 hours at 98,000 g at 4°C. Fractions were collected from below, and fractions corresponding to densities 1.15-1.20 were pooled. The RNA was extracted from this as described above. This RNA preparation contained putative viral RNA and membrane associated RNA deriving from material with density properties of 1.15-1.20 g/ml. Figures 6 and 7A show that in this particular set of hybridizations only the RNA isolated from tumor preparation with density properties of 1.15-1.20 g/ml or "virus" region contains RNA homologous to the ^{3}H-DNA synthesized from the RNA of human milk cores.

The hybridizations between this same ^{3}H-DNA probe synthesized from the RNA of human milk cores and the RNA extracted from analogous fractions of human benign breast tumors (Figure 7B), leukemic and sarcoma tumors were negative with SD values of 0-1.2.

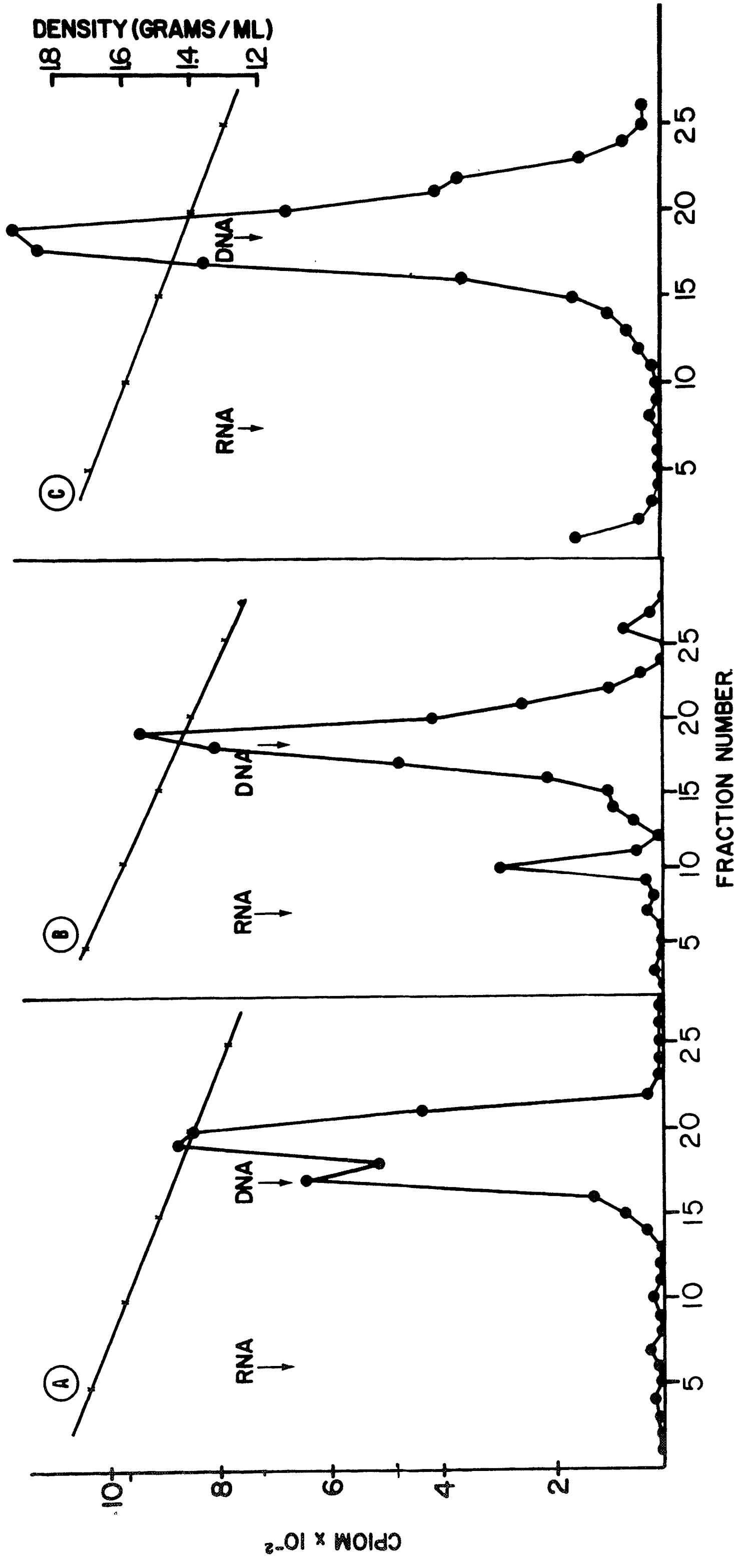

FIG. 6. Cs_2SO_4 equilibrium density gradient centrifugation of human milk ^{3}H-DNA after annealing to human breast carcinoma RNA from (A) pellet obtained after pelleting tumor homogenate through 20% glycerol, (B) tumor sample with density property of 1.15-1.20 g/ml, (C) polysomal pellet.

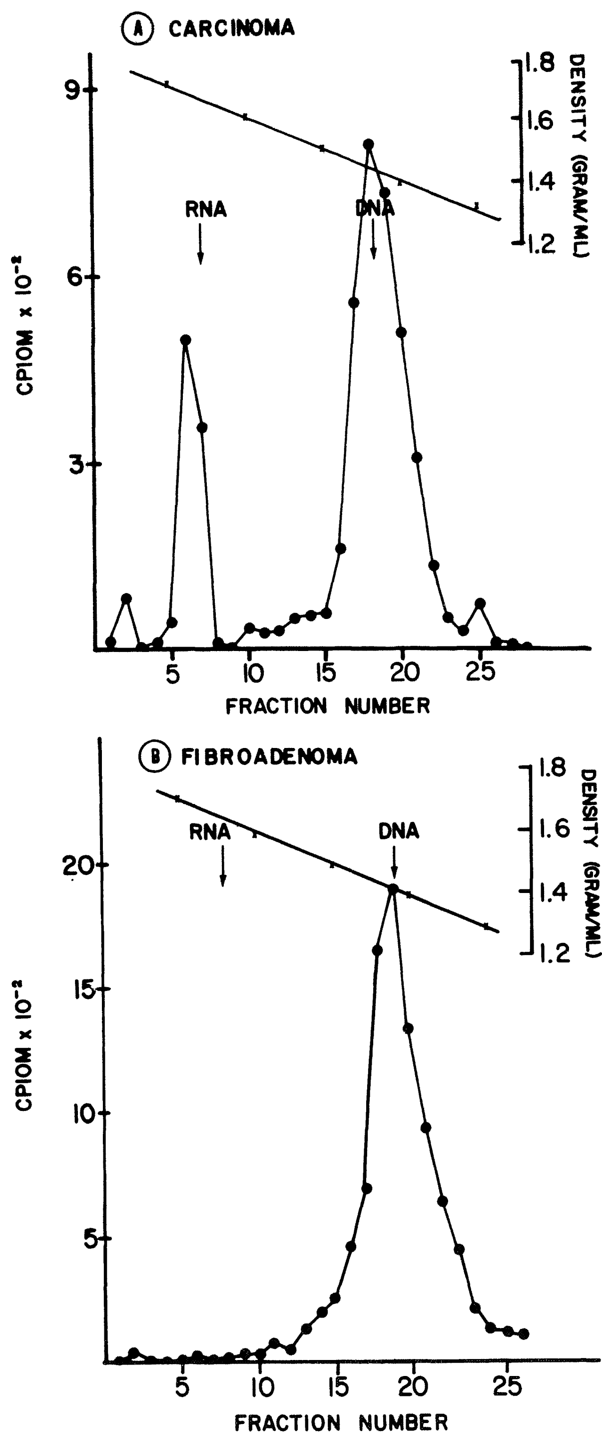

FIG. 7. Cs_2SO_4 equilibrium density gradient centrifugation of hybridization of human milk particles ^{3}H-DNA to human breast tumor RNA from (A) adenocarcinoma (B) fibroadenoma.

These data show that this ^3H-DNA probe synthesized from the RNA of human milk cores detects sequences in the RNA which are specific for malignant breast tumors. Because we did not estimate the extent of transcription of the RNA of the human milk particles into a ^3H-DNA probe, nor the extent of homology of this probe with the RNA from human malignant breast tumors, we can only indicate that there is a degree of homology in nucleic acid sequences between both. The possibility that a different, but closely related particle to the human milk particle is involved in human breast cancer is not ruled out by these studies.

The studies described here provide additional evidence implicating a viral involvement in human breast cancer. They also provide a comparable molecular basis for murine and human mammary adenocarcinoma.

ACKNOWLEDGEMENTS

Supported by the National Institutes of Health, the National Cancer Institute, Special Virus Cancer Program Contract NO 1-CP-43223 and 70-2049 and by Research Grant CA 02332. We also acknowledge the assistance of David Colcher, Janet Young, Jane Castrop, J. Greenfield, O. Posen, and V. D'Elia in these studies. We thank Dr. Michael Brennan and Dr. Marvin Rich of the Michigan Cancer Foundation for graciously providing human milk used in these studies.

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USE OF PSEUDOTYPES OF VESICULAR STOMATITIS VIRUS TO STUDY CELLULAR RESISTANCE TO MURINE RNA TUMOR VIRUSES

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ABSTRACT

Pseudotypes of vesicular stomatitis virus (VSV) genomes coated by the surface envelope from an N-tropic or a B-tropic tumor virus grow equally well in cells homozygous for either the *Fv-1^n* or *Fv-1^b* alleles. Therefore, the product of the *Fv-1* locus, which restricts growth of murine RNA tumor viruses, must act on an intracellular aspect of tumor virus replication, a step following attachment and penetration.

Hartley and colleagues (Hartley, Rowe, and Huebner, 1970) and Lilly (1967) described a major genetic locus of resistance in mice to RNA tumor viruses. This locus, designated Fv-1, maps on chromosome 4 (linkage group VIII) (Rowe, Humphrey, and Lilly, 1973). It has two alleles, Fv-1^n and Fv-1^b. When mice are homozygous for the Fv-1^n allele they are permissive for N-tropic RNA tumor viruses but are non-permissive to B-tropic RNA tumor viruses. Mice homozygous for the Fv-1^b allele are permissive for B-tropic but not N-tropic RNA tumor viruses. The heterozygous host is resistant to both N- and B-tropic viruses (Hartley *et al.*, 1970; Pincus, Hartley, and Rowe, 1971; Pincus, Rowe, and Lilly, 1971).

To determine whether the resistance is at the cell surface or due to some intracellular restriction, pseudotypes of VSV were used. Zavada (1972a; 1972b) found that co-infection of cells with vesicular stomatitis virus (VSV) and RNA tumor virus results in the formation of pseudotypes containing VSV genomes coated by antigens of the RNA tumor virus. These pseudotypes have the surface properties of the RNA tumor virus. Previous studies on phenotypic mixing between VSV and a paramyxovirus SV5 showed that glycoproteins alone were responsible for the mixing phenomenon (Choppin and Compans, 1970; McSharry, Compans, and Choppin, 1971). Pseudotypes of VSV with coats from N-tropic or B-tropic murine leukemia viruses (MuLV) could be used to differentiate between surface and intracellular resistance by determining their ability to form plaques on murine cells homozygous for either the Fv-1^n or Fv-1^b allele. The expected results are shown in Table 1. If the resistance were a surface phenomenon, the pseudotypes would not attach to and penetrate into the resistant cells and no plaques would result. If the resistance were an intracellular one, both kinds of pseudotypes would be able to get into both permissive and non-permissive cells and result in VSV plaques. VSV grows well on both kinds of murine cells (Huang _et al._, 1973). An additional control to indicate that plaques on murine cells were initiated by pseudotypes of VSV(MuLV) is to show that the same preparation of VSV(MuLV) does not form plaques on monolayers of Chinese hamster ovary (CHO) cells. VSV(MuLV) presumably does not grow readily on CHO cells because its properties prevent attachment and penetration into the cells (Huang _et al._, 1973).

TABLE 1. Predicted growth of VSV(N-MuLV) and VSV(B-MuLV) pseudotypes on murine cells homozygous for the Fv-1 alleles.

	NIH/3T3 Fv-$1^{n/n}$*	JLS-V9 Fv-$1^{b/b}$*
Surface resistance		
VSV(N-MuLV)	+	-
VSV(B-MuLV)	-	+
Intracellular restriction		
VSV(N-MuLV)	+	+
VSV(B-MuLV)	+	+

*Genetic composition at the Fv-1 locus has been determined for NIH/3T3, derived from a NIH/Swiss mouse (Jainchill, Aaronson, and Todaro, 1969) and for JLS-V9 cells, derived from a BALB/c mouse (Wright _et al._, 1967).

N-tropic and B-tropic MuLV stocks were obtained from Drs. Janet Hartley and Wallace P. Rowe. When tested in the XC assay in our laboratory they had titers of a hundred- to a thousandfold higher on permissive cells than on non-permissive cells. Preparations containing VSV(MuLV) pseudotypes were made by co-infecting cells permissive for B-tropic or N-tropic MuLV with the respective MuLV and with VSV. Details of infection have been presented elsewhere (Huang et al., 1973). Progeny from these cells presumably contain the parental types VSV and MuLV and, also, pseudotypes--VSV(MuLV) and MuLV(VSV)--as well as particles with mixed coats. Particles containing MuLV genomes would not be detected in our VSV plaque assays. Parental-like VSV(VSV) would be neutralized by antiserum against VSV. VSV(MuLV) pseudotypes would be the only particles expected to initiate plaques after complete neutralization of VSV(VSV) with high concentrations of anti-VSV antibody.

In Figure 1, the neutralization curves are plotted to indicate the fraction of plaque-forming units (PFU/PFU_o) initiated by VSV after neutralization by increasing concentrations of antiserum against VSV. Plaques were assayed on NIH/3T3 ($Fv\text{-}1^n/Fv\text{-}1^n$), JLS-V9 ($Fv\text{-}1^b/Fv\text{-}1^b$) and CHO cells. Figure 1a shows that VSV(N-MuLV) formed as many plaques on NIH/3T3 cells as on JLS-V9 cells, whereas no non-neutralizable fraction was detectable on CHO cells. The $> 10^3$ difference on murine cells compared to CHO cells indicates the presence of VSV(N-MuLV). Thus, it can be concluded that VSV(N-MuLV) attached and penetrated equally well into cells homozygous for either of the Fv-1 alleles.

Similarly, Figure 1b shows that detectable plaques were formed by VSV(B-MuLV) on both types of murine cell but not on CHO cells. With both preparations of pseudotypes there was a slight but reproducible difference in the efficiency with which they initiated plaques on the two types of murine cells. VSV(N-MuLV) formed slightly more plaques on NIH/3T3 than on JLS-V9 cells, whereas VSV(B/MuLV) formed significantly more plaques on JLS-V9 cells than on NIH/3T3 cells. These small differences may indicate some preferential attachment and penetration of the pseudotypes to permissive cells. This may relate to some other host genetic control over the replication of RNA tumor viruses in murine cells.

Nevertheless, the growth of both VSV pseudotypes with coats from either N-tropic or B-tropic MuLV on cells homozygous for the Fv-1 alleles leads to the conclusion that resistance determined by the Fv-1 locus is an intracellular one and may be a specific restriction for some step during the replication of murine RNA tumor viruses. Using a methodology similar to ours, Krontiris, Soeiro, and Fields (1973) have obtained comparable results.

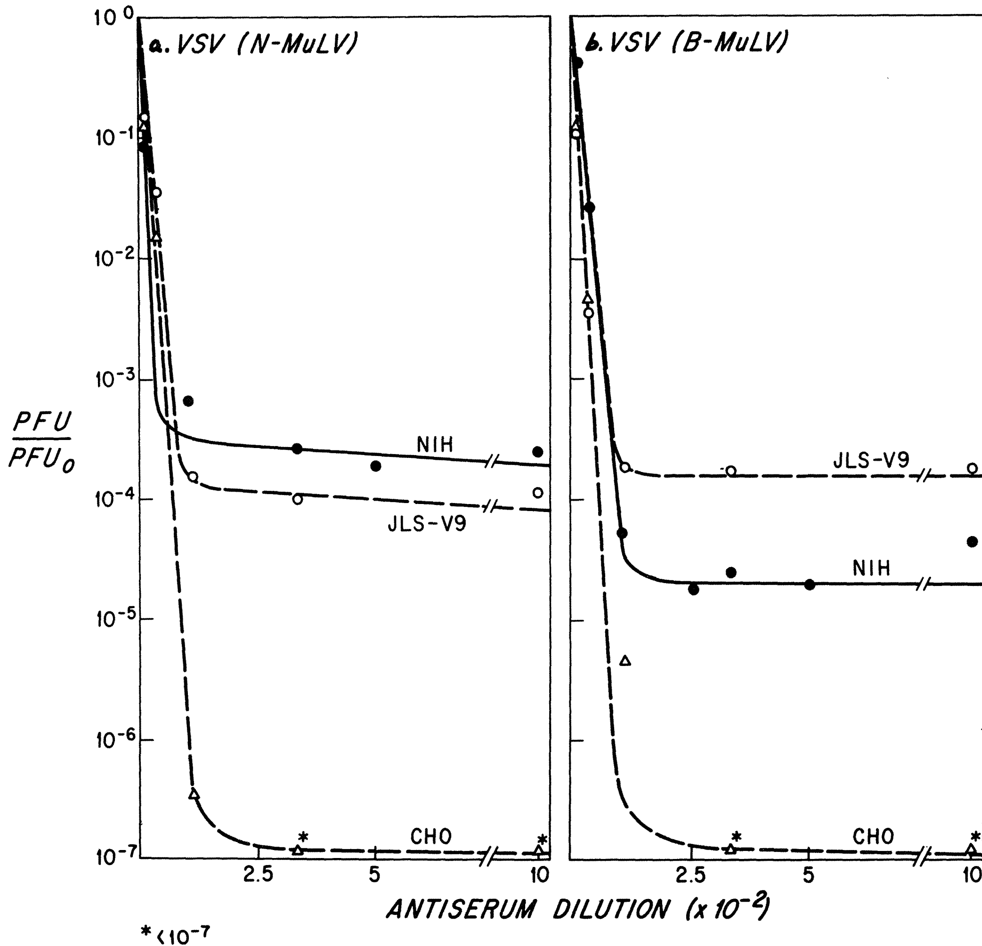

FIG. 1. Growth of VSV(N-MuLV) and VSV(B-MuLV) on NIH/3T3, JLS-V9 and CHO cells. VSV(N-MuLV) was made by co-infecting NIH/3T3 cells with VSV and N-tropic MuLV. VSV(B-MuLV) was made by co-infecting JLS-V9 cells with VSV and B-tropic MuLV. The resultant titers of VSV on CHO cells were 1.00 x 10^8 PFU/ml and 1.88 x 10^8 PFU/ml, respectively. Similar titers were obtained when the preparations were assayed on NIH/3T3 and JLS-V9 cells. Details on cell growth, infection of cells, preparation of antiserum and neutralization are found elsewhere (Huang et al., 1973).

Studies using approaches other than pseudotypes have also suggested that the gene product of the Fv-1 locus acts intracellularly (Fenyo et al., 1973; Yoshikura, 1973; Eckner, 1973). The use of VSV pseudotypes to study surface properties of RNA tumor viruses is now widely used in other contexts (Huang et al., 1973; Krontiris et al., 1973; Love and Weiss, 1974).

ACKNOWLEDGEMENTS

We thank Ms. Tazwell Wilson and Ms. Louise Chu for excellent technical assistance. We are especially grateful to Dr. Janet Hartley for supplying N-tropic and B-tropic MuLV. This work was supported by a National Science Foundation Research Grant GB 34266, American Cancer Society research grants VC-63 and VC-4D and a contract from the Viral Cancer Program. A. S. H. is a Research Career Development Awardee of the U.S. Public Health Service. P. B. was supported by the Schweizerische Krebsliga. D. B. is an American Cancer Society Professor of Microbiology.

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HUMAN ACUTE LEUKEMIA: INCREASING EVIDENCE FOR INVOLVEMENT OF TYPE-C RNA TUMOR VIRUSES

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INTRODUCTION

Type-C RNA tumor viruses have been associated with the development of leukemia/lymphoma in birds, rodents, cats, and subhuman primates. In man, however, morphologically identifiable virus particles have not been definitively identified in cells from patients with these diseases despite extensive searches by skilled electron microscopists. In recent years remarkable progress has been made in the biochemical and immunological characterization of RNA tumor viruses, and by these more sensitive parameters, "footprints" of RNA tumor viruses were first identified in human leukemic cells (Gallo, Yang, and Ting, 1970). The viral "footprints" so far identified consist of the two essential replicative components of RNA tumor viruses, intact 70S (or subunit 35S) virus-specific RNA (Baxt, Hehlman, and Spiegelman, 1972; Gallo *et al.*, in press; Gallagher *et al.*, in press) and virus-related RNA-directed DNA polymerase (reverse transcriptase) (Sarngadharan *et al.*, 1972; Todaro and Gallo, 1973; Gallagher *et al.*, submitted for publication).

It has been our working hypothesis that RNA tumor virus information is importantly related to leukemogenesis in man as in animals. In order to support this hypothesis, two developments in this field of investigation have required a more precise definition of the "viral components" in human leukemic cells. First, DNA polymerases have been identified in a variety of mammalian cells, including normal cells, which resemble viral reverse transcriptase

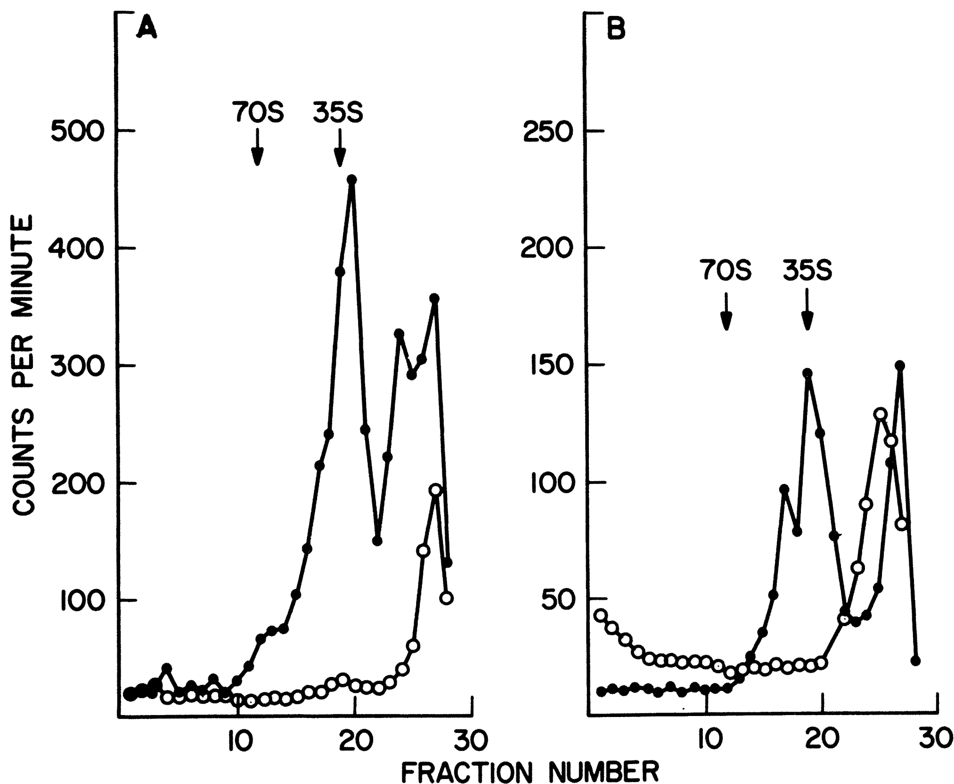

FIG. 1. Velocity glycerol gradient analyses of endogenous DNA products from acute myelogenous leukemic cells. Hybridization data for DNA product from this patient (HL-7) are presented in Table 1. Procedures for the synthesis, purification and analysis of the DNA product are described elsewhere (Gallagher et al., in press). (A) Endogenous DNA product from the "crude" microsomal fraction: ●——●, all four trinucleotides present in the reaction mixture; o——o, dGTP omitted from the reaction mixture. The absence of 35S DNA complex in the gradient from the minus dGTP reaction indicates that DNA was not synthesized by a terminal addition reaction (Baxt et al., 1972). (B) Endogenous DNA product from the microsomal fraction after banding in an equilibrium density sucrose gradient (active fraction density = 1.15-1.17 g/ml): ●——●, untreated; o——o, after RNase treatment.

in some biochemical functions (Bolden et al., 1972; Lewis et al., submitted for publication; Livingston et al., in press). Second, reverse transcriptase and 70S RNA have been identified in apparently non-oncogenic RNA "tumor" viruses (Temin, in press; Parks et al., 1971) and, possibly, in uninfected embryonic cells (Kang and Temin, 1973). Our studies, summarized here, clearly distinguish reverse transcriptase from other cellular DNA polymerases and specifically relate human 35S/70S RNA and reverse transcriptase to oncogenic mammalian type-C viruses.

LIKE RNA TUMOR VIRUSES, CYTOPLASMIC PARTICLES FROM HUMAN LEUKEMIC CELLS SUPPORT RNA-DIRECTED, ENDOGENOUS DNA SYNTHESIS

Before presenting specific evidence for the presence of viral-related macromolecules in human leukemic cells, it will be useful to discuss briefly "endogenous DNA synthesis." In RNA tumor viruses, reverse transcriptase and 70S RNA are intimately associated in the virus nucleoid, and they can, in the absence of added primer or template, "endogenously" synthesize DNA from deoxynucleoside triphosphate precursors. Two tests have been frequently utilized to analyze further this reaction. First, it may be shown that the reaction is eliminated or markedly reduced by pre-incubating the reaction mixture with ribonuclease (Sarngadharan et al., 1972; Sarin and Gallo, in press). Although this is a useful screening procedure, it does not discriminate between the possibilities of destruction of RNA template or of RNA primer for a DNA-directed reaction (Bobrow et al., 1972; Reitz et al., submitted for publication). Second, since some of the DNA reaction product remains associated with the RNA template, it has been possible to detect RNA-DNA hybrids with the discrete size of viral 35S/70S RNA (Schlom and Spiegelman, 1971). The most critical control in this test is determining that the DNA reaction product will back hybridize to specific RNA template.

In 1972 our laboratory (Sarngadharan et al., 1972) and Spiegelman's laboratory (Baxt et al., 1972) identified a post-mitochondrial, cytoplasmic pellet fraction from human leukemic cells which supports endogenous DNA synthesis. As in RNA tumor viruses, the reaction was sensitive to pre-treatment with RNase, and DNA product-RNA hybrids were found with the size of viral RNA. Regarding the latter, Spiegelman and associates initially observed that the native (endogenous) template was 70S or 35S in size, and we have now confirmed this in a high percentage of acute myelogenous leukemia patients (Gallagher et al., in press), particularly detecting DNA product-RNA complexes which are 35S in size (Figure 1). Recently, by combined physical and enzymatic techniques we have conclusively determined that the leukemic cell endogenous reaction is both RNA-primed and RNA-templated (Gallo et al., in

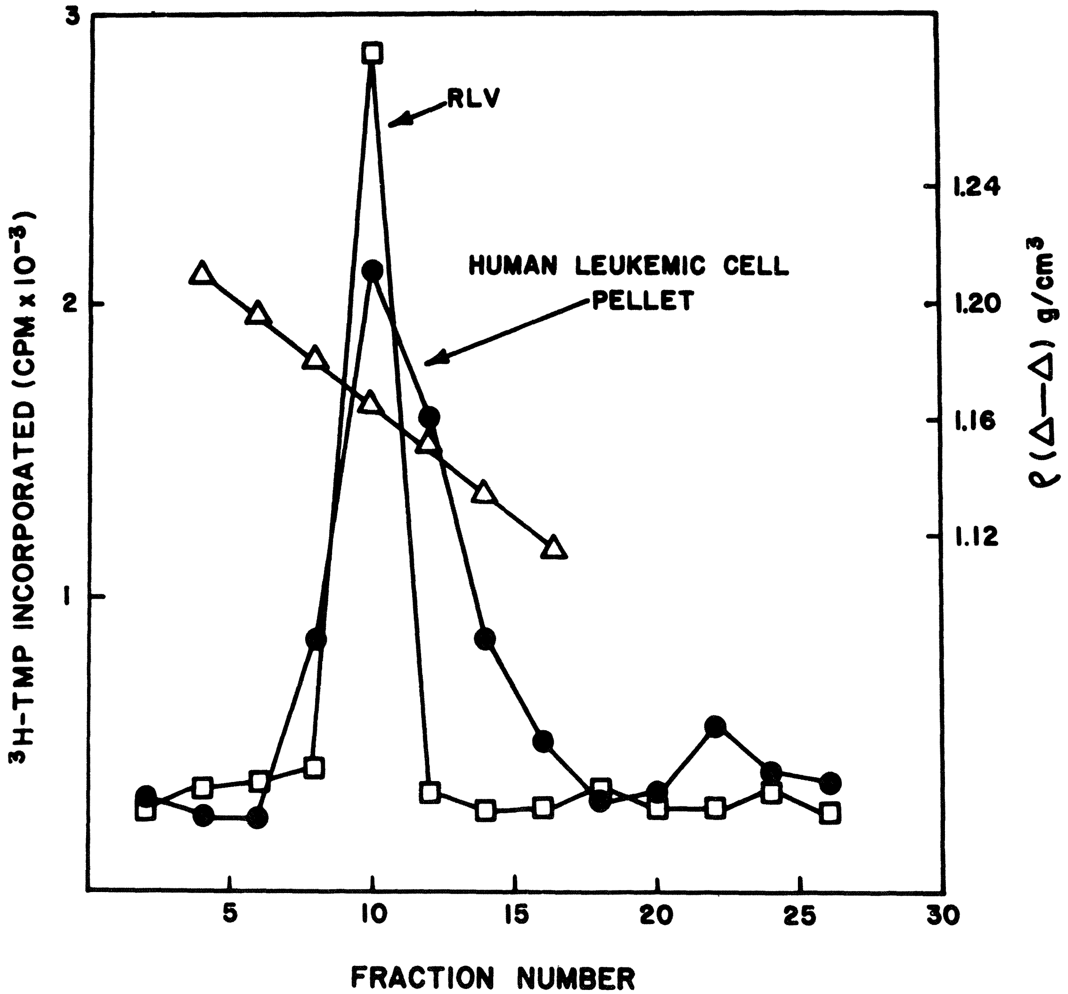

FIG. 2. The endogenous DNA synthesis activity of human leukemic "particles" and murine leukemia virus (Rauscher) after equilibrium sucrose density centrifugation. Technical procedures utilized for this analysis are detailed elsewhere (Todaro and Gallo, 1973). ●——●, human leukemic particles; □—□, murine leukemia virus.

press). Thus, RNA-directed DNA polymerase extracted from this cytoplasmic complex has the same "endogenous" reaction properties as true viral reverse transcriptase. Similar studies with normal leukocytes (phyto-hemagglutinin-stimulated lymphocytes) indicate that endogenous DNA synthesis, which we previously reported (Bobrow et al., 1972), is primarily related to the presence of mitochondria and is RNA-primed but DNA-templated (Reitz et al., submitted for publication).

An interesting attribute of the endogenous DNA synthesizing complex from human leukemic cells is that it bands in sucrose equilibrium density gradients at the same density as intact RNA tumor viruses (Figure 2; Baxt et al., 1972; Gallo et al., in press; Todaro and Gallo, 1973). This implies the existence of a discrete particle. We have extended this notion by demonstrating that the DNA-synthesizing particle is not disaggregated by physical manipulation, and we have used this property to further purify the "particle" by repeated sucrose density centrifugation (Gallo et al., in press) and by macromolecular sieve chromatography (Gallagher et al., submitted for publication). Present studies in our laboratory are directed toward morphologically identifying these biochemically active forms and testing them for possible biological activity. Also, an explanation is required for the observation that the leukemic cell "particles," which have the same density as type-C viruses and which contain 70S RNA and reverse transcriptase related to these same molecules in type-C viruses (vide infra), are apparently intracellularly located (in association with microsomes), whereas in type-C viruses the characteristic form and density are acquired and 70S RNA is assembled at the time of viral budding from the cell membrane (Duesberg, Canaani, and Helm, 1973; Chang et al., 1972; Gallo and Ting, 1972).

DNA SYNTHESIZED BY LEUKEMIC CELL PARTICLE HAS SEQUENCE HOMOLOGY TO PRIMATE TYPE-C VIRUS 70S RNA

Baxt et al. (1972) first reported that DNA synthesized endogenously by the microsomal fraction of human leukemic cells hybridized to 70S RNA from mouse leukemia virus (Rauscher) but not to 70S RNA from mouse mammary tumor virus or from avian myeloblastosis virus. These results indicate that the human DNA product (and, therefore, the template, cytoplasmic RNA) has sequences which are genetically related specifically to mouse leukemia virus. However, the fraction of the DNA probe cross-hybridizing in this fashion was very low. Recent studies conducted in our laboratory indicate a significantly greater degree of homology with 70S RNA from the primate type-C Simian sarcoma Virus (SiSV) and to the Kirsten strain of murine sarcoma virus (Table 1, Gallo et al., in press). Both of these viruses are a mixture of sarcoma and leukemia genetic elements.

TABLE 1. Hybridization of [^{3}H]-DNA product from human leukemic cytoplasmic particles and from primate type-C virus to 70S RNA isolated from RNA tumor viruses*

RNA source+	% hybridization of ^{3}H-DNA from: Patient HL-7	Patient HL-8	SiSV
SiSV (NRK)	53	23	57
MuSV (Kirsten)	19	41	45
MuLV (AKR)	5	14	37
MuLV (Rauscher)	5	12	2
FeSV (Gardner)	N.T.**	5	2
FeLV (Rickard)	N.T.	0	<1
AvLV (AMV)	0	0	0

*Methods for the preparation of viral RNA and of ^{3}H-DNA product and for hybridization on filter discs are described elsewhere (Gallo et al., in press).

+The viruses used as a source of RNA are abbreviated as follows: SiSV (NRK) = Simian sarcoma virus grown in normal rat kidney cells (NRK); MuSV (Kirsten) = a sarcoma-leukemia virus complex grown in NRK cells which originated by repeated infection of rats with cross-type mouse leukemia virus; MuLV (AKR) = a cross-type mouse leukemia virus grown in mouse fibroblast cells and originating spontaneously from AKR mice; MuLV (Rauscher) = mouse leukemia virus, strain Rauscher. MuLV (Rauscher) shows less than 10% genetic homology with cross-type mouse leukemia viruses in assays like those presented here. FeSV (Gardner) = feline sarcoma-leukemia virus complex, Gardner strain; FeLV (Rickard) = feline leukemia virus, Rickard strain; AvLV (AMV) = avian leukosis virus, strain avian myeloblastosis virus.

**N.T., not tested.

DNA synthesized endogenously by SiSV hybridized to viral RNA's in a very similar pattern to the human DNA product (Table 1). From these results, it would be predicted that SiSV DNA product should hybridize to RNA in the microsomal fraction of human leukemic cells, and this was found to be the case for the patients presented in Table 1 (see Gallo et al., in press, Table 4).

The greater homology to the primate virus demonstrated by these studies might have been anticipated from evolutionary considerations; however, the results with Kirsten murine sarcoma virus were surprising. In this regard, it may be quite significant that the Kirsten virus was originally isolated from mice which had received repeated injections of extracts from human leukemic cells (Kirsten et al., 1967). An alternative possibility is that the human DNA

product hybridizes with sarcoma-specific sequences in these sarcoma-leukemia viral complexes, although extensive cross-hybridization to feline sarcoma virus was not observed (Table 1). If this were to be the case, it would require revision of our concepts of the possible role of RNA tumor viruses in human leukemogenesis and alteration of our experimental approaches to demonstrating biological activity for presumptive human "leukemia" viruses.

BIOCHEMICAL CHARACTERISTICS OF REVERSE TRANSCRIPTASE FROM CYTOPLASMIC PARTICLES OF HUMAN LEUKEMIC CELLS

A comprehensive review of reverse transcriptase from RNA tumor viruses and leukemic cells has been presented elsewhere (Sarin and Gallo, in press). Here, we will consider only those biochemical features which distinguish leukemic cell reverse transcriptase from the three major cellular DNA polymerases (Table 2). By these and other biochemical criteria, the leukemic cell polymerase is virtually identical to reverse transcriptase from mammalian type-C viruses.

As previously discussed, leukemic cell reverse transcriptase can catalyze endogenous DNA synthesis which is RNA templated and RNA primed. This criterion is the _sine qua non_ for a true reverse transcriptase, although this reaction could, of course, be missed if the enzyme were dissociated from its natural RNA template. Its location in a particle which bands at the density of RNA tumor viruses and its association with 35S or 70S RNA with sequence homology to RNA tumor viruses strengthen the analogy to but do not prove relatedness to viral reverse transcriptase. As previously mentioned, this association of leukemic cell reverse transcriptase with a distinct "particle" has been utilized to purify reverse transcriptase from other cellular DNA polymerases by one of two procedures: (a) direct extraction of the microsomal fraction after preparation by differential centrifugation with further purification of the extracted polymerases by column chromatography (Sarngadharan _et al._, 1972) and (b) successive purification of the virus-like "particle" (Gallagher _et al._, submitted for publication). Regarding the latter, we find in some cases that gel chromatography has been an excellent method of separating membrane-associated reverse transcriptase from contaminating soluble DNA polymerases (Figure 3). While this work was in progress, Garrett and Bollum (1973) similarly reported the utilization of agarose chromatography to dissociate normal cellular DNA polymerases from cytoplasmic particulate components. This method does not "work" in cells from a significant proportion of leukemic patients, suggesting that in these cases the "particles" do not have a sufficient degree of integrity or resistance to dissociation or destruction by catabolic factors to permit this mode of purification. Therefore, we are also investigating

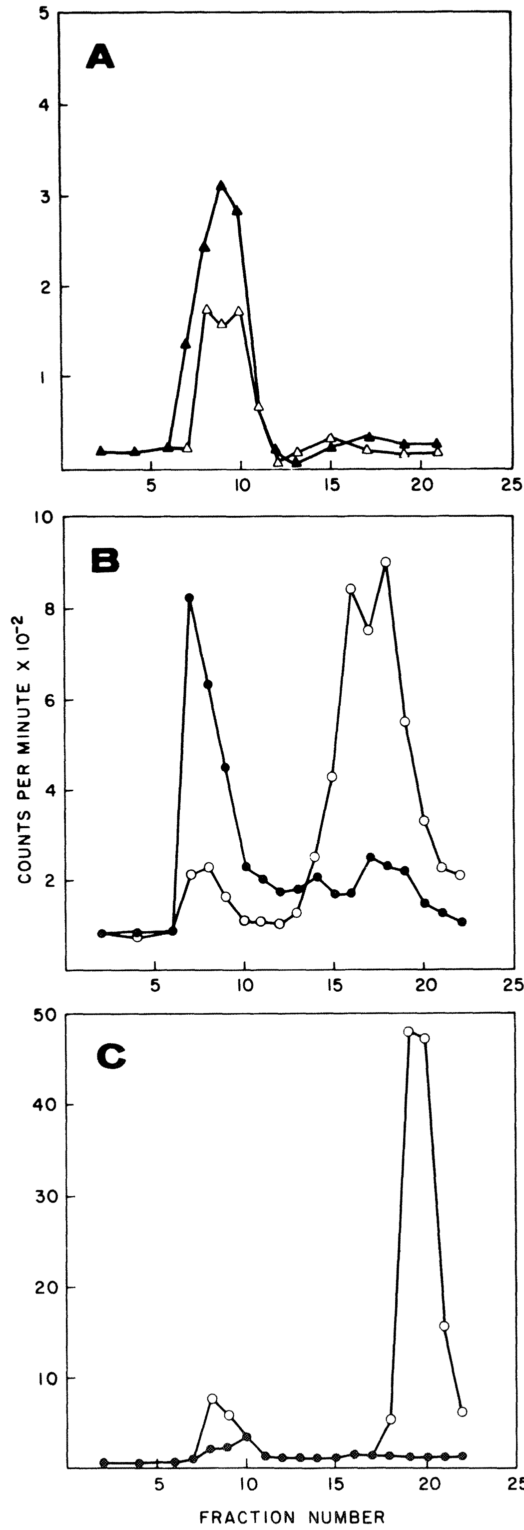
A
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FIG. 3. DNA polymerase activity from the microsomal fraction of human leukemic and normal peripheral white blood cells after sepharose 4B chromatography. The post-mitochondrial, 100,000 x g cytoplasmic pellet fraction (microsomal fraction) was applied to a 0.9 cm x 30 cm column containing sepharose 4B (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM KCl, 1 mM dithiothreitol and 0.5 mM EDTA. The void volume of such a column is 6 ml with an exclusion size of 20 x 10^6 daltons. The column was eluted with the same buffer at a flow rate of 0.25 ml-0.5 ml per minute at 4°C and 1 ml fractions collected. Fractions were assayed for DNA polymerase activity in the absence (endogenous activity) or presence of added synthetic primer-templates (exogenous activity). (A) Endogenous DNA polymerase activity from a case of acute myelogenous leukemia: ▲—▲, untreated; △—△, pre-incubated with ribonuclease. The partially RNase-sensitive endogenous DNA polymerase activity is localized in the column void volume indicating a minimum molecular size for the DNA synthesizing complex of 20 x 10^6 daltons. (B) Exogenous DNA polymerase activity from a case of acute myelogenous leukemia: ●—●, with oligo dT·poly rA; o—o, with oligo dT·poly dA. The DNA polymerase activity preferring the RNA template is localized in the void volume, indicating an association with a large complex, whereas the DNA polymerase activity preferring DNA template is included in the gel, indicating a lack of particle association. (C) Exogenous DNA polymerase activity from phytohemagglutinin-stimulated (normal, proliferating lymphocytes: ●—●, with oligo dT·poly rA; o—o, with oligo dT·poly dA. Almost all DNA polymerase activity is included in the gel, and the DNA template is preferred in all fractions.

TABLE 2. Biochemical features of human leukemic reverse transcriptase which are the same as reverse transcriptase from mammalian type-C viruses and which distinguish it from other cellular DNA polymerases

Feature	Classification of DNA polymerase				
	Leukemic cell reverse transcriptase	Type-C virus reverse transcriptase	Cellular DNA Pol I	Cellular DNA Pol II	Cellular DNA Pol III
Intracellular location	Cytoplasmic; membrane-associated	--	Cytoplasmic soluble	Cytoplasmic and nuclear	N.D.
Endogenous reaction	+	+	0	0	0
Molecular size	70,000	70,000	150,000	30,000	>90,000
Response to distinguishing templates					
(a) 70S viral RNA	+	+	0	0	0
(b) dG·rC	+	+	0	0	0
(c) dT·rA>dT·dA	+	+	0	0	0

the efficacy of directly solubilizing all possible DNA polymerases from whole cells and attempting to resolve all polymerases by successive chromatographic procedures. This method has been shown to give excellent resolution of viral reverse transcriptase from the three major cellular DNA polymerases in human white blood cells infected with simian sarcoma virus (Lewis et al., submitted for publication). This method has the advantage of being able to compare polymerases which are subjected to the same conditions during extraction and purification but it is not yet established if it will provide adequate sensitivity for the detection of reverse transcriptase which is apparently present in only low amounts in leukemic cells.

When dissociated from its natural RNA template, the leukemic cell reverse transcriptase has in several instances now been demonstrated to have an approximate molecular weight of 70,000 daltons, which is the same as that for type-C virus reverse transcriptase (Abrell and Gallo, 1973; Ross et al., 1971) and distinctly different from that of the three major cellular DNA polymerases (Smith and Gallo, 1972; Lewis et al., in preparation) (Table 2). Further, like viral enzyme, the leukemic cell polymerase prefers the primer-template oligo dT·poly rA to oligo dT·poly dA. However, this does not distinguish reverse transcriptase from cellular DNA polymerase III (Lewis et al., submitted for publication), the so-called R-DNA polymerase described by Weissbach and his colleagues (Bolden et al., 1972; Table 2). Since these synthetic primer-templates are frequently used in preliminary tests to detect reverse transcriptase, it is important always to include other criteria to insure that R-DNA polymerase or possibly other poly-A directed, poly-dT synthesizing enzymes (Livingston et al., in press) are not being detected. Also it should again be mentioned, since so many false detections of "reverse transcriptase" have been reported, that the homopolymeric hybrid poly dT·poly rA is totally non-specific and may be efficiently utilized by many DNA polymerases (Sarin and Gallo, in press; Gallo, 1971). Additionally, leukemic cell reverse transcriptase can transcribe viral 70S RNA (Sarngadharan et al., 1972; Bhattacharyya et al., 1973) and poly rC primed with oligo dG (Sarngadharan et al., 1972; Todaro and Gallo, 1973; Gallagher et al., submitted for publication). Utilization of these templates has otherwise been demonstrated only with, and hence may be specific for, viral reverse transcriptase (Gallagher et al., submitted for publication; Livingston et al., in press; Baltimore and Smoler, 1971). Finally, leukemic cell reverse transcriptase does not utilize primer in the absence of template (Todaro and Gallo, 1973; Gallagher et al., submitted for publication) and therefore it is not terminal deoxy-nucleotidyltransferase, which has been reported in the leukemic cells from one case of acute lymphocytic leukemia (McCaffery, Smoler, and Baltimore, 1973).

TABLE 3. Relative inhibitory ability of anti-polymerase antibodies (IgG) to inhibit the human leukemic reverse transcriptase

Antibody to DNA polymerases prepared from*	Relative inhibitory effect on human leukemic reverse transcriptase**
gibbon ape virus	1-2
Woolly monkey virus (SSV)	2-5
mouse leukemia virus	5-10
cat leukemia virus	>10
RD 114	>20
Mason-Pfizer Mammary Tumor Virus	>10
Avian Myeloblastosis Virus	>50
normal lymphocyte DNA polymerase I	>50

*The experiments with antibodies to viral reverse transcriptase have been published elsewhere by Todaro and Gallo (1973) and by Gallagher et al. (submitted for publication). The antibody to normal lymphocyte DNA polymerase I has been described elsewhere by Smith et al. (submitted for publication).

**The numbers refer to the μg of purified IgG anti-polymerase antibody to give 30 to 40% inhibition of the human leukemic reverse transcriptase. Controls were IgG purified from pre-immunized animals and do not inhibit the enzyme.

REVERSE TRANSCRIPTASE FROM HUMAN LEUKEMIC CELLS AND PRIMATE TYPE-C VIRUS ARE IMMUNOLOGICALLY CLOSELY RELATED

Table 3 summarizes the results which we have observed in four cases of acute myelogenous leukemia when the leukemic cell reverse transcriptase was tested for inhibition by antisera to reverse transcriptase from various RNA tumor viruses. These studies were conducted in collaboration with Dr. George Todaro, NCI, Bethesda, Maryland (Todaro and Gallo, 1973; Gallagher et al., submitted for publication). The antisera to the two primate type-C viruses, gibbon ape virus (Kawakami et al., 1972), and simian sarcoma virus (Wolfe et al., 1971) inhibited the leukemic cell reverse transcriptase almost as effectively as they inhibited their homologous enzymes. Antisera to mouse, cat and avian type-C virus polymerase and to a non-type-C primate virus (Mason Pfizer monkey virus) showed no or much lesser degrees of inhibition. These results are quite similar to those reported in a study of the immunologic relationships of reverse transcriptase from primate RNA tumor viruses (Scolnick, Parks, and Todaro, 1972). In that study antisera to type-C virus reverse transcriptase was also demonstrated not to inhibit reverse transcriptase from "foamy" viruses, so it may be inferred

that the leukemic cell enzyme has no immunologic relatedness to DNA polymerase from these non-oncogenic viruses, although this was not directly tested. In addition it might be mentioned that there is at present no known human foamy virus. Finally, antisera to simian sarcoma virus did not inhibit any of the three cellular DNA polymerases and, conversely, antisera to cellular DNA polymerase I did not inhibit viral or leukemic cell reverse transcriptase (Smith _et al._, submitted for publication).

CONCLUSIONS

From our studies summarized here, we conclude that some and perhaps all human acute leukemia cells contain reverse transcriptase and high molecular weight cytoplasmic RNA, which are related in general biochemical function to analogous molecules in mammalian oncogenic type-C viruses and which are related by more exact primary structural criteria (nucleic acid hybridization and immunologic cross-reactivity) to analogous molecules specifically from closely related primate species. At the present time our specific evidence is restricted to cases of acute myelogenous leukemia, or chronic myelocytic leukemia in an acute "blastic" phase, although biochemically-defined reverse transcriptase has also been detected in acute lymphoblastic leukemia cells. In addition, we have many "negatives" but as technology has improved so has the percentage of positive cases. Despite extensive efforts we have not detected endogenous RNA-directed DNA synthesis or reverse transcriptase in normal leukocytes, although this could be related to inadequate sensitivity of the techniques or to the unavailability of normal myeloblastic or lymphoblastic stem cells. Temin's laboratory has reported the presence of reverse transcriptase in chick embryo and in rat tissue culture cells (Temin, in press; Kang and Temin, 1973); however, in both of these instances it is difficult to know if these cells are free of virus infection. In any event, Mitzutani and Temin (1973) have reported that these "cellular" enzymes are not immunologically related to reverse transcriptase from known tumor viruses.

These studies raise many interesting questions for future investigations, including the following: Is the viral information in human leukemic cells derived from endogenous genetic information or from exogenous sources? Are the reverse transcriptase-35S/70S RNA particles in leukemic cells infectious for secondary cells or for secondary hosts? Are these molecules really specific for leukemic cells and, as such, can sensitive probes which detect them be used for diagnostic and prognostic tests? In remission is the viral information lost or is it simply repressed? By controlling expression of this information can the leukemic cell be converted to phenotypically normal white cells? The answers to these questions

could be crucial to devising rational approaches to anti-leukemia therapy. Our theoretical considerations relative to some of these questions have been presented elsewhere (Gallo, in press).

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HERPES- AND HERPESVIRUS-RELATED VIRUSES

HERPES SIMPLEX VIRUS: ASPECTS OF STRUCTURE AND REGULATION OF VIRAL RNA AND PROTEIN SYNTHESIS

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INTRODUCTION

This paper summarizes briefly two aspects of Herpesvirus research of interest to us: the structure and composition of the Herpesvirion and the mechanism by which Herpesviruses regulate their own replication.

The anatomy of the Herpesvirion has been recently reviewed in detail (Roizman and Furlong, 1974). All of the currently available information strongly suggests that the Herpesvirion consists of 4 architectural components, i.e., (i) a core consisting of DNA arranged in the form of a toroidal body surrounding a protein mass, (ii) a bilayered capsid the outer surface of which consists of 162 capsomeres, (iii) an amorphous coat which appears to consist of fibrillar material surrounding the capsid and (iv) a trilaminar membrane, the envelope, with spikes protruding from its outer surface. The structural components of the virion identified to date other than DNA and protein are lipids and polyamines. In this paper we shall deal with two aspects of the structure of the virus, i.e., a first approximation of the number of polypeptide species and the amounts of each contained in the Herpesvirion and with the variation in the structural protein observed among different Herpes simplex I strains.

In principle, it is reasonable to view regulation of viral macromolecular metabolism as a four-bodied problem; i.e., that it is determined by the physiologic state of the cell before and during

infection, by transcription and post-transcriptional processing of the RNA, by translation of viral mRNA, and, lastly, by viral DNA synthesis which could either amplify the template available for transcription or present modified templates for transcription. This paper deals with current studies on transcription and translation and these are presented intermixedly. The following facts are pertinent to the data presented in this paper:

(i) The duration of the reproductive cycle of Herpes simplex 1 (HSV-1) in human cells at multiplicities of 5 to 80 PFU/cell is from 17 to 19 hours at 37°C (Hoggan and Roizman, 1959a; Roizman, Aurelian, and Roane, 1963). Viral DNA synthesis is first detected around 3 hours post infection and nearly 90 percent of the progeny viral DNA is made by 9 hours post infection (Roizman and Roane, 1964; Roizman, 1969; Cohen, Vaughn, and Lawrence, 1971). Infected cell protein synthesis is required for the onset of viral DNA synthesis, and exposure of cells to inhibitors of protein synthesis from the time of infection precludes viral DNA synthesis (Roizman and Roane, 1964). Infectious viral progeny is first detected between 5 and 6 hours post infection (Hoggan and Roizman, 1959a; Roizman et al., 1963). Inhibitors of DNA synthesis do not prevent the synthesis of structural proteins (Levitt and Becker, 1967; Nii et al., 1968; Roizman, 1972).

(ii) Viral RNA is made in the nucleus (Wagner and Roizman, 1969; Roizman et al., 1970). Viral transcripts present in the nucleus range in size from 4S to greater than 60S, whereas transcripts present in the cytoplasm are at most 35S (Wagner and Roizman, 1969; Roizman et al., 1970). Nuclear transcripts sedimenting faster than 45S contain sequences arising from at least 40 percent of the DNA, suggesting that the bulk of the stable transcripts is made as high molecular weight RNA which is cleaved prior to transport from the nucleus (Jacquemont and Roizman, manuscript in preparation). A large fraction estimated to range from 96 to 99 percent of total viral transcripts accumulating in the infected cell and arising from 22 to 25 percent of viral DNA has been shown to be extensively adenylated (Silverstein et al., 1973). The adenylation of viral RNA appears to be a post transcriptional event preceding the transport of RNA from the nucleus into cytoplasm (Bachenheimer and Roizman, 1972). Viral polypeptides are made in cytoplasmic free and bound polyribosomes (Sydiskis and Roizman, 1966 and 1967), and differ widely in their function. Some are transported into the nucleus (Olshevsky, Levitt, and Becker, 1967; Spear and Roizman, 1968); others are incorporated into the cellular membranes, become glycosylated, and at least in part, form the specialized membranes which envelop the virus (Spear, Keller, and Roizman, 1970; Spear and Roizman, 1970; Roizman, 1971).

MATERIALS AND METHODS

Virus and Cells

The experiments described in this paper were done with the F strain of human Herpesvirus 1 [Herpes simplex 1 (strain F), HSV-1 (F)]. The virus was isolated at the University of Chicago Hospitals and passaged a maximum of 4 times in HEp-2 cells at low multiplicity. The procedures for preparation of the virus, infection and maintenance of infected cells have been published elsewhere (Ejercito, Kieff, and Roizman, 1968; Roizman and Spear, 1968; Spear and Roizman, 1972; Heine, Spear, and Roizman, 1972; Heine _et al._, 1974; Kieff, Bachenheimer, and Roizman, 1971; Kieff _et al._, 1972; Roizman _et al._, 1970; Frenkel and Roizman, 1971; Gibson and Roizman, 1971, 1972, 1973, and 1974; Roizman and Furlong, 1974). The properties of HSV-1 (mP, MP, VR3, HFEM, 13v, B4) and HSV-2 (G) used in comparative studies with HSV-1 (F) have been described elsewhere (Heine _et al._, 1974; Ejercito _et al._, 1968). All of these strains have a history of numerous passages outside the human host.

Experimental Procedures

The procedure for purification of HSV virions was that described by Spear and Roizman (1972) as amended by Heine _et al._ (1974). In all of the experiments described in this paper, the timing of infection begins from the moment of exposure of HEp-2 cells to virus. The composition of medium used for _in vivo_ labeling of polypeptides with ^{14}C amino acids, the procedures for solubilization, electrophoretic separation of polypeptides in high resolution polyacrylamide gel slabs containing sodium dodecyl sulfate, autoradiography of the dried gels, and computer-aided planimetry of both gels and autoradiograms have been reported in detail by Spear and Roizman (1972) and by Honess and Roizman (1973). The procedures for preparation of viral DNA from viral capsids were as described by Kieff _et al._ (1971), except that after banding in sucrose density gradients, the DNA was further deproteinized with phenol.

RNA-DNA Hybridizations

The technique of Frenkel and Roizman (1972) was designed to determine the fraction of DNA whose transcripts accumulate in the cell. It involves measuring the rates of conversion of trace amounts of labeled viral DNA into DNA-RNA hybrid in the presence of excess unlabeled RNA extracted from either whole cells, nuclei, cytoplasm or polyribosomes. The reaction was monitored by subjecting the hybridization mixture to digestion with single strand-specific

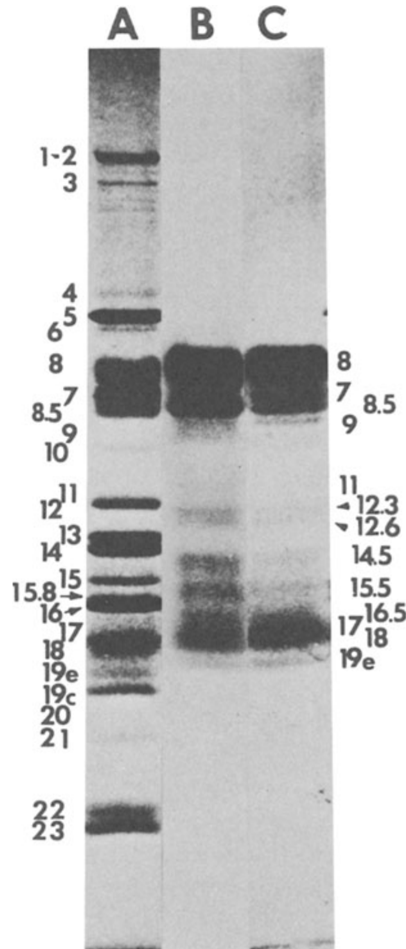

FIG. 1. Autoradiograms of HSV-1 (F) polypeptides separated on polyacrylamide gels. A. polypeptides from virions labeled with ^{14}C-amino acids. B, C. polypeptides from virions labeled with ^{14}C-D-glucosamine and ^{14}C-L-fucose, respectively, subjected to electrophoresis on an 8.5% gel slab crosslinked with DATD. A, B and C are from adjacent sample slots of a single slab gel, but different exposure intervals were used in printing the photographs for this figure. Coomassie Brilliant Blue stained polypeptides in A, B, and C were identical in amount and electrophoretic mobilities. The trace bands between VP1-2 and 3, and between VP3 and 4 on Figure 1A were seen only in occasional preparations; together they account for only 0.3% of the total absorbance of this autoradiogram, i.e., well within the range of estimated amounts of host contaminants in our preparations of purified virions (Spear and Roizman, 1972). Band 15.8 and 16 were clearly separated only on 7.5% DATD crosslinked gels.

nucleases of Neurospora crassa, prepared aften Rabin, Preiss, and Fraser (1971), which hydrolyzes the DNA remaining single stranded but not the DNA in hybrid. Theoretical details of the hybridization procedure are given in Frenkel and Roizman (1972). Specific conditions of hybridization are given in the legends to figures and tables.

RESULTS

The Structural Polypeptides of HSV-1 Virions

The structural polypeptides of HSV-1 (F). Figure 1 shows autoradiograms of polyacrylamide gels crosslinked with N,N'-diallyltartardiamide (DATD) and containing electrophoretically separated structural polypeptides of HSV-1 (F) virus labeled *in vivo* with ^{14}C-amino acids, ^{14}C-glucosamine or with ^{14}C-fucose. The information obtained from absorbance scans of Coomassie Brilliant Blue stained gels or of autoradiograms of these gels may be summarized as follows:

(i) We have resolved 33 species of polypeptides and this must be regarded as a minimal number. It does not include polypeptides less than 25,000 or take in consideration the possibility that some of the bands contain more than one species of polypeptides. Of this number, at least 14 polypeptide species are glycosylated.

(ii) The molecular weights of virion polypeptides were estimated by subjecting to electrophoresis polypeptides of known molecular weight along with virion polypeptides (Figure 2). The current designations, molecular weights and properties of the virion polypeptides are summarized in Table 1.

(iii) The average number of copies of each polypeptide in the virion may be computed from the molecular weight of the polypeptide, the total virion protein mass, and the fraction of the total virion protein mass constituted by each polypeptide. The virion protein mass was estimated from determinations of protein and DNA content of several purified virus preparations (Heine *et al.*, 1974). Based on the determined protein to DNA ratio of 10.70 ± 0.96 and assuming a molecular weight of 10^8, the total aggregate mass of the virion is 11.7×10^8 daltons and its weight is 19.4×10^{-16} gm.

The estimates of the percentage of total virion protein contributed by each polypeptide are based on computer-aided planimetric analysis both of autoradiograms of polyacrylamide gels containing electrophoretically separated ^{14}C-amino acid labeled polypeptides (Table 1, column c) and of Coomassie Brilliant Blue stained gels

TABLE 1. Enumeration, quantitation and glycosylation of virion polypeptides of HSV-1 [F1]

Virion polypeptide	Molecular weight x 10^{-3} a	b	% virion protein mass c	d	Molecules per particle e	f	Glycosylation Glucos-amine	Fucose
1-2	260-275	>260	3.3(10)	3.5(5)	150	150	-	-
3	260	>260	1.0 (8)	1.3(4)	40	60	-	-
4	184	177	1.1 (8)	0.9(7)	64	60	-	-
5	155	157	10.9(10)	13.5(7)	810	1000	-	-
6	146	149	1.0(10)	-	80	-	-	-
6.5	-	130	-	0.4(2)	-	40	-	-
7	126	126	4.8 (8)	-	450	-	+?	+
8		129	6.5 (8)	-	590	-	+	+
8.5	-	119	4.1 (8)	-	400	-	+	+
(7+8+8.5)			18.3(10)	11.8(7)	2140	1100		
9	112	115	-	0.8(5)	-	86	+	+
10	98	100	-	0.9(3)	-	100	-?	-?
(9+10)			2.0 (6)	1.7(5)	220	180		
11	93	94	-	2.5(2)	-	310	+	+
12	87	91	-	2.2(2)	-	280	-	-
(11+12)			3.8 (9)	4.7(5)	480	600		
12.3	-	88	N.D.	N.D.			+	+
12.6	-	86	N.D.	N.D.			+	+
13	78	82	5.0 (6)	-	710	-	-	-
14	78	80	6.5 (6)	-	950	-	-	-
(13+14)			11.5(10)	13.0(7)	1660	1880		
14.5	-	76	N.D.	N.D.			+	+
15	71	73	2.9 (8)	3.6(2)	460	580	-	-
15.5	-	70	N.D.	N.D.			+	+
15.8	-	69	-	-	-	-	+?	+?
16.0	65	68	-	-	-	-	-	-
(15.8+16.0)			6.9 (8)	5.9(2)	1200	1010		
(15+15.8+16.0)			9.8(10)	11.1(7)				
17	59	62	3.6 (7)	3.5(2)	680	660	+	+
18	57	59	5.4 (7)	4.1(2)	1020	810	+	+
19E	53	57	3.2 (7)	2.5(2)	660	510	+	+
(17+18+19E)			12.3(10)	10.3(5)				
19C	53	55	3.8 (8)	4.2(2)	808	890	-	-
20	50	51	1.5 (6)	1.8(7)	350	410	+	+
21	44	47	-	1.1(4)	-	270	-	-
21h			-	2.2(4)	-	570	-	-
22	37	39	4.9 (6)	8.8(2)	1470	2640	-	-
23	33	36	5.3 (6)	4.5(2)	1720	1460	-	-
(22+23)			9.9 (8)	11.8(7)				
24	25	-	-	-	-	-		

TABLE 1. Legend.

Column a: Molecular weights of virion polypeptide as determined by Spear and Roizman (1972) by electrophoresis on polyacrylamide gels crosslinked with methyl bis acrylamide and calibrated with proteins of known molecular weight.

Column b: Molecular weights of virion polypeptides determined by electrophoresis on calibrated polyacrylamide gels crosslinked with DATD (Figure 2).

Columns c and d: Contribution of each polypeptide to total virion protein mass determined by computer aided planimetry (Honess and Roizman, 1973) of absorbance scans of (c) autoradiograms and (d) absorbance scans of Coomassie Brilliant Blue stained gels. The number of determinations on different gels contributing to each mean value is indicated in parentheses. Note that in some instances for groups of closely spaced polypeptides (e.g., VP7, 8, 8.5 and VP13, 14) the contribution of the group of components could be estimated under conditions where resolution was inadequate to allow accurate estimation of the amounts of each species within the group. Therefore the mean values for the contribution of these groups of components were derived from a greater number of estimations than was the number of estimations of each polypeptide within the group. N.D. not accurately estimable, all these values were <1% of protein mass.

Columns e and f: The number of molecules of each polypeptide or group of polypeptides per virion was calculated from their percentage in columns c(e) and d(f), respectively, based on the relationship:

$$\text{Molecules per virion} = \frac{\text{total protein weight of virion} \times \text{percentage contribution of polypeptide to total virion protein}}{\text{polypeptide molecular weight}}$$

For groups of more than one polypeptide the total number of molecules of all polypeptides within the group was calculated using the independently estimated values for the percentage contribution of the group to total virion protein and a value for the molecular weight which was the average of the molecular weights of the separated polypeptides. The number of molecules refers to values calculated for extreme values of $(19.4)\cdot 10^{-16}$ g/virion for the weight of the virion protein determined as described in the text. (Data from Heine et al., 1974.)

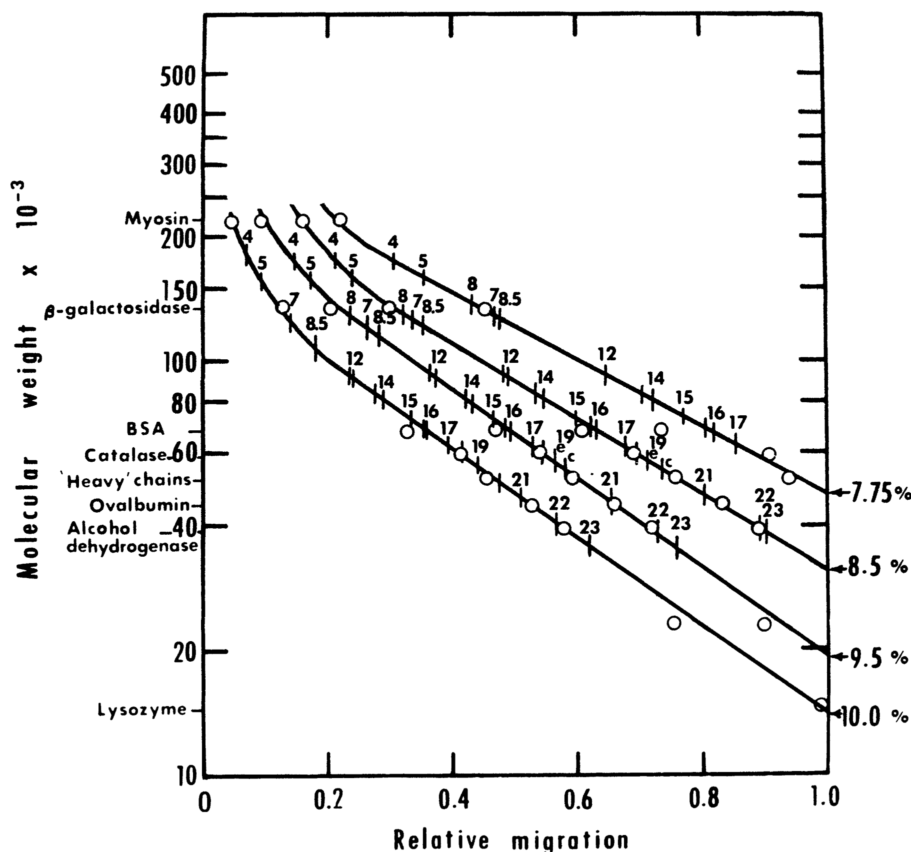

FIG. 2. Molecular weights of HSV-1 (F) virion polypeptides determined from their migration relative to protein standards of known molecular weight (open circles, identified at the appropriate molecular weight on the ordinate) on 7.75, 8.5, 9.5 and 10.0% polyacrylamide gels crosslinked with DATD. The position of virion polypeptides on the various gels is indicated by short vertical lines intersecting the curve connecting standard proteins and annotated with the appropriate numerical designation. The positions of VP1-3 are not shown since at all of the gel strengths employed their mobilities were less than that of myosin and the relationship between distance of migration and log molecular weight was not linear in this range of mobilities. The positions of minor glycosylated polypeptides 9, 12.3, 12.6, 14.5 and 15.5 are not shown to simplify the figure; however their molecular weights were estimated in an identical fashion to those shown and are summarized in Table 1 together with the estimates obtained for other polypeptides. The migrations of standard proteins and of virion polypeptides were expressed as a fraction of the distance migrated by bromophenol blue dye. Data from Heine et al., 1974.

(Table 1, column d). The computed number of copies based on each of these estimates is shown in columns e and f, respectively. Two comments should be made in connection with these data. A priori, both methods of estimation suffer from systematic errors. Radiochemical analyses suffer from two sources of error, i.e., errors due to the fact that the structural polypeptides do not all belong to the same coordinately regulated groups and, as documented elsewhere, they belong to classes whose temporal patterns of synthesis differ (Honess and Roizman, 1973, 1974). This source of error can be minimized by beginning the labeling interval early in infection. The second error would arise if polypeptides had large differences in the molar percentage of the amino acids used for labeling. However, no large differences were observed between autoradiograms of electrophoretically separated viral polypeptides labeled with a mixture of thirteen ^{14}C-amino acids and those labeled with the three amino acids (Honess and Roizman, 1973). Analyses of Coomassie Brilliant Blue stained gels are free of these errors but suffer from the fact, documented elsewhere (Gibson and Roizman, 1974; Spear and Roizman, 1972), that virion polypeptides differ in their affinity for this stain. For most polypeptides the agreement between the estimates obtained by the two techniques is quite good and it is notable that the exceptions are polypeptides 7, 8, 8.5 and 22 which, as documented elsewhere (Gibson and Roizman, 1974; Spear and Roizman, 1972), differ from other virion polypeptides with respect to their affinity for Coomassie Brilliant Blue.

In general these estimates provide an explicit statement of the molecular complexity of the Herpesvirion. In particular they indicate that a number of major virion components are present in comparable molarities, i.e., VP5, VP19C, VP14 and VP15.8+16.0, and that while many components are present in more than 400 copies per virion, with the possible exceptions of VP22 and 23, none are represented in more than about 1000 copies per particle. Based on either absorbance of autoradiograms or of Coomassie Brilliant Blue stained gels (Table 1) we calculated that the polypeptides known to be in the virus capsid, VP5, 19C, 21, 23 and 24 (Gibson and Roizman, 1972, 1974) make up 20-23 percent of the protein content of the virion. Of the remainder, 33 to 39 percent is made up of glycosylated polypeptides presumed to be in the virion envelope and 33 to 39 percent of non-glycosylated non-capsid polypeptides whose location is uncertain (Roizman and Furlong, 1974).

Comparison of virion polypeptides of several strains of HSV-1. The structural and biochemical studies reported from this laboratory in the recent years have been done with strain HSV-1 [F1] annotated in Materials and Methods. Previous studies from our laboratory have shown HSV-1 [F1] differs from many virus strains propagated serially in the laboratory for many years with respect to immunologic specificity, certain physical properties, viral

FIG. 3. Autoradiograms of a polyacrylamide gel slab containing electrophoretically separated polypeptides from purified virions of HSV-1 (F1), HSV-1 (mP), HSV-1 (MP), HSV-1 (HFEM) and HSV-1 (13 v B4) labeled with ^{14}C-amino acids. The intact autoradiogram is shown on the right together with the designations of HSV-1 (F1) virion polypeptides. To facilitate comparisons, the autoradiograms of each gel are also shown on the left. The arrows on the (-) side of each gel pinpoint the position of HSV-1 (F) polypeptides absent from the various HSV-1 strains examined. The arrows on the (+) side indicate the polypeptides in virions of these other strains for which there is no electrophoretic analogue in the HSV-1 (F1) virions. The arrows against the gel containing the electrophoretically separated HSV-1 (F1) polypeptides indicate all polypeptides whose electrophoretic analogue is absent in one or more of the other HSV-1 strains tested. Designation of polypeptides is the same as shown in Figure 1. Data from Heine _et al._, 1974.

development, and effect of the virus on the social behavior of infected cells (Roizman, 1971; Roizman and Spear, 1971). It was therefore of interest to determine the extent of differences in the structural polypeptides of virus strains differing in their isolation and passage histories. The basis for this comparison was the observation that the electrophoretic profiles of virion polypeptides obtained from numerous independently purified preparations of HSV-1 [F1] in the same type of gel were identical. Moreover, HSV-1 (F1) could not be differentiated from another isolate with a similar history HSV-1 (F5) with respect to the relative amounts and electrophoretic mobility of the virion polypeptides (Heine *et al.*, 1974). In contrast, the autoradiogram of electrophoretically separated virion proteins of 4 HSV-1 strains (HSV-1 [13 v B4], HSV-1 [HFEM], HSV-1 [mP] and HSV-1 [MP]) (Figure 3) showed that the structural polypeptides of strains characterized by long histories of passage in cell culture, and in some instances in experimental animals, differed extensively from those of the HSV-1 [F1] strain. The autoradiograms in Figure 3 are presented as they appeared on the intact gel slab and separately to aid in pinpointing those polypeptides which were either missing in one or more strains or which did not coincide in electrophoretic mobility with those of HSV-1 [F1]. It is noteworthy that the differences were confined to non-capsid proteins and that the strains with a history of numerous laboratory passages differ not only from the limited passage strains but also among themselves.

Studies on the Regulation of Viral RNA and Protein Synthesis

Fraction of DNA represented in RNA transcripts accumulating in different compartments of the cell during the viral reproductive cycle. In this series of experiments, cells were fractionated and RNA extracted at 2, 3, 8 and 14 hours post infection. The results of the hybridization experiments may be summarized as follows:

(i) By conventional definition, "early" events occur before the onset of viral DNA synthesis. We therefore sought to determine the amount of DNA which serves as a template at 2 hours after exposure of cells to virus; i.e., approximately one hour before new viral DNA synthesis begins. The experiments done so far have not provided a definitive answer. In the first study (Frenkel and Roizman, 1972), 45 percent of viral DNA was driven into DNA-RNA hybrid by 2 hour RNA. In more recent studies, illustrated in Figure 4A, it became apparent that the amount of viral RNA accumulating in infected cells was multiplicity dependent. Thus the RNA sequences present in 2 hour infected cells infected at a multiplicity of 40 PFU/cell were roughly equivalent to the amount present at 3 hours after infection with a multiplicity of 10 PFU/cell. Judging by the rate of hybridization, the amount of RNA present at 2 hours after

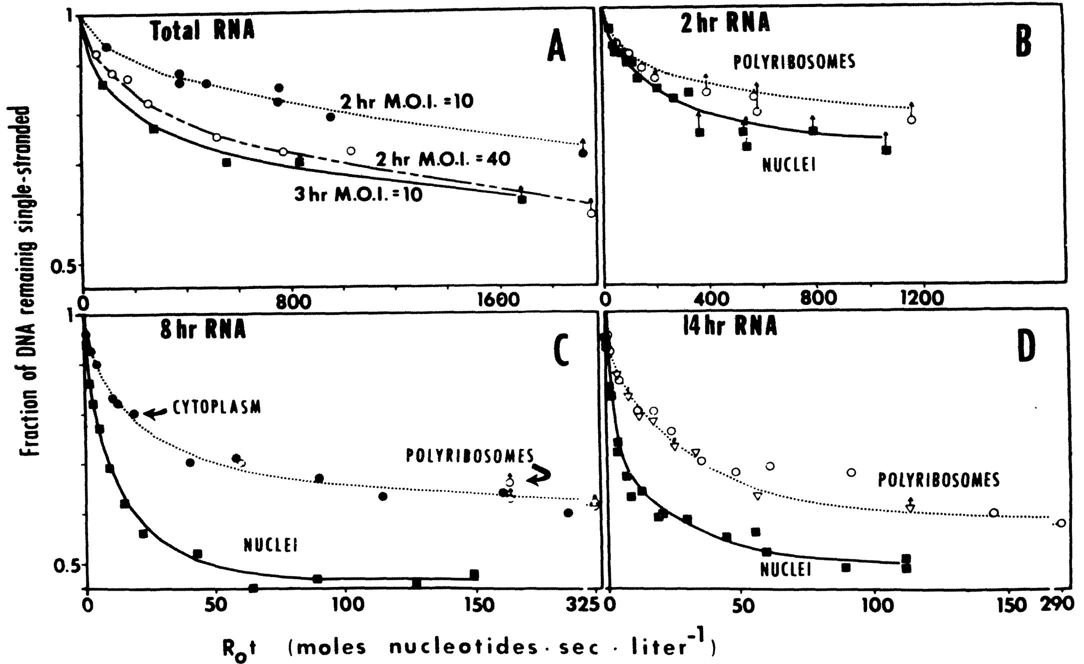

FIG. 4. Hybridization of ^3H-labeled Herpesvirus DNA with excess unlabeled RNA extracted from infected cells. Viral DNA was labeled *in vitro* with ^3H TTP in a repair replication (Kelly *et al.*, 1970) according to the procedures of Nonoyama and Pagano (1973). Viral RNA, extracted from cells infected with 10 PFU/cell unless otherwise indicated, was purified from whole cells or from subcellular fractions by three cycles of treatment with pancreatic DNase followed by extraction with SDS and phenol at 56°C. Polyribosomes were obtained by sedimentation through sucrose gradients containing 0.01 M Tris Cl (pH 7.5), 0.05 M KCl, 0.0025 M $MgCl_2$ and 0.0002 M EDTA. Purified RNA preparations were dialyzed against 0.04 M $NaPO_4$ (pH 6.8) prior to hybridization. The concentration of the reagent in the hybridization mixture ranged from 0.01 to 0.05 μg of DNA and 1 to 15 mg of RNA per ml of buffer consisting of 0.04 M $NaPO_4$ (pH 6.8) and 0.23 M NaCl. Hybridization mixtures were incubated in sealed glass capillary pipets at 75°C for various lengths of time, up to 24 hr. Following incubation, the fraction of DNA converted to hybrid form was assayed by digesting with the *Neurospora crassa* single-strand specific nuclease. Arrows associated with points in the figure indicate the extent of DNA reassociation that occurred in parallel reactions with HEp-2 RNA, incubated for the same length of time as the experimental point. For 14 hr. polyribosomes, the two kinds of symbols represent two separate polyribosome preparations. Data from Roizman *et al.*, 1975.

infection with a multiplicity of 10 PFU/cell was much less than in cells infected for the same amount of time at the higher multiplicity. In neither instance did the hybridization reach completion. It seems obvious that these attempts to measure the amounts of DNA transcribed early in infection suffer from two problems. At relatively low multiplicities sufficient to infect all or nearly all cells, the amounts of viral RNA accumulating in 2 hour infected cells were too low to detect the full amount of viral DNA that might have been transcribed before the onset of its duplication. Although the data predict that sufficient viral RNA might accumulate at 2 hours in cells infected at very high multiplicities of infection to drive 40 to 50 percent of viral DNA into DNA-RNA hybrid, the effect of multiplicity of infection on the time of onset of viral DNA synthesis is not known, and hence no conclusion can yet be drawn from these experiments regarding the extent of early transcription of viral DNA. Alternative approaches--for example, analyses of viral RNA sequences in cells infected and maintained in the presence of drugs which preclude viral DNA synthesis--indicate that transcripts arising from 45 to 50 percent of the DNA accumulate in cells maintained in medium containing cycloheximide (described below and by Frenkel et al., 1973).

(ii) The transcripts extracted from nuclei or whole cells late in infection drove 50 to 55 percent of the DNA into DNA-RNA hybrid. Beyond this point, hybridization levels off and no more DNA was driven into hybrid even at much higher R_0t values.

(iii) The total amount of DNA whose transcripts were represented in polyribosomes ranged from at least 20 percent at 2 hours post infection to a maximum of 40 to 42 percent at 8 and 14 hours post infection. The data shown in Figure 4C suggest, and further summation experiments (not shown) have confirmed, the conclusion that the RNA sequences contained in 8 hour-infected cell cytoplasm were all represented in polyribosomes.

(iv) RNA sequences estimated in different experiments to arise from 8 to 15 percent of viral DNA, accumulated in the nucleus and could not be detected in the cytoplasm at 8 hours post infection. We do not know whether these sequences are selectively retained in the nucleus or whether they are transported and then rapidly degraded.

Synthesis of Viral Polypeptides

Identification and enumeration of primary viral translational products. The objectives of these studies were to enumerate the polypeptides made throughout the viral reproductive cycle, determine whether they are host or virus specific and whether rapid post translational cleavages of these polypeptides take place. The

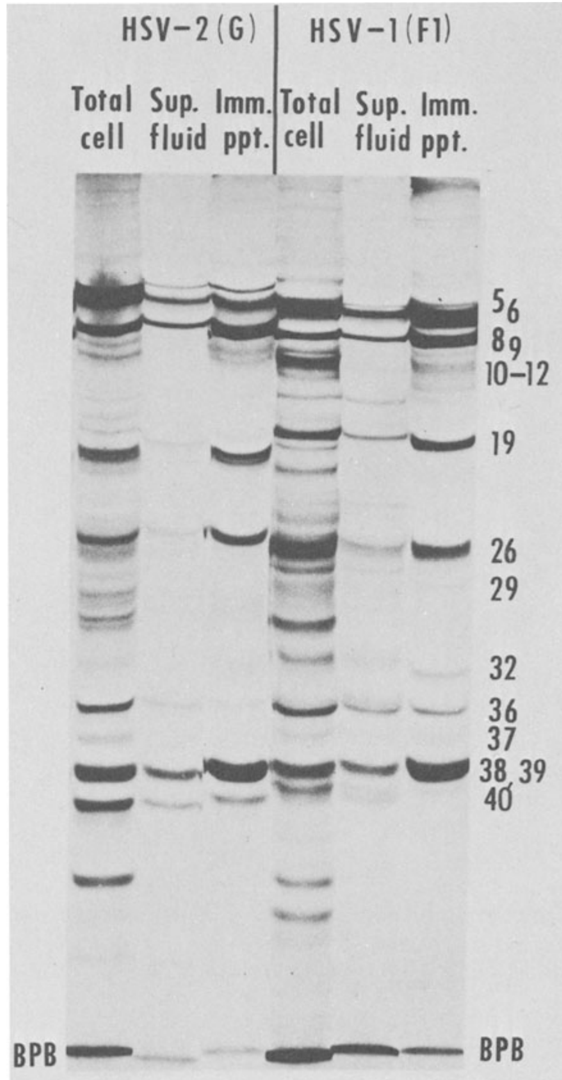
HSV-2(G)
HSV-1(F1)
Total cell
Sup. fluid
Imm. ppt.
Total cell
Sup. fluid
Imm. ppt.
5 6
8 9
10-12
19
26
29
32
36
37
38 39
40
BPB
BPB

FIG. 5. Immune precipitation of labeled polypeptide from infected cells with virus specific immune sera. In this experiment HEp-2 cells were labeled with ^{14}C-amino acids from 4.25 to 6.5 hours after infection with either HSV-1 (F) or HSV-2 (G). The cells were then homogenized and the homogenates sedimented at 35,000 rpm for 1 hour in a Beckman SW40 rotor. The supernatant fluids were then mixed with an excess of rabbit hyperimmune antisera specific for Herpesvirus antigens and incubated at 4° overnight. The resulting immune precipitates were then sedimented at 3500, washed thoroughly by resuspension and sedimentation in ice-cold phosphate buffered saline, and finally resuspended and solubilized by heating in 2 percent SDS, 5 percent β-mercaptoethanol, and 0.05 M tris-hydrochloride, pH 7.0. Detailed descriptions of the preparation and properties of these immune sera and their use in immune precipitation reactions are available elsewhere (Watson *et al.*, 1966; Honess *et al.*, 1974; Honess and Watson, 1974). The polypeptides in the solubilized immune polypeptide precipitate were then separated by electrophoresis of 8.5 percent polyacrylamide gels in parallel with solubilized samples of the unfractionated infected cell homogenates (total cell) and the supernatant fluids (sup. fluid). The autoradiogram shows from left to right: (i) the electrophoretically separated polypeptides made in HSV-2 (G) infected cells, (ii) the polypeptides from the same cells available for immunoprecipitation, (iii) the HSV-2 polypeptides immunoprecipitated by homologous antiserum, (iv) the electrophoretically separated polypeptides made in HSV-1 (F) infected cells, (v) the polypeptides from the same cells available for immunoprecipitation, (vi) the HSV-2 polypeptides immunoprecipitated by homologous antiserum. The difference in intensity of images of the polypeptides in samples of the supernatant fluid and corresponding immune precipitates is due to the fact that the volume of supernatant fluid mixed with antibody was approximately sixfold higher.

The hyperimmune sera specifically and efficiently precipitate a subset of the labeled polypeptides from the homologous infected cells, but give no significant precipitation of polypeptides from uninfected cells (Honess and Watson, 1974). Data from Honess and Roizman, 1973.

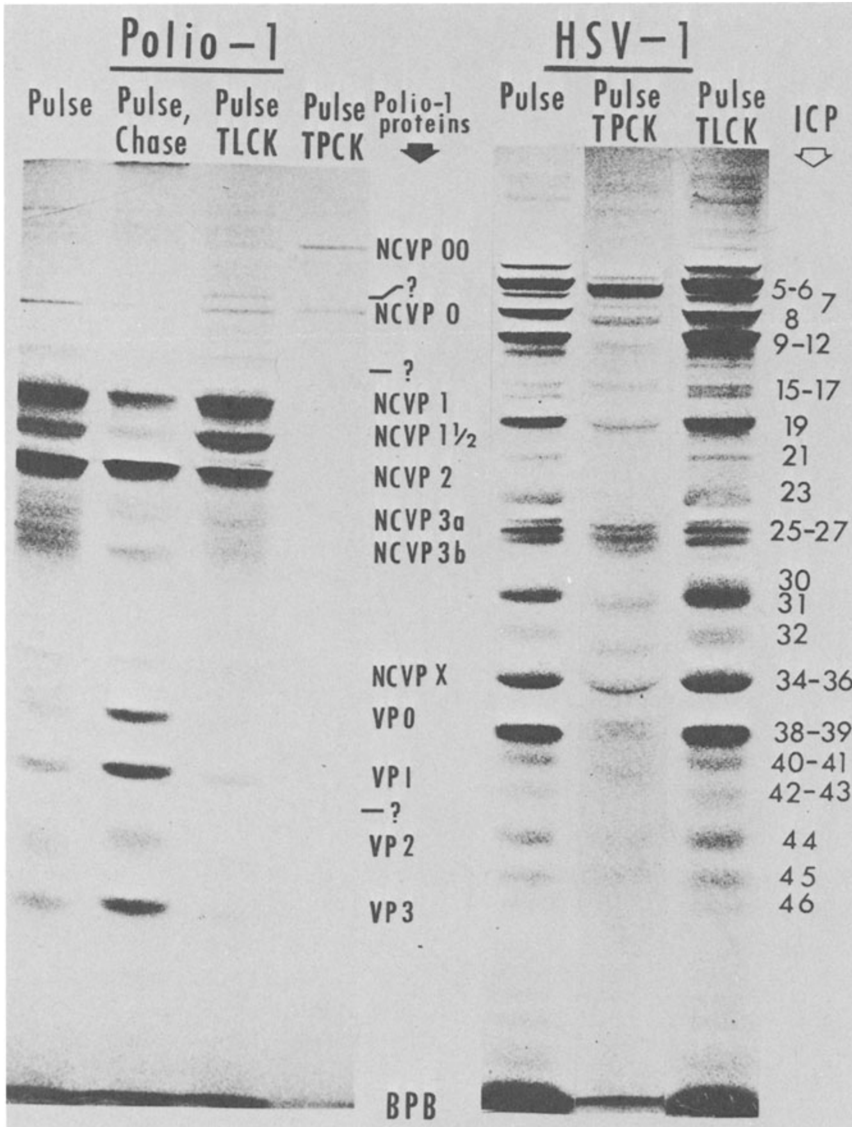
Polio-1
HSV-1
Pulse
Pulse, Chase
Pulse TLCK
Pulse TPCK
Polio-1 proteins
Pulse
Pulse TPCK
Pulse TLCK
ICP
NCVP 00
?
NCVP 0
?
NCVP 1
NCVP 1½
NCVP 2
NCVP 3a
NCVP 3b
NCVP X
VP0
VP1
?
VP2
VP3
BPB
5-6
7
8
9-12
15-17
19
21
23
25-27
30
31
32
34-36
38-39
40-41
42-43
44
45
46

FIG. 6. Test for rapid post-translational cleavages in the synthesis of HSV-1 (F) infected cell polypeptides. Autoradiogram of a 9.0 percent polyacrylamide gel slab containing electrophoretically separated polypeptides labeled with ^{14}C-amino acids in HEp-2 cells infected with poliovirus type 1 and with HSV-1 (F), respectively, in the presence and absence of specific inhibitors of trypsin (tosyl-lysyl chloromethyl ketone, TLCK) and chymotrypsin (tosyl phenylalanyl chloromethyl, TPCK) proteases. Left, polypeptides synthesized in poliovirus infected cells labeled with ^{14}C-amino acids for 15 minutes from 5 hours post infection in the absence of inhibitors (pulse), labeled for 15 minutes and then incubated for a further 40 minutes with medium lacking labeled precursors in the absence of inhibitors (pulse chase), and labeled for 15 minutes in the presence of 10^{-4} M TLCK (pulse TLCK) and TPCK (pulse TPCK). Right, polypeptides synthesized in HSV-1 (F) infected cells during a similar 15 minute labeling interval from 5 hours post infection in the absence (pulse) or presence of the inhibitors (pulse TPCK, pulse TLCK). The poliovirus polypeptides were designated according to Jacobson, Asso, and Baltimore (1970). Selected polypeptides made in HSV-1 (F) infected cells were numbered according to Honess and Roizman (1973).

The inhibitor of chymotrypsin, TPCK, drastically reduced overall protein synthesis in both poliovirus and HSV-1 (F) infected HEp-2 cells, but whereas it almost abolished the appearance of secondary products of rapid post-translational cleavage of poliovirus proteins and led to the accumulation of polyprotein precursors (NCVP 00 and NCVP 0) there was little qualitative effect on the HSV-1 (F) ICP. Notably there was no accumulation of any polypeptide larger than those detected at the same time in untreated cells. Data from Honess and Roizman (1973).

experimental design employed in these experiments involved pulse labeling infected cells with ^{14}C-amino acids at different intervals throughout the reproductive cycle. The cells were then solubilized and the polypeptides were separated by electrophoresis on high resolution polyacrylamide gels. The criteria for classification of the polypeptides as virus specific and the results based on scanning of both Coomassie Brilliant Blue stained gels and of autoradiograms of these gels may be summarized as follows:

(i) Polypeptides were classified as virus-specific on the basis of three criteria. First, since overall host protein synthesis declines after infection (Roizman, Borman, and Kamali-Rousta, 1965; Sydiskis and Roizman, 1966, 1967), polypeptides synthesized at increasing rates at the time of decline of host polypeptide synthesis could therefore be expected to be virus-specific. Second, antisera prepared in rabbits immunized with infected rabbit kidney cells specifically precipitate a number of polypeptides from infected BHK-21, HEp-2 or RK13 cells but do not give significant precipitation of uninfected cell polypeptides (Honess and Watson, 1974). Polypeptides reactive with these antisera (Figure 5) could be considered virus-specific if they also increase in rates of synthesis after infection. Lastly, it has been observed that in cells infected with variants of HSV-1 or with HSV-2 some polypeptides are either over-produced, under-produced or replaced with polypeptides differing in electrophoretic mobilities (Honess and Roizman, 1973). Thus, differences in the electrophoretic mobilities of several polypeptides made in HEp-2 cells infected with HSV-1 (F) and HSV-1 (G) are readily apparent in Figure 5. The polypeptides whose production is under genetic control of the virus may also be considered virus-specific provided that they are absent from uninfected cells and are synthesized at increasing rates after infection. On the basis of these criteria, approximately 48 polypeptides separated in high resolution polyacrylamide gels have been classified as virus-specific (Honess and Roizman, 1973, 1974). They consist of both structural components of the virion and polypeptides for which a counterpart in the virion has not been found and which were therefore classified as nonstructural polypeptides.

(ii) Meaningful analysis of the rates of synthesis of these 48 polypeptides depends on knowing whether they are primary products of translation or whether they undergo rapid post translational cleavage. Polypeptides accumulating in infected cells were studied in three types of experiments: (a) during short and long labeling intervals, (b) during a short pulse followed by a period of chase, and (c) during treatment of infected cells with inhibitors of proteolytic enzymes known to be involved in post translational cleavages. These experiments produced no evidence that virus-specific polypeptides undergo rapid post translational cleavage (Figure 6) even though delayed cleavages of a number of polypeptides

possibly related to virus assembly were readily demonstrable (Honess and Roizman, 1973; Honess and Roizman, unpublished data). Based on this evidence, the 48 virus-specific polypeptides could be considered as primary translational products. The aggregate molecular weight of these polypeptides corresponds to a sequence of 41,000 amino acids (Honess and Roizman, 1973). Given DNA of molecular weight of 10^8 of which a maximum of 50 percent could serve as template for transcripts functioning as mRNA, the polypeptides could account for about 75 percent of the total genetic information of the virus.

Evidence for coordinate regulation of viral polypeptide synthesis. Evidence for coordinate regulation of viral polypeptide synthesis emerged from measurements of the rates of synthesis of polypeptides throughout the viral reproductive cycle. The design of these experiments involved computer-aided planimetry of autoradiographs of polyacrylamide gel slabs containing electrophoretically separated polypeptides from cells pulse labeled at different times after infection. The conditions of labeling of the polypeptides (Honess and Roizman, 1973) were such that variation in amino acid composition of the various polypeptides would not affect significantly the estimates of the amounts of the various polypeptides made during the pulse. The relative molar rates of synthesis of the polypeptides was determined from the absorbance of the autoradiographic image of the band, the molecular weight of the polypeptides, and the relative rates of overall protein synthesis at the time the cells were pulse labeled (Honess and Roizman, 1973). These studies (Figure 7) indicate that in parasynchronously infected cells all polypeptides could be fitted into five classes (A to E) with differing temporal patterns of synthesis. Class E contained polypeptides which could not be identified as virus-specific and which were synthesized at declining rates throughout infection. Polypeptides in classes C and D reached maximum rates of synthesis early in infection and declined afterwards. The two classes differed solely in the time at which maximal rates were attained. However, the polypeptides identified as belonging to class C were mostly structural whereas those identified as belonging to class D were both structural and nonstructural. The rates of synthesis of B class polypeptides reached a maximum early in infection and remained constant thereafter. The polypeptides of class A correspond in electrophoretic mobility to major structural components of the virion and were synthesized at increasing rates until at least 12 hours after infection. Analysis of the rates of polypeptide synthesis in the parasynchronously infected cells also indicated that although the temporal patterns of synthesis of polypeptides in each class were similar, suggesting that they were coordinately regulated, individual polypeptides within a given class were not generally synthesized at identical rates suggesting that a polypeptide abundance control was superimposed on the coordinate, temporal

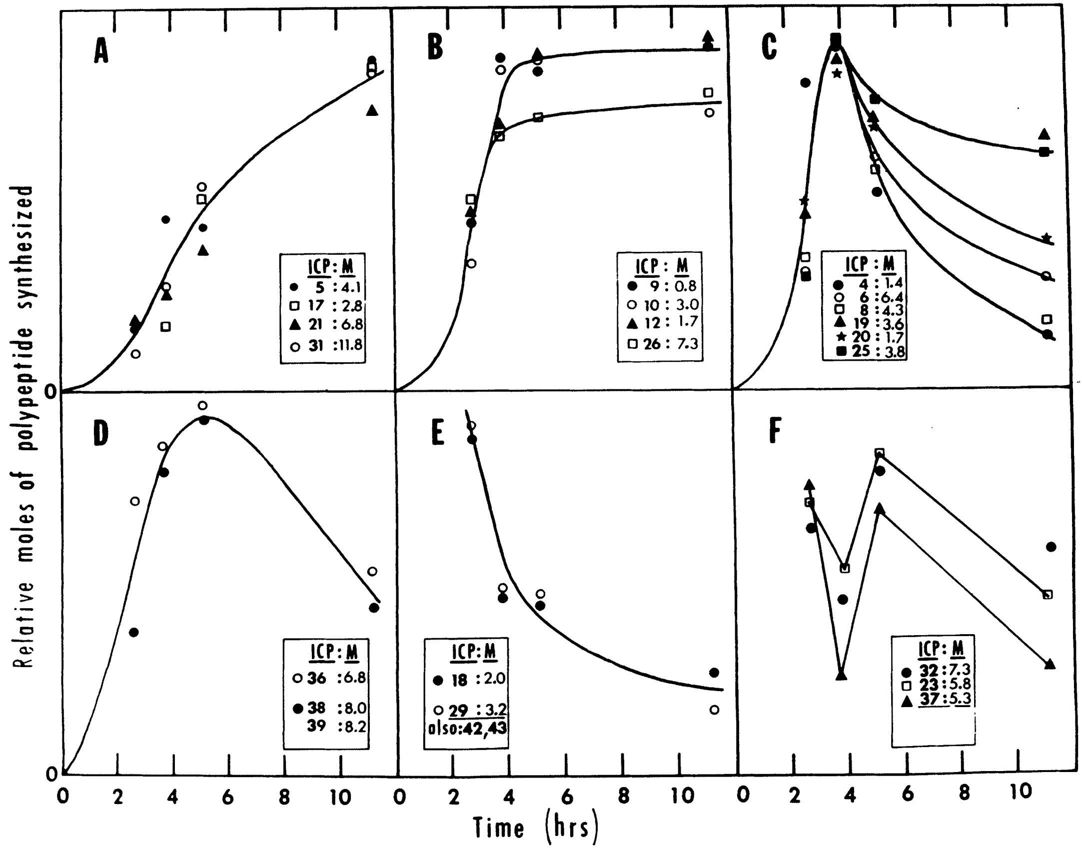
A
ICP: M
5 : 4.1
17 : 2.8
21 : 6.8
31 : 11.8
B
ICP : M
9 : 0.8
10 : 3.0
12 : 1.7
26 : 7.3
C
ICP : M
4 : 1.4
6 : 6.4
8 : 4.3
19 : 3.6
20 : 1.7
25 : 3.8
D
ICP: M
36 : 6.8
38 : 8.0
39 : 8.2
E
ICP: M
18 : 2.0
29 : 3.2
also: 42,43
F
ICP: M
32 : 7.3
23 : 5.8
37 : 5.3
Relative moles of polypeptide synthesized
0
0
0 2 4 6 8 10
0 2 4 6 8 10
0 2 4 6 8 10
Time (hrs)

FIG. 7. Temporal patterns of synthesis of HSV-1 (F) polypeptides. The data presented in this figure are based on three sets of experiments (Honess and Roizman, 1973). In the first, it was established that the variation in the amount of various infected cells polypeptides detected by autoradiography was not affected significantly by the choices of ^{14}C-amino acid mixtures used in this study. In the second, the rates of overall amino acid synthesis were determined by pulse labeling with ^{14}C amino acids replicate cultures of cells infected with 20 PFU/cell for fixed intervals at different times after infection. The value Kt, used in subsequent calculations, expresses the rate of overall protein synthesis normalized with respect to the maximum rate observed in the infected cells. In the third series of experiments, the cells infected at the same multiplicity were pulse labeled for short intervals at different times throughout the reproductive cycle. Labeled polypeptides in cell lysates were then separated by electrophoresis on polyacrylamide gel slabs. Autoradiograms of these gels were scanned in a Gilford spectrophotometer connected to a GA 16/45 computer which integrated the amount of absorbance in the autoradiographic images of the various bands. The relative molar rate of synthesis (RM_i) of the polypeptide during each labeling interval was calculated from the equation

$$RM_i = 10^7 \cdot K_t \cdot \frac{A_i}{M_i \, \Sigma(A_1 \ldots A_n)}$$

where A_i is the absorbance of the image produced by polypeptide (i) of molecular weight M_i, $\Sigma(A_1 \ldots A_n)$ is the sum of absorbances of all autoradiographic images in the gel, K_t is the normalized overall rate of protein synthesis for the pulse labeling interval t, and the constant (10^7) is a number of convenience inserted to avoid working with very small numbers.

The figure shows the data obtained for a number of HSV-1 (F) polypeptides, clustered into classes (A-F) on the basis of the observed patterns of variation in molar rates of synthesis. Polypeptides making up each class are identified in the boxed legends in the figure. Since the RM for the polypeptides within each class are not the same the plots were normalized. However, the largest value of RM for the polypeptides shown is given under column M in the boxed legend next to the number and symbolic representation of the polypeptides. As might be expected, some of the bands contained more than one polypeptide. Examples of patterns obtained for such bands are shown in F. By varying the gel strength, the individual polypeptides in bands shown in F were sufficiently separated to permit reclassification of these polypeptides in classes A to E. Data from Honess and Roizman (1973).

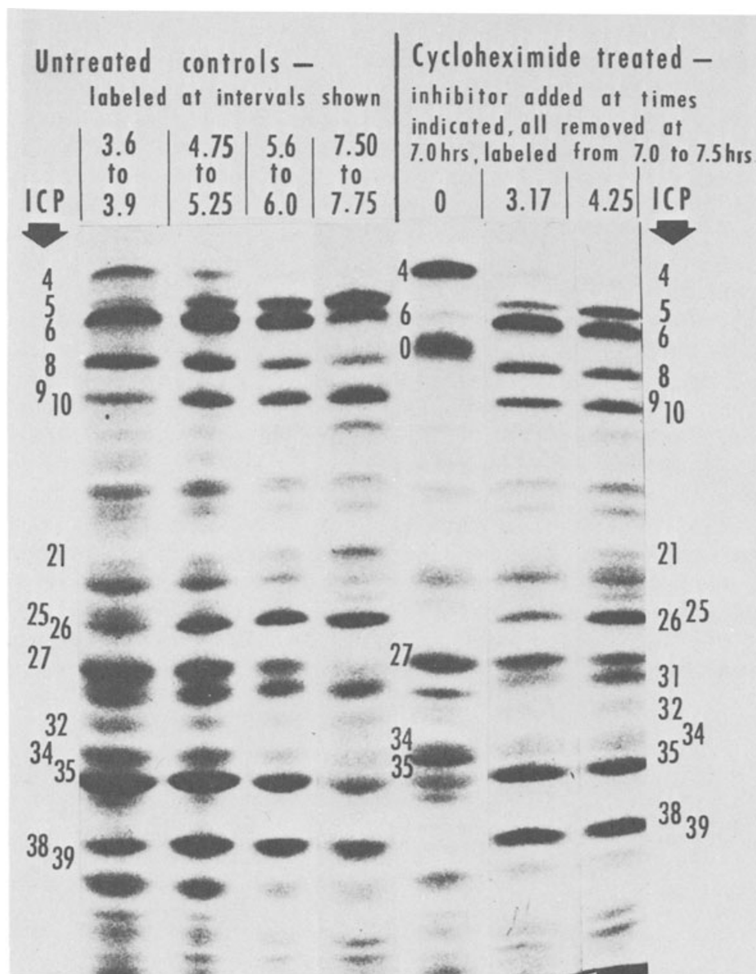

FIG. 8. Polypeptide synthesis in untreated HSV-1 (F) infected HEp-2 cells and in cells immediately after treatment with cycloheximide for various intervals after infection. Autoradiogram of a polyacrylamide gel slab containing electrophoretically separated polypeptides from cell lysates of HEp-2 infected with HSV-1 (F) and labeled with ^{14}C-amino acids at intervals during the first 8 hours of the viral reproductive cycle (left) untreated control samples or from 7.0 to 7.5 hours post infection after the removal of 50 μg/ml of cycloheximide added at either 0, 3.17, or 4.25 hours (right). Note that only host and α viral polypeptides are made immediately following the removal of the inhibitor added at the time of infection. Addition of cycloheximide at 3.25 hours, i.e., after some infected cell protein synthesis has taken place results in the synthesis of both β and γ viral polypeptides immediately after the removal of the drug. Data from Honess and Roizman (1974).

control of synthesis. Another interesting finding is that structural and nonstructural polypeptides could not be differentiated with respect to their range or their average molar rates of synthesis.

Evidence for sequential ordering of coordinately regulated viral polypeptide synthesis. These experiments were based on the observation that transcripts arising from at least 45 percent of viral DNA accumulate in cells treated with inhibitory concentrations of cycloheximide (Frenkel et al., 1973) or of puromycin (Silverstein and Roizman, unpublished data) for 2 or more hours from the time of exposure of cells to virus. Based on the possibility that all of the transcripts accumulating in treated cells might be available for translation, the question arose whether all viral polypeptides or only a specific class of polypeptides would be made immediately after withdrawal of the drug. The design of the experiments summarized in this section was as follows. Cells were exposed to either cycloheximide or puromycin, either at the time of exposure to virus or at intervals thereafter. At times indicated in the text or legends to figures, the cells were pulse labeled with ^{14}C-amino acids for a brief interval either immediately after withdrawal of the drug or at intervals thereafter. The results obtained with cycloheximide and with puromycin were similar. Analysis of the polypeptides made after withdrawal of inhibitors of protein synthesis revealed four aspects of the regulation of viral protein synthesis.

First, viral polypeptides could be readily segregated into three groups designated α, β and γ. Polypeptides in group α consisted of one minor structural (#4) and several nonstructural polypeptides (#0, 27). The synthesis of these polypeptides required no prior infected cell protein synthesis; i.e., they were made immediately after withdrawal of inhibitors of protein synthesis added to the medium at the time of infection or 1 hour later (Figures 8, 9). The initial rates of synthesis of these polypeptides immediately following withdrawal of the drugs increased as the duration of drug treatment increased (Figure 9), indicating that the mRNA specifying these polypeptides was stable and accumulated in the presence of inhibitors of protein synthesis. In untreated cells, these polypeptides were synthesized at maximal rates between 3 and 4 hours post infection and at decreasing rates thereafter. The β group (e.g., #6, 8, 36) also consisted of minor structural and nonstructural polypeptides. In untreated cells they were synthesized at maximal rates between 5 and 7 hours after infection and, like the α polypeptides, at decreasing rates thereafter. β group polypeptides were made immediately after withdrawal of inhibitors of protein synthesis only if addition of the inhibitors was postponed until after 1 to 1.5 hours post infection; i.e., until after α polypeptides had been synthesized (Figures 9, 10). The initial rates of synthesis of β polypeptides after withdrawal of inhibitors exceeded those

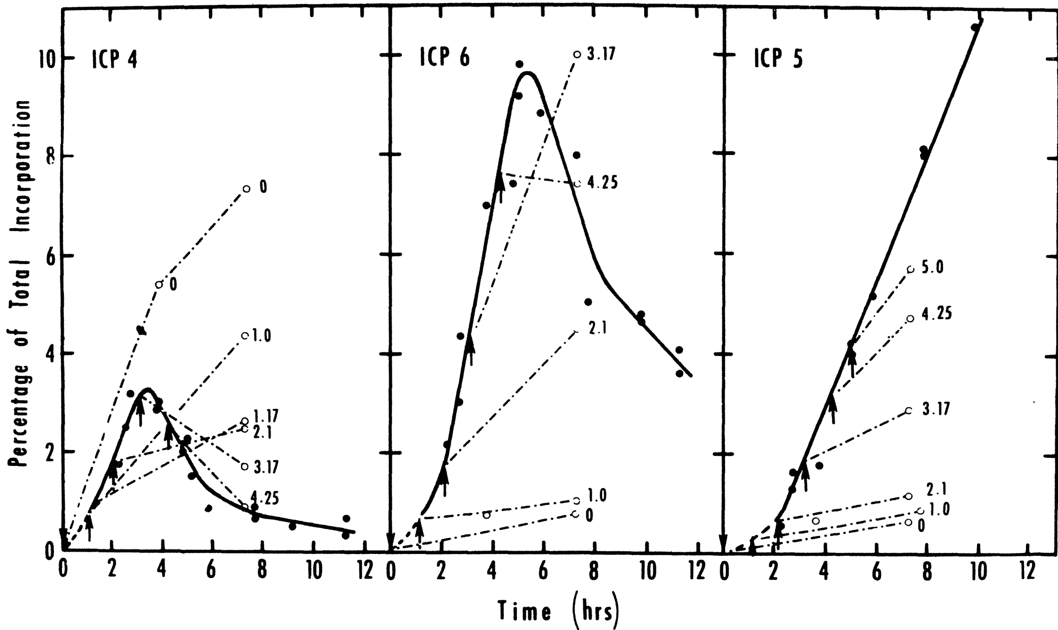

FIG. 9. The synthesis of infected cell polypeptides (ICP) representative of α (ICP4), β (ICP 6) and γ (ICP 5) groups of viral polypeptides during the normal virus growth cycle and after different intervals of treatment with cycloheximide. The amounts of each polypeptide made are expressed in terms of their percentage contribution to the total incorporation of ^{14}C-amino acids into protein during 0.25 to 0.5 hour labeling intervals. The filled circles connected by the solid line represent measurements of the synthesis of polypeptides immediately after the removal of cycloheximide. The drug was added at the times indicated both by arrows and by the arabic numbers next to the open circles. Corresponding times of addition and removal are interconnected by dashed lines. The data were derived by planimetry of autoradiograms of electrophoretically separated polypeptides similar to those shown in Figure 5. Similar data were obtained for other polypeptides of each group, notably for ICP 0 (α group) and ICP 8 (β group) shown in Figure 7. Although the data are expressed in percentages of total amino acid incorporation, they reflect real differences in molar rates of synthesis. Specifically in four experiments the specific activities of samples derived from cells labeled immediately after withdrawal of cycloheximide present in the medium from 0 to 7 hours post infection were all 50 percent of those of untreated cells labeled at the same time. Yet four- to tenfold differences were observed in the percentage of incorporation of amino acids into α polypeptides of the samples from treated and untreated cultures. Data from Honess and Roizman (1974).

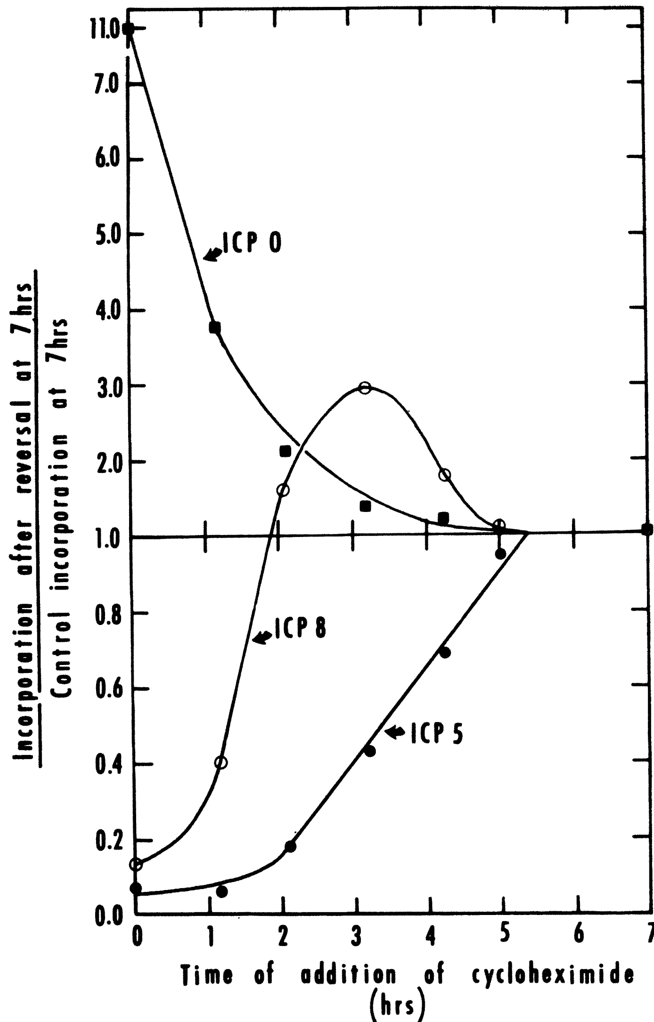

FIG. 10. Initial rates of α (ICP 0) (filled squares), β (ICP 8) (open circles), and γ (ICP 5) (filled circles) polypeptides observed on removal of cycloheximide added at different times and removed simultaneously at 7.0 hours post infection. The data are expressed as the ratio of the percentage of total radioactivity incorporated during a 30 minute labeling interval immediately after removal of cycloheximide to the percentage of incorporation into the same polypeptide in untreated cultures labeled from 7.5 to 7.75 hours. Data points are plotted as a function of the time of addition of the inhibitor and are based on analyses of autoradiograms similar to that shown in Figure 5. Almost identical plots were obtained for other polypeptides of each group, notably for ICP 4 (α group) and ICP 6 (β group) shown in Figure 6. Data from Honess and Roizman (1974).

observed in untreated cells only when cycloheximide was added between 3 and 4 hours post infection, i.e., after synthesis of α polypeptides. The γ group consisted largely of the major structural polypeptides (e.g., #5, 21, 31) contained in the A class, i.e., in untreated infected cells they were synthesized at progressively increasing rates. In treated infected cells, the γ group polypeptides were synthesized immediately after removal of inhibitors of protein synthesis only if addition of the drug to the medium was delayed sufficiently to permit some of both α and β polypeptides to be made. However, at no time did the initial rates of synthesis of γ polypeptides exceed those of untreated cells (Figures 9, 10).

The second point concerns the requirements for α and β polypeptide synthesis and two comments should be made. The function supplied by α polypeptides which enabled β polypeptides to be made was stable. This is evident from the observation that β polypeptides were made immediately after withdrawal of cycloheximide present in the medium from 3 to 7 hours post infection, i.e., α polypeptides made prior to cycloheximide addition persisted in the infected cells during the four hours of cycloheximide inhibition, and were then able to mediate β polypeptide synthesis upon removal of the cycloheximide. Furthermore, the data presented in Table 2 indicate that the presence of α polypeptides alone was not sufficient for the synthesis of β polypeptides which seem also to require new RNA synthesis. This conclusion is based on the observation that addition of actinomycin D at the time of removal of cycloheximide present in the medium from the time of infection allowed the continued synthesis of α polypeptides, but precluded the synthesis of β polypeptides. Similarly, addition of actinomycin D at the time of maximal rates of synthesis β polypeptides precluded the synthesis of γ polypeptides. It is worth noting that in the presence of actinomycin D ongoing synthesis of α and β polypeptide synthesis appeared to decline significantly more slowly than γ polypeptide synthesis, raising the possibility that the half-life of γ mRNA is shorter than those of the mRNAs specifying α and β polypeptides.

The third observation relates to cessation of α and β polypeptide synthesis late in infection. The data summarized in Table 2 show that α polypeptide synthesis declined very rapidly when β polypeptides were made and more slowly when β polypeptide synthesis was blocked by actinomycin D. A similar relationship was found between β and γ polypeptide synthesis. It is noteworthy that host polypeptides (e.g., #34 and 35), which, like α polypeptides, were also made immediately after withdrawal of inhibitors of protein synthesis added at the time of infection, were also shut off once β polypeptides were made. The host polypeptides differed from α polypeptides in two respects. Unlike α polypeptides, their initial rate of synthesis following withdrawal of the drugs declined as the duration of cycloheximide treatment was prolonged. Moreover, the

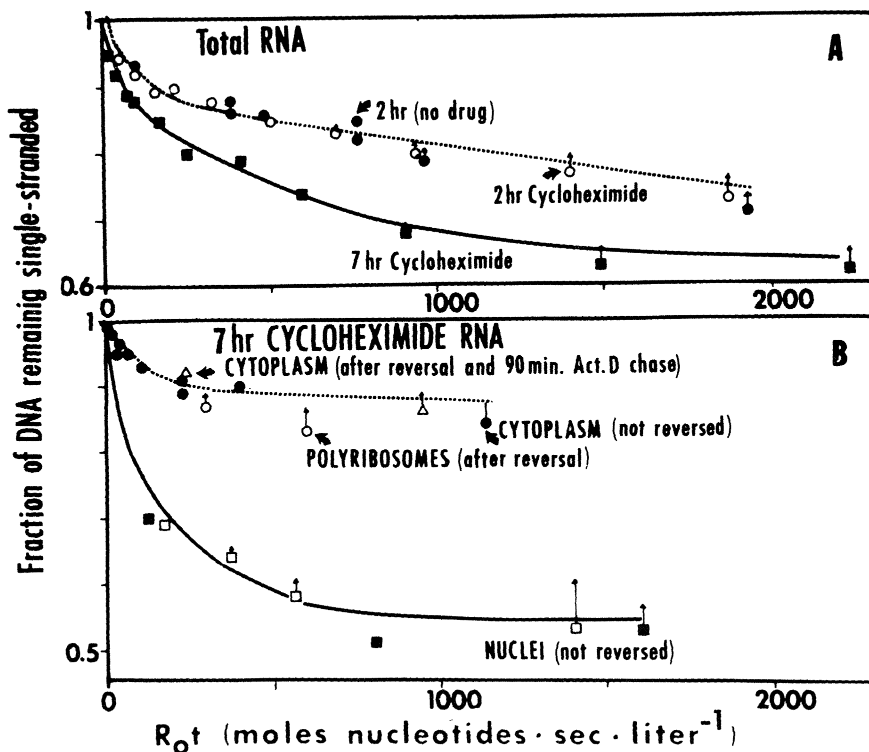

FIG. 11. Hybridization of labeled Herpes DNA with excess RNA extracted from cells infected at a multiplicity of 10 PFU/cell in the presence of cycloheximide (50 to 100 μg/ml). Where indicated, cycloheximide inhibition was reversed by rinsing the cell monolayer four times with 25 ml aliquots of warm, antibiotic-free medium. Actinomycin was used, where indicated, at a final concentration of 10 μg/ml. Background DNA reassociation is indicated as in Figure 4. For 7 hr cycloheximide nuclear RNA, the two kinds of symbols represent two different RNA preparations. Data from Roizman et al., 1975.

TABLE 2. Effects of actinomycin D on synthesis of polypeptides in untreated infected cells and after removal of cycloheximide

Group	Infected cell polypeptide number	Amounts of polypeptide synthesized[a] (1) Untreated cells Pulse 7.0 to 7.5	(2) Untreated cells Pulse 9.5 to 10.0	(3) Actinomycin D 7.0 to 9.5 Pulse 9.5 to 10.0	(4) Cycloheximide treated from 1.0 to 7.0 h Pulse 7.0 to 7.5 h	(5) Cycloheximide treated from 1.0 to 7.0 h Pulse 9.5 to 10.0 h	(6) Cycloheximide treated from 1.0 to 7.0 h Actinomycin D 7.0 to 9.5 Pulse 9.5 to 10.0 h
α	4	2.2	1.3[c]	1.6	7.4	2.9	6.8
	0	-	-	1.6	13.9	1.5	5.1
β	6	13.7	7.1	10.6	3.0	5.4	2.5
	8	9.8	5.1	5.8	1.7[c]	3.7	1.7[c]
γ	5	12.5	13.6	6.7	1.5[c]	2.8	1.1[c]
Host	34,35	9.5	4.2	9.7	14.9	7.7	10.1
Relative incorporation of ^{14}C-amino acids into total protein in sample[b]		1.54	1.27	1.16	1.68	1.00	1.38

[a]In this experiment 12 replicate cultures of HEp-2 cells were infected at a multiplicity of 10 to 20 PFU/cell. Cycloheximide (50 μg/ml) was added to six of these cultures at 1.0 hours post infection and removed at 7.0 hours. Two treated and two untreated infected cultures were labeled with ^{14}C-amino acids from 7.0 to 7.5 hours. At the time of removal of cycloheximide, actinomycin D (10 μg/ml) was added to each of two treated and two untreated cultures and these cultures together with those not treated with actinomycin D were labeled from 9.5 to 10.0 hours post infection. Labeled polypeptides from lysates of each of these samples were then separated on polyacrylamide gel slabs and the amounts of the polypeptides of interest made during the above labeling intervals were quantitated by computer-aided planimetry of the absorbance tracings of the autoradiograms. The data were first expressed as the percentage of total amino acid incorporation contributed by each polypeptide and then corrected for differences in the total incorporation of labeled amino acids so that amounts of polypeptide synthesized in different samples could be compared.

TABLE 2. Legend--continued.

[b]Relative incorporation of ^{14}C-amino acids into total protein in various samples, expressed as the ratios of the total integrated absorbance of autoradiograms from electrophoretically separated samples. Note that the amount of polypeptide synthesized is the product of the relative incorporation into the sample and the percentage of total integrated absorbance due to a given polypeptide. These data are shown to indicate the extent of variation in total amino acid incorporation observed in these samples.

[c]Values are maximum estimates for amounts of these minor components.

[d]Data from Honess and Roizman (1973).

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initial rate of synthesis of α polypeptides increased as the multiplicity of infection increased, whereas host polypeptides were made at decreasing rates when a higher multiplicity of infection was used (Honess and Roizman, 1974).

The last observation is that inhibitors of DNA synthesis did not affect the transition from α to β polypeptide synthesis but did reduce the amounts of γ polypeptides made by up to 50 percent (not shown). The data suggest that parental viral DNA is capable of supplying mRNA for all three groups of polypeptides.

Viral RNA Synthesis and Transport in Cells Treated with Cycloheximide

As noted previously, studies published elsewhere (Frenkel *et al.*, 1973) showed that at least 45 percent of viral DNA was transcribed in the presence of cycloheximide added from the time of infection. However, the studies described in the preceding section of this paper showed that only a subset of viral polypeptides, i.e., only the α group, was made immediately after withdrawal of cycloheximide and that the synthesis of β and γ polypeptides required new RNA synthesis. The objective of the experiments described below was to determine the nature and distribution of viral RNA made in the presence of cycloheximide. In this section we are reporting three series of experiments. In the first, we measured hybridization of ^{3}H-labeled DNA to unlabeled RNA extracted from cells infected in the presence of cycloheximide and maintained in the presence of the drug for 2 and 7 hours, respectively. The data (Figure 11) show that the hybridization of RNA extracted at 2 hours post infection from treated and untreated cells cannot be differentiated with respect to the rate at which it drives viral DNA into hybrid. In this study, the hybridization did not reach completion,

and hence the amount of the DNA whose transcripts accumulated in the cells is not known. It is noteworthy that the amount of viral RNA present in cells treated for 7 hours increased relative to that present in 2 hour treated cells (Figure 11). However, comparison of the kinetics of hybridization shows that the amount of viral RNA in 7 hour treated cells was substantially lower than in untreated 8 hour infected cells (Figures 4, 11) in which a considerable amount of viral DNA synthesis had already taken place.

The second series of experiments dealt with the distribution of viral RNA sequences in cells treated with cycloheximide for 7 hours from the time of infection. The data (Figure 11B) show that before the withdrawal of cycloheximide the nuclei contained RNA sequences arising from 45 to 50 percent of viral DNA whereas the cytoplasm contained RNA arising from only 10 percent of the DNA. RNA sequences arising from the same limited portion of DNA were found in the polyribosomes formed immediately after removal of cycloheximide and this amount did not increase during a 90 minute chase with actinomycin D. The data thus indicate that (i) in the presence of cycloheximide, transcripts arising from only 10 percent of the DNA were transported from the nucleus to the cytoplasm and were functional as judged by their presence in polyribosomes following cycloheximide reversal. (ii) Transcripts arising from an additional 35 to 40 percent of viral DNA were present in the nucleus but were not transported into the cytoplasm in the presence of the drug. (iii) The α polypeptides made in the presence of actinomycin D following cycloheximide withdrawal did not facilitate transport of the RNA which had not been transported from the nucleus prior to the synthesis of these polypeptides. The last finding could be interpreted in one of three ways: (i) α polypeptides play a role in transport of mRNA not defined by these experiments, (ii) the α polypeptides function only if present in the nucleus during the synthesis of RNA or (iii) the unexported RNA sequences arising from 35 to 40 percent of viral DNA cannot be transported because they are defective either because of improper transcription or because of subsequent limited degradation.

The third experiment in this series was designed to determine whether the same DNA sequences were transcribed in the presence of cycloheximide as in its absence.

The summation hybridization experiment summarized in Table 3 showed that the amount of viral DNA hybridized in the presence of RNA from both 7 hours cycloheximide-treated infected cells and 8 hour-infected untreated cells did not significantly exceed the amount of hybrid formed when each RNA was tested individually. Furthermore, preincubation of the two RNA preparations together under conditions appropriate for annealing did not reduce the amount of DNA subsequently driven into hybrid by the combined RNAs,

indicating absence of complementary sequences between the two RNA preparations. Thus, the bulk of the sequences transcribed in the presence and absence of cycloheximide are identical.

DISCUSSION

The Structural Proteins of Herpes Simplex Virus

The number of polypeptide species in the HSV-1 virion. In an earlier paper (Spear and Roizman, 1972) our laboratory reported that polypeptides contained in HSV-1 (F) virions formed 24 bands on electrophoresis through polyacrylamide gels crosslinked with N, N'-methylbisacrylamide (MBA). Analysis done in polyacrylamide gels crosslinked with DATD (Heine et al., 1974) resolved several additional species of polypeptides. The separations of non-glycosylated polypeptides obtained in those gels were in general comparable to those previously obtained on MBA crosslinked gels, and the agreement between the molecular weights estimated in this and preceding studies was excellent (Table 1, columns a and b). The main advantage of the DATD crosslinked gels was their ability to separate a number of minor, glycosylated proteins from the non-glycosylated polypeptides with which they comigrated on MBA crosslinked gels. These analyses on DATD gels have shown that the HSV-1 virion contains at least 33 electrophoretically distinct species of polypeptides. Several comments should be made in connection with this observation. Although the 33 polypeptides do not exceed the potential informational content of HSV-1 DNA, we cannot exclude the possibility that not all polypeptide moieties of the 33 glycosylated and non-glycosylated polypeptides are primary gene products. It may be that the virion contains both a non-glycosylated precursor and its glycosylated product or the precursor and products from cleavage of a glycosylated polypeptide. However, the improved separation of minor glycosylated polypeptides has shown that they do not differ significantly in the extent of their glycosylation from that of the major glycoproteins. Thus if minor glycosylated polypeptides represent a glycosylated subset of other non-glycosylated virion polypeptides, it is apparent that glycosylation is as extensive on this subset as on the major glycoproteins.

Variability in the structure of the HSV-1 virions. The observed variation in the polypeptide composition of the virions from these strains has a number of implications. First, it is evident that the observed variations are not random, but rather that certain polypeptides vary among the virus strains whereas others are invariant in all strains so far examined. The variable polypeptides are chiefly non-capsid components. This is perhaps not surprising since the constraints on the conformation of non-

TABLE 3. Analysis of viral RNA made in the presence of cycloheximide

Model			Prediction
1	7 h	Cyclo	No summation, no RNA reassociation
	8 h		
2	7 h	Cyclo	Summation, RNA reassociation
	8 h		
3	7 h	Cyclo	Partial summation, RNA reassociation
	8 h		

Preincubation of RNA	RNA* present during hybridization			Percent DNA in hybrid			
	7 h	Cycloheximide	8 h	Observed	Predicted by model 1	2	3
Alone	1,063		---	35;36			
Alone	1,275		---	33;36			
Alone	---		172	31;35			
Alone	---		400	46			
Alone	1,063		172	40;41	46	70	>46
Together	1,144		187	41;46	46	≪36	<36
Together, then denatured	1,144		187	42;45	46	70	>46

TABLE 3. Legend.

The models being tested are shown at the top. The parallel lines represent viral DNA. The heavy lines represent the regions of the DNA transcribed. Model 1 assumes that the same sequences are transcribed in both presence and absence of cycloheximide. Model 2 assumes that DNA transcribed in the presence of cycloheximide is complementary to the template transcribed in the absence of the drug. Model 3 assumes that the sequences transcribed in the presence and absence of the drug are in part homologous, in part complementary.

The lower portion of the table shows the results of hybridizations designed to test the models.

The RNAs were preincubated, individually or together, for 10 hours. Denatured, ^{3}H-labeled viral DNA was then hybridized with one of both RNAs for 12 hours. Each condition was tested in duplicate. There was 6% DNA reassociation during a parallel 12 hours incubation with HEp-2 RNA.

*Numbers indicate R_0t. Data from Roizman et al., 1975.

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capsid polypeptides, which would include those of the envelope, may be lower than those of capsid polypeptides. Although it is apparent from these studies that the pressures selecting these variations are not uniform for all polypeptides, the nature of the selection processes are not presently known. The observation that a number of laboratory strains differ from two isolates of HSV-1 passaged a limited number of times in culture may mean that the initial isolates were different or that the laboratory strains represent variants selected for some advantageous property in the cells in which they were passaged. The first hypothesis implies that wild strains of HSV-1 vary both in time and geographically. Although this may well be the case, definitive data that would substantiate or refute this hypothesis are lacking. However, several observations support the alternative. Thus, HSV-1 (MP) arose spontaneously in a culture infected with HSV-1 (mP) and has a selective advantage in cultures infected at low multiplicities in that it spreads from infected to uninfected cells more rapidly than the parent strain (Hoggan and Roizman, 1959b). Another example is the selection of spontaneous mutants (HSV-1 (MPdk$^+$lp) and HSV-1 (MPdk$^+$sp) capable of multiplying in dog kidney cells from cultures of these cells abortively infected with the HSV-1 (MP) virus (Roizman and Aurelian, 1965). The mutants differed from the parent HSV-1 (MP) with respect to immunologic specificity, buoyant density in CsCl solution and stability at 40°C--all of which are consistent with altered virion structure.

Finally, the data have some important practical implications since concurrent with, or related to the variations in the non-capsid components, are two other properties of the virus. Thus, the virion may exhibit altered immunologic specificity as has been clearly demonstrated for HSV-1 (MP) which is neutralized equally well by both anti HSV-1 and HSV-2 sera whereas the parent HSV-1 (mP) virus is more readily neutralized by anti HSV-1 than by anti HSV-2 sera (Ejercito et al., 1968; Plummer et al., 1970). Moreover, cells infected with the laboratory strains differ from those infected with HSV-1 (F1) in details pertaining to development as evident from electron microscopic studies (Schwartz and Roizman, 1969) and from the nature of the social interaction (i.e., tight clumps, loose clumps, polykaryocytosis) of infected cells among themselves (Ejercito et al., 1968; Roizman, 1971; Keller, Spear, and Roizman, 1970; Roizman and Spear, 1971). The obvious implications are that virus strains used as antigenic reagents in seroepidemiologic studies must be shown to be identical with the strains circulating in the population being analyzed since misleading data may otherwise result. Moreover, biochemical studies should include sufficient information on the virus strain being used to assess the validity of comparisons of data generated in different laboratories.

Regulation of Viral RNA and Protein Synthesis

The data presented in this paper indicate that synthesis of both viral RNA and proteins is regulated at several levels. The sites of regulation of the functions of viral RNA revealed in these studies are listed in Table 4. Several additional comments should be made.

Controls of transcription of viral DNA. We may in principle invoke three kinds of controls. These are temporal controls which define the regions of the DNA transcribed at different times in the reproductive cycle, abundance controls which define the amounts of viral RNA transcribed from each region, and functional controls which determine whether a particular set of RNA sequences contained in a transcript will ever be translated. We have at present no evidence that temporal controls are effected in HEp-2 cells infected with HSV-1. The reasons for this are as follows: First, all measurements to date have been done on RNA accumulating in infected cells rather than on RNA made at particular times in the reproductive cycle. The only interval suitable for an analysis based on current methods, therefore, is early in infection, i.e., before the onset of DNA synthesis. In this instance the failure to see as much transcription as occurs late in infection could be the consequences of two factors; namely, low viral RNA concentrations and differences in abundance of the RNA. In the final analysis, we cannot operationally differentiate between a temporal control and an abundance

TABLE 4. Apparent events regulating the synthesis of viral gene products

Process	Regulation	Determinants
Transcription:	Synthesis of transportable and nontransportable transcripts	Availability of α and β proteins
Processing and transport:	Selective adenylation	Appropriate 3' signals in RNA
	Selective transport	Translatable RNA and/or the presence of viral proteins
Translation:	Coordinate onset and cessation of synthesis	Cognate signals in DNA and mRNA
	Cascade regulation of α, β, and γ proteins	Presence of specific viral proteins

control. Evidence for differences in abundance of RNA accumulating in cells has been noted elsewhere (Frenkel et al., 1973). We have, however, no evidence that abundance is mediated at the level of transcription rather than at a post-transcriptional level. Furthermore, analyses of RNA abundance are rendered more complex by the recent finding (Kozak and Roizman, manuscript in preparation) that a stable fraction of viral RNA arising from at least 15 percent of viral DNA can self-anneal under the conditions employed for hybridization. Currently the best evidence that any controls operate at the level of transcription arises from functional differences in the RNA made both in the presence and absence of inhibitors of protein synthesis. Thus in untreated infected cells the RNA detected on polyribosomes arises from a maximum of 42 percent; transcripts arising from an additional 8 to 14 percent of the DNA are retained in the nucleus and are not found in the cytoplasm. In cycloheximide treated cells, RNA arising from as much as 30 to 35 percent of DNA is selectively retained in the nucleus and cannot be detected in the cytoplasm. Since the evidence indicates that the RNA retained in the nucleus during cycloheximide treatment contains the same sequences as the RNA present in the cytoplasm of untreated cells late in infection, and since these sequences cannot be detected in the cytoplasm even after α proteins are made during an actinomycin D chase following cycloheximide removal, a plausible interpretation of the data is that these sequences are contained in nonfunctional molecules. The molecular difference between functional and nonfunctional transcripts is not known. They may differ in size, in initiation and termination sequences, in extent of adenylation, etc.

Controls of processing and transport. Among the known post-transcriptional alterations of RNA transcripts are cleavage of message precursor, base modification, adenylation, de-adenylation and transport. For HSV transcripts, cleavage of RNA was deduced from the discrepancy in the size of homologous sequences in nuclei and cytoplasm (Wagner and Roizman, 1969; Roizman et al., 1970; Jacquemont and Roizman, manuscript in preparation). The presence of poly A in viral transcripts (Bachenheimer and Roizman, 1972) and decrease in the size of poly A segment with time (Bachenheimer and Roizman, unpublished data) have been described. It has also been shown that transcripts arising from specific regions of viral DNA differ at least in the extent of adenylation (Silverstein et al., 1973). In this paper we would like to focus specifically on transport. Two observations are pertinent here. First, at 8 hours post infection, all of the viral RNA sequences present in the cytoplasm were also represented in the polyribosomes. Additional sequences, presumably non-translatable and arising from 8 to 14 percent of the DNA, accumulated in the nucleus but could not be detected in the cytoplasm. This picture is even more accentuated in cells treated with cycloheximide for 7 hours from the time of infection, in which case RNA sequences arising from only 10 percent of the viral DNA accumulated in the cytoplasm and were represented in polyribosomes following cycloheximide removal. Yet the nuclei of these cells contained sequences arising from an additional 35 to 40 percent of the DNA. We cannot at this time determine whether sequences which we fail to detect in the cytoplasm are retained in the nuclei or are transported and rapidly degraded. The hypothesis that these sequences are retained in the nuclei is tantalizing since it would suggest that the nucleus filters out only functional RNA.

Translation of viral mRNA. Analyses of the synthesis of virus-specific polypeptides have revealed three phenomena; namely, coordinate regulation of viral polypeptide synthesis, sequential ordering of the synthesis of coordinated groups, and a superimposed polypeptide abundance control. It is convenient to discuss the first two together.

Evidence of coordinate regulation of viral polypeptide synthesis emerged from two independent series of experiments, i.e., from analyses of rates of synthesis of viral polypeptides in untreated infected cells (Figure 7 and Honess and Roizman, 1973) and from the sequential ordering of polypeptide synthesis obtained in infected cells treated with cycloheximide (Figures 8-10 and Honess and Roizman, 1974). The evidence for sequential synthesis of polypeptides emerges clearly from comparisons of polypeptides made early (2.5 to 3 hours) and late (11 to 12 hours). These comparisons show that viral polypeptides made in large amounts early give way to another set of polypeptides made in large amounts late.

The evidence that synthesis of the three groups of polypeptides is interdependent emerged from the studies utilizing the inhibitors of protein synthesis and actinomycin D. Several points should be made in connection with these data.

First, we do not know how many coordinately regulated groups there are. The first approximation, based on the temporal patterns of their synthesis, suggested four classes (A-D, Figure 7). A second approximation, based on the sequential ordering of polypeptides, suggests three groups (Figures 9, 10). However, the β group may not be homogeneous since the rates of synthesis of the β polypeptides do not decay uniformly in the presence of actinomycin D. A better definition of the number of coordinately regulated groups might emerge from studies of ts mutants. Second, sequential synthesis of coordinately regulated groups has two predictive implications arising from the fact that the timing of both initiation and termination of synthesis of viral polypeptides comprising a coordinately regulated group must be determined. Based on our data, we may conclude that initiation of synthesis of at least α and β polypeptides is determined by the availability of competent viral RNA in the cytoplasm. Thus functional transcripts specifying α polypeptides are available immediately after infection and are not dependent on prior infected cell protein synthesis. Functional transcripts for β polypeptide synthesis become available by <u>de novo</u> synthesis only after α polypeptides are made. The requirements for the appearance of functional transcripts specifying γ polypeptides are less clear largely because γ polypeptide synthesis is unstable in the presence of actinomycin D. However, the data do suggest that this is the case and that the synthesis of γ polypeptides requires new RNA synthesis in addition to the presence of the β polypeptides. At the other end of the spectrum, it is more difficult to explain the decline in the rates of synthesis of α and β polypeptides by the mere availability of functional RNA. As we have already noted, ongoing α polypeptide synthesis does not decrease as rapidly in the presence of actinomycin D as in its absence, suggesting that its half-life in the absence of β polypeptides is long relative to that of γ mRNA. We are thus led to believe that the shut off of α polypeptide synthesis cannot be accounted for by a lack of flow of new transcripts specifying these polypeptides, and that the regulation is at some post transcriptional level, possibly at the level of translation. The point that we wish to stress is that both production of functional RNA molecules and cessation of function of these RNAs in a coordinate manner implies that both the RNA templates giving rise to these transcripts and the transcripts themselves must carry cognate signals which initially serve to specify proper transcription and which subsequently specify that a particular set of transcripts is no longer to be translated. The apparent heterogeneity of β polypeptide synthesis suggests that appearance of functional mRNA and termination

of translation are each specified by a different set of cognate signals, but more extensive studies of the polypeptides comprising this group will have to be carried out before even tentative conclusions can be reached.

Lastly, the molar rates of synthesis within each coordinately regulated polypeptide group vary as much as tenfold or more--certainly more than the error of the methods employed in our measurements. This observation suggests that a polypeptide abundance control is superimposed on top of everything else. We cannot at the moment explain this phenomenon; conceivably the answer may come from comparisons of purified mRNAs from strains which produce "normal" amounts of polypeptides with those of strains which cause specific polypeptides to be over-produced.

ACKNOWLEDGEMENTS

These studies were done under the auspices of the Chicago Cancer Research Center Project (CA-14599), and were aided by grants from the American Cancer Society (VC 103 H), the United States Public Health Service (CA 08494) and the Leukemia Foundation.

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MALIGNANT TRANSFORMATION BY HERPESVIRUSES

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Determination of the etiology of human neoplasia has presented a formidable challenge. Since Ellerman and Bang (1908) and subsequently Rous (1911) demonstrated early in this century that a transmissible agent could cause leukemia and sarcoma of chickens, scientists have engaged in a frustrating quest for comparable human viruses. In the past decade, demonstration that DNA containing papovaviruses can lytically infect some cells and can both transform cells in culture and produce tumors in experimental animals (Butel, Tevethia and Melnick, 1972) has initiated investigation of multipotential viruses indigenous to human populations.

Increased suspicion that human herpesviruses may be etiologic agents in certain human neoplasias has recently intensified efforts to demonstrate their oncogenic potential. Animal herpesviruses have been linked to tumor etiology in chickens, monkeys, and Leopard frogs, and two human herpesviruses--Epstein-Barr virus and herpes simplex virus--have been more or less definitively associated (Table 1) with human tumors (Rapp, 1973).

ONCOGENIC HERPESVIRUSES OF ANIMALS

Marek's disease of chickens is characterized by lymphoid tumors and neuropathy, notably paralysis of the extremities (Purchase, 1972). The etiologic agent, Marek's disease herpesvirus (MDV), replicates with aquisition of its lipid envelope only in the feather follicle epithelium. Consequently, it is readily spread by contact, dust,

TABLE 1. Herpesviruses with *in vitro* transforming ability or *in vivo* oncogenic potential

Virus	Host	*In Vitro* Cell Transformation	Host Cell DNA Stimulation	Oncogenicity
Marek's disease virus	Chicken	Unknown	Yes	Lymphoma (Chicken)
Herpesvirus saimiri and ateles	Monkey	Unknown	Unknown	Lymphoma (Monkey)
Lucké virus	Frog	Unknown	Unknown	Adenocarcinoma (Frog)
Herpesvirus sylvilagus	Rabbit	Unknown	Unknown	Lymphoma (Rabbit)
Guinea pig herpes-virus	Guinea pig	Unknown	Unknown	Suspected leu-kemia (Guinea pig)
Epstein-Barr virus	Man	Leukocyte	Yes	Suspected lym-phoma (Man)
Herpes simplex viruses	Man	Hamster, mouse and human fibroblasts	Unknown	Adenocarcinoma and Fibro-sarcoma (Hamster)
Cytomegalovirus	Man	Hamster embryo fibroblast	Yes	Fibrosarcoma (Hamster)

and dander to young chickens and probably initiates infection in the upper respiratory tract (Adldinger and Calnek, 1973). Development of lymphoid tumors has been repeatedly prevented by vaccination of day-old chicks with a non-pathogenic, antigenically related herpesvirus of turkeys. The effectiveness of such immunization appears to depend on activation of the chicken's immunological response prior to natural infection by wild type MDV. The turkey virus and then the virulent MDV replicate and stimulate antibody synthesis with the result that malignant transformation of susceptible leukocytes is blocked. Thus, vaccination of chickens has confirmed the virus etiology of Marek's disease and has suggested a possible approach to the etiology and control of human neoplasia. Further, Marek's disease and its causative agent have many parallels with Burkitt lymphoma of man and the Epstein-Barr virus.

Fatal lymphomas and leukemias have been produced by two monkey viruses in animals that do not normally harbor these agents. Herpesvirus saimiri (HVS) and Herpesvirus ateles (HVA), isolated from the squirrel and spider monkey, respectively, vary in their host range for cytopathology and oncogenicity (Table 2), but do not appear to cause tumors in their natural hosts (Meléndez et al., 1972). Koch's postulates have been fulfilled for Herpesvirus saimiri. Marmoset monkeys infected with partially purified virus developed fatal lymphoma, and virus cultured from tumorous lymph nodes again produced lymphomas when inoculated into fresh marmosets

TABLE 2. Properties of two oncogenic monkey herpesviruses: Herpesvirus saimiri (HVS) and Herpesvirus ateles (HVA)

Properties	HVS	HVA
Plaques (in vitro)		
Owl Monkey Kidney	yes	no
African Green Monkey Kidney (Vero)	yes	no
Squirrel Monkey Fetal Lung	yes	yes
Hamster Heart	no	yes
Tumors		
Squirrel Monkey	no	no
Owl Monkey	yes	±[a]
Cotton-Top Marmoset	yes	yes
Spider Monkey	yes	no
New Zealand Rabbit	yes	no

[a]± = lymphoreticuloproliferative disorder

(Laufs and Fleckenstein, 1972). Recent data on the immunological response to Herpesvirus saimiri suggest that resistance to the oncogenic potential of this virus may depend on the speed with which the humoral, and perhaps cellular, response is activated. The non-susceptible squirrel monkey, for example, develops antibodies to HVS 2 to 4 times faster than do owl and marmoset monkeys, animals that succumb to the lymphoma (Klein *et al.*, 1973).

Renal adenocarcinoma of Leopard frogs (Lucké tumor) has been linked to the Lucké frog herpesvirus (Granoff, 1972). Failure to replicate the virus in cell culture, combined with the routine detection of additional viruses in tumor cells, has made proof of etiology difficult. The Lucké virus is temperature-sensitive and appears to be activated *in vivo* and *in vitro* only at low temperature (4-9°C in the laboratory). Consequently, only tumors from frogs collected in winter or early spring or held at low temperature demonstrate virus. Recently, virus-specific messenger RNA was detected in tumor cells from frogs collected in the summer (Collard *et al.*, 1973), evidence that the virus genome is latent in "summer" tumors. This latent, temperature-sensitive virus poses many intriguing questions about the role of the virus in initiation and maintenance of transformation.

Preliminary evidence suggests that the lymphoma experimentally produced in wild cottontail rabbits (Hinze and Wegner, 1973) and the leukemia naturally produced in strain 2 guinea pigs (Hsiung, Fong, and Gross, 1973) may be caused by herpesviruses. In both systems, herpesvirus particles have been detected in cultured tumor cells but not in fresh tumor material.

Whether the particles expressing oncogenicity are defective viruses normally present in the population or whether they are an independently evolving subpopulation of tumor viruses is unknown. Since most herpesviruses replicate with the production of a large percent of defective particles, the former hypothesis is favored.

LINKS BETWEEN HUMAN HERPESVIRUSES AND CANCER

Burkitt lymphoma, a tumor of the jaw region that is prevalent in certain African populations, may be etiologically linked to the Epstein-Barr (EBV) virus (Klein, 1973), causative agent of infectious mononucleosis. Although the virus has not been detected in tumor biopsy material, both tumor cells (Hausen *et al.*, 1970) and tumor-derived lymphoblastoid cells that have been established into permanent lines (Nonoyama and Pagano, 1971; Hausen *et al.*, 1972) contain hybridizable EBV DNA. In addition, a small percent of the cultured cells spontaneously produce virus precursors (virus-specific antigens detectable by immunofluorescence) and infectious

EBV particles. Recently, Reedman and Klein (1973) have reported the presence of a complement fixing nuclear antigen, detectable by an anticomplement immunofluorescence test, in more than 90% of the lymphoblastoid cells in a given population. They have proposed that this antigen is analogous to the T antigen of transforming papovaviruses. Coupled with the prognostic rise and fall of anti-EBV antibodies in patients with Burkitt lymphoma (Henle et al., 1971) and the repeated demonstration that this virus can transform human and marmoset leukocytes to proliferating lymphoblasts containing EBV markers (Miller and Lipman, 1973), these observations support the hypothesis that the Epstein-Barr virus may be the etiologic agent of Burkitt lymphoma. For a more comprehensive discussion of its oncogenic potential, refer to the section by H. zur Hausen in this volume.

Herpes simplex virus type 2 (HSV-2) has been associated with cervical carcinoma by seroepidemiological studies (Adam et al., 1972; Josey, Nahmias and Naib, 1968; Rawls, Tompkins, and Melnick, 1969) showing that patients with the tumor have a higher incidence of neutralizing antibody to HSV-2 than do matched controls. While some of these data are equivocal (Rawls et al., 1970), the report (Frenkel et al., 1972) of an HSV-2 genome fragment in virus free cells from one cervical carcinoma and the isolation of a virus immunologically identified as HSV-2 in degenerating (virus free) cultured carcinoma cells (Aurelian et al., 1973) are compatible with the hypothesis that HSV-2 can establish latency and transform cervical cells. Demonstration of such virus properties *in vitro* is necessary to confirm these observations.

Recent reports (Aurelian et al., 1973; Hollinshead and Wegner, 1973; Sabin and Tarro, 1973) that only sera from patients with various cancers contain complement-fixing antibody to herpesvirus nonvirion antigens are provocative. If these investigators have detected nonvirion antigens and their corresponding antibodies, their further characterization should contribute insight to mechanisms operative in herpesvirus transformation and offer markers useful for diagnosis and prognosis of some virus-associated tumors.

HERPESVIRUS TRANSFORMATION OF MAMMALIAN CELLS TO MALIGNANCY

The herpesviruses are ubiquitous, commonly produce subclinical infections in natural hosts, and are multipotential in their interactions with molecules, cells and whole organisms. These viruses can initiate replication, effecting almost total inhibition of host macromolecular synthesis a few hours after adsorption. Alternatively, herpesviruses can enter a latent state in which no virus particles or precursors are detectable but from which the virus can be activated, evidence that the complete virus genome has been con-

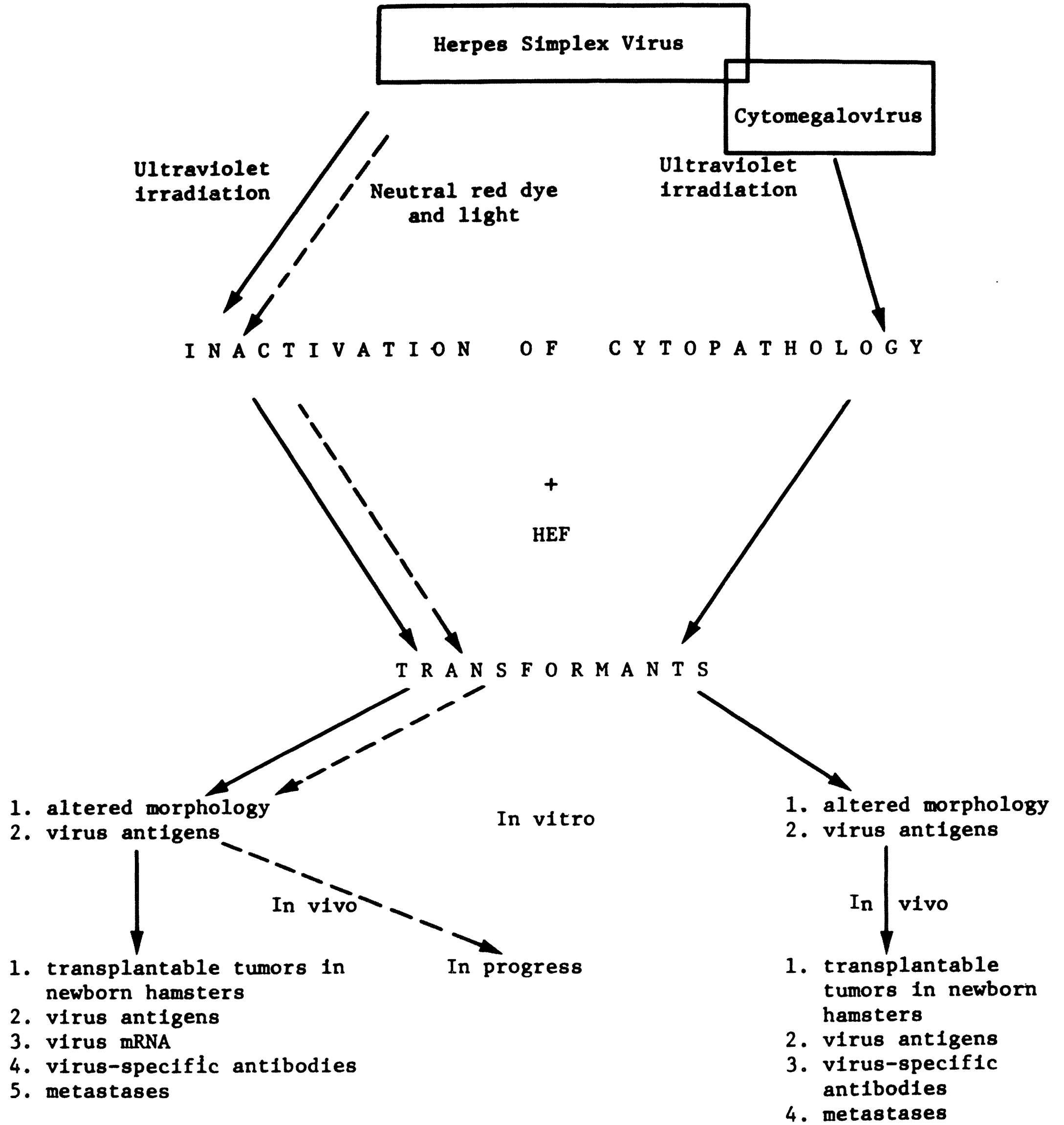

FIG. 1. Transformation of hamster embryo fibroblasts to malignancy by inactivated human herpesviruses.

served. More recently, the herpesviruses have been included among DNA viruses with transforming activity as virus-mediated transformation of cultured cells has been demonstrated by various techniques (Figure 1).

Direct demonstration of the oncogenic potential of human herpesviruses has not been possible because of the extreme virulence of these viruses in newborn rodents. Likewise, virus induced cytopathology obliterated any cell transforming activity that might have been expressed in cells permissive for virus replication. To circumvent the dual problems of virulence and cytopathology, herpesvirus infectivity was successfully inactivated by two methods, permitting virus transformation in cell culture. Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and human cytomegalovirus (CMV) exposed to ultraviolet (UV) irradiation morphologically transformed hamster embryo fibroblasts (HEF) (Albrecht and Rapp, 1973; Duff and Rapp, 1971 and 1973). In a similar fashion, HEF were transformed by HSV-1 and HSV-2 that had replicated in neutral red treated cells and were subsequently inactivated by exposure to light (Rapp, Li, and Jerkofsky, 1973). Transformants produced by UV-irradiated HSV and CMV formed stable lines that were oncogenic as evidenced by the formation of primary tumors and metastases when the cells were transplanted into inbred hamsters. HSV-2 and CMV tumors were identified pathologically as fibrosarcomas. More significantly, one HSV-1 tumor (Duff and Rapp, 1973) and a recently observed HSV-2 tumor (Rapp, n.d.) were described as adenocarcinomas, a pathology commonly observed in human neoplasias. Determination of the oncogenicity of the dye-light inactivated virus transformants is in progress.

What evidence supports the hypothesis that these hamster cells were in fact transformed to malignancy by herpes simplex virus and cytomegalovirus? Until virus DNA is detected by molecular hybridization or until the virus genome and virus particles are induced in transformed cells, designation of these cells as virus-transformed will be based on circumstantial evidence. Since the manipulations employed to inactivate virus infectivity in all probability destroyed complete genomes, thus enabling the new defective particles and those normally present in the virus pool to transform without competition from infectious particles, rescue of intact genomes or infectious virus from these cells may be impossible.

However, support for virus-mediated transformation is derived from several observations summarized in Table 3. A significant percent of HSV and CMV transformants demonstrated virus-specific antigens both in the cytoplasm and on the cell surface (Tables 4 and 5). These antigens, detected by immunofluorescence, were sufficiently immunogenic to elicit the synthesis of virus neutralizing antibodies in hamsters that received transplants of transformed

TABLE 3. Hamster embryo fibroblasts (HEF) transformed *in vitro* by ultraviolet-irradiated herpesviruses

Properties	Transforming virus HSV-1[a]	HSV-2[b]	CMV[c]
Transformants *in vitro*			
Altered morphology	+	+	+
Immortality	+	+	+
Virus antigens	+	+	+
Virus mRNA	Not done	+	Not done
Hamster leukosis virus antigens (gs)	-	-	-
Complete virus particles	-	-	-
Oncogenicity in hamsters	Andenocarcinoma	Fibrosarcoma Adenocarcinoma	Fibrosarcoma
Tumor cells (in hamsters)			
Virus antigens	+	+	+
Virus mRNA	Not done	+	Not done
Hamster C-type particles	-	-	-
Metastatic	+	+	+
Elicit synthesis of herpesvirus-specific antibodies in tumor-bearing hamsters	+	+	+

[a]HSV-1 = herpes simplex virus type 1

[b]HSV-2 = herpes simplex virus type 2

[c] CMV = cytomegalovirus

TABLE 4. Detection of herpes simplex virus (HSV)-specific antigens in HSV-transformed and infected hamster embryo fibroblasts (HEF) and in tumor cells by immunofluorescence methods

Cell Type	Sera			
	anti-HSV-1 or anti-HSV-2[a]	HSV-1 tumor[b]	HSV-2 tumor[c]	Normal Hamster
HEF Transformants				
UV-HSV-1[d]	+	+	+	-
UV-HSV-2	+	+	+	-
NR-HSV-1[e]	+	Not done	+	-
NR-HSV-2	+	Not done	+	-
Tumor cells				
UV-HSV-1	+	+	+	-
UV-HSV-2	+	+	+	-
HEF infected with				
HSV-1	+	+	+	-
HSV-2	+	+	+	-
HEF	-	-	-	-

[a]Pooled sera from hamsters immunized with HSV-1 or HSV-2.

[b]Pooled sera from hamsters bearing tumor induced by UV-inactivated HSV-1 (14-012-8-1).

[c]Pooled sera from hamsters bearing tumors induced by UV-inactivated HSV-2 (333-8-9).

[d]UV-HSV-1 = HEF transformed by ultraviolet light inactivated HSV-1.

[e]NR-HSV-1 = HEF transformed by neutral red-light inactivated HSV-1.

TABLE 5. Herpesvirus antigens on the surface of hamster embryo fibroblast (HEF) transformants and tumor cells

Cell type	Percent of cells showing surface fluorescence when exposed to the following sera: anti-HSV-1	anti-HSV-2	anti-CMV
UV-HSV-2[a] (333-8-9)	61	60	-
HEF	12	9	-
UV-CMV[b] (Cx-90-3B)	-	-	47
UV-CMV, T-1[c]	-	-	17

[a]UV-HSV-2 = HEF transformed by ultraviolet light-inactivated herpes simplex virus type 2.

[b]UV-CMV = HEF transformed by ultraviolet light-inactivated human cytomegalovirus.

[c]UV-CMV, T-1 = Cells from one primary tumor that developed after UV-CMV were transplanted into weanling hamsters.

cells and subsequently developed tumors. Sera from HSV-1 tumor-bearing hamsters neutralized HSV-1 and HSV-2 with equal efficiency, while sera from HSV-2 tumor-bearing hamsters were more effective against HSV-2 than against HSV-1. Since approximately 50% of the HSV-1 and HSV-2 DNA sequences are homologous (Collard, Thornton, and Green, 1973; Kieff *et al.*, 1972; Ludwig, Biswal and Benyesh-Melnick, 1972), such cross-reactivity is expected. More significantly, sera from tumor bearing hamsters (HSV and CMV) reacted in immunofluorescence with the corresponding parental transformants only. Demonstration that tumor-bearing hamsters synthesized antibodies that reacted both with the transforming virus and with virus-specific antigens in the (parental) transformants is compatible with the hypothesis that the virus originally transformed the hamster embryo fibroblasts. It is significant that complete herpesvirus particles, hamster leukosis virus antigens (gs), or C-type particles have not been detected in these cells (Glaser, Duff, and Rapp, 1972; Rapp *et al.*, 1972).

In vitro cell transformation by herpesviruses has been demonstrated independently in other systems (Table 6). 1) Mouse L cells deficient in genes for thymidine kinase were able to synthesize virus thymidine kinase following transformation by UV-irradiated HSV-1 and HSV-2 (Munyon *et al.*, 1971; Munyon, n.d.). 2) When cells from an established hamster line were morphologically transformed by UV-irradiated HSV-2, transformants that synthesized virus antigens were highly oncogenic (Kutinová, Vonka, and Brouček, 1973).

TABLE 6. *In vitro* transformation of mammalian cells by human herpesviruses

Investigators	Virus	Cell type transformed	Criteria for virus-mediated cell transformation
Munyon *et al.* 1971 and 1972	UV-irradiated HSV-1 and HSV-2[a]	Mouse L-cells (LTK^-)[b]	Thymidine kinase positive transformants (LTK^+)
Duff and Rapp 1971 and 1973	UV-irradiated HSV-1 and HSV-2	Hamster embryo fibroblasts	Transformants with virus antigens, virus mRNA, immortal, oncogenic
Kutinová *et al.* 1973	UV-irradiated HSV-2	Hamster cell line	Transformants with virus antigens, highly oncogenic
Darai and Munk 1973	Non-irradiated HSV-2 (Incubation of cells and virus at 42°C post-adsorption)	Human embryo lung fibroblasts	Transformants with virus antigens, immortal
Rapp *et al.* 1973	Neutral red+light treated HSV-1 and and HSV-2	Hamster embryo fibroblasts	Transformants with virus antigens, immortal
Albrecht and Rapp 1973	UV-irradiated human cytomegalovirus	Hamster embryo fibroblasts	Transformants with virus antigens, immortal, oncogenic

[a]UV-irradiated HSV-1 and HSV-2 = ultraviolet irradiated herpes simplex virus types 1 and 2.

[b]LTK^- = mouse L cell genetically deficient in thymidine kinase synthesis.

3) Non-irradiated herpes simplex virus type 2 morphologically transformed human embryonic lungs cells that were shifted to a temperature nonpermissive for virus replication (42°C) after adsorption (Darai and Munk, 1973). This observation is intriguing and might be explained if the transforming viruses were temperature-sensitive (ts) mutants with 42°C the permissive temperature for a virus function critical to transformation. It will be instructive to learn whether these cells contain virus mRNA as do the virus free summer cells from the Lucké frog tumor.

Further evidence for a portion of the HSV-2 genome in (UV-inactivated) HSV-2 transformants is derived from molecular hybridization data showing that messenger RNA equivalent to 10-13% of the HSV-2 genome and cross-reactive with HSV-1 DNA is present in these cells (Collard et al., 1973). Whether the virus genome is fragmented, present in multiple copies, integrated, or loosely associated with the cellular DNA is unknown.

How the virus initiates and maintains the transformed state is subject to conjecture. If parallels can be drawn at this time between the transforming papovaviruses and herpesviruses, it is likely that virus gene products are necessary for both initiation and maintenance of transformation. Temperature-sensitive mutants that transform at permissive, but not at nonpermissive temperatures can potentially answer this question in a manner analogous to the polyoma (Benjamin, 1972) and Rous sarcoma virus (Martin, 1970) mutants that have shown transformation to be virus-dependent.

How virus replication is limited to early virus functions (pre-DNA synthesis) is potentially more complex. The virus may code for a repressor analogous to the λ phage repressor. Alternatively, the cell may synthesize a virus inhibitor or repressor or may simply lack some product, such as an enzyme, that the virus requires to complete its replication. The last two would be termed nonpermissive cells.

Experiments compatible with the virus-coded and cell-coded repressor or inhibitor hypotheses are as follows: 1) Extracts of HSV-2 transformants were able to reduce or prevent HSV-2 plaque formation in susceptible recipient cells that had been treated with the extract prior to virus adsorption (Rapp, n.d.). 2) In addition, these transformants were resistant to superinfection by low multiplicities of HSV-2 (Doller, Duff, and Rapp, 1973). Both observations suggest the presence of a virus-coded repressor or inhibitor. 3) Pretreatment of permissive human embryo lung cells (Table 7) and nonpermissive human embryo kidney cells with iododeoxyuridine (IUdR) enhanced CMV replication in the former (St. Jeor and Rapp, 1973a) and permitted CMV replication in the latter (St. Jeor and Rapp, 1973b). These important observations suggest

TABLE 7. The effect of IUdR on plaque formation by cytomegalovirus in permissive human embryo lung cells

Treatment	Days Post-Infection: Cyto-pathology	Small Plaques	Countable Plaques	Maximum CMV Titer (pfu/ml)
No drug	2	7	9	1.0×10^4
50 γ IUdR	1	3	6	5.0×10^4
100 γ IUdR	1	3	6	1.3×10^5

that IUdR interfered with a cellular inhibitor of virus replication. 4) In a similar manner, IUdR treatment of virus-free somatic hybrid cells, formed by fusion of normal human cells with producer Burkitt lymphoma cells (P3J-HR-1), induced virus antigens, virus DNA, and virus particles (Glaser and Nonoyama, 1973; Glaser et al., 1973; Glaser and Rapp, 1972). Again, these results suggest that the non-lymphoid cell chromosomes in the hybrid produced a virus repressor or inhibitor that was obstructed in some manner by IUdR. The inhibitors or repressors must be isolated to confirm these hypotheses.

ONCOGENICITY OF HERPESVIRUS TRANSFORMANTS

The factors that determine whether a transformed cell will be oncogenic in vivo is a question of unknown magnitude. The tumor mass must increase to a size that exceeds the antigen recognition and cytolytic capacities of both the immune lymphoid cells and humoral antibody plus complement. If cellular immunity to tumor-specific transplantation antigens is blocked, i.e., by blocking antibodies, antigen excess, or antigen-antibody complexes, the transformed cells can escape detection and proliferate beyond control. Preliminary studies with HSV-2 transformed hamster embryo fibroblasts (HEF) yielded data (Table 8) compatible with the blocking antibody hypothesis (Duff, Doller, and Rapp, 1973). Briefly, weanling hamsters preimmunized with HSV-1 prior to challenge with HSV-2 transformants developed primary tumors and a markedly increased number of metastases, particularly to the lung. In contrast, prior immunization with SV40 did not prevent primary tumor growth, but did reduce or prevent tumor metastases. The failure of pre-immunization with HSV-1 to cause rejection of the tumor cell transplant is evidence that herpesvirus-transformed cells lack classical transplantation antigens leading to immunity. Extrapolating to human situations where childhood HSV infections have stimulated the synthesis of herpes-specific antibodies and where virus DNA with

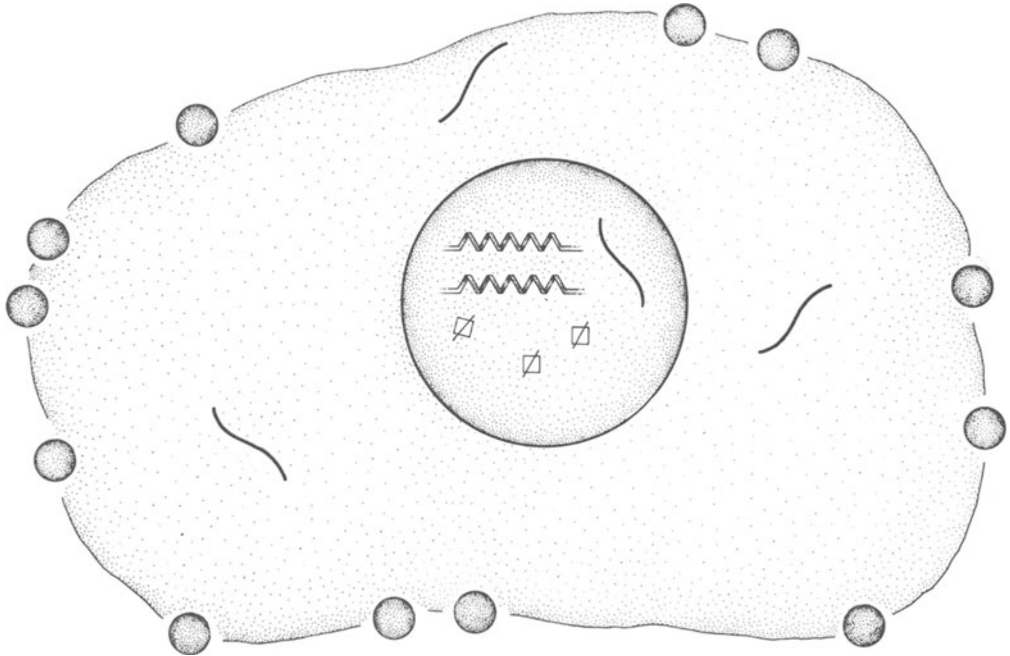

FIG. 2. Partial inhibition of the expression of the virus genome in transformed cells.

Ø Postulated inhibitor of expression of virus genome.

Virus and cell DNA.

Virus messenger RNA.

Virus-specific proteins at the cell surface.

TABLE 8. The effect of preimmunization on the appearance of primary tumors and metastases in weanling hamsters inoculated with HSV-2 transformed hamster embryo fibroblasts

Preimmunization	Number of hamsters	% with primary tumor	% with lung metastases
HSV-1	27	100	48
SV40	40	100	0
Medium control	34	100	21

transforming potential has been conserved in latently infected cells, herpesvirus-specific blocking factors could enhance proliferation of transformed cells as they appear to in hamsters. This HSV-hamster system may offer a sensitive model that can be manipulated to study parameters affecting the immune response to herpesvirus transformed cells.

RECAPITULATION

From this discussion it is evident that human herpesviruses can transform mammalian cells to malignancy with retention and conservation of at least a portion of the virus genome. Figure 2 details a model herpesvirus transformed cell with limited virus expression. The presence of virus-specific mRNA and antigens in some transformants is evidence that virus genes can function in these cells. Whether virus transcription and translation occur in all transformants is central to whether a herpesvirus gene product is required to initiate and/or to maintain the transformed state. That these transformants are oncogenic, forming primary tumors and metastases in newborn hamsters, is highly significant in light of recent demonstrations that lymphomas of chickens and monkeys are caused by oncogenic animal herpesviruses. These studies make plausible the hypothesis that common human herpes-viruses with transforming ability may be etiologic agents in certain human neoplasias. If this is so, intensive vaccination of controlled human populations, an approach that has so successfully eliminated Marek's disease from chicken populations, should reduce the incidence of those human cancers for which human herpes-viruses are the suspect etiologic agents.

ACKNOWLEDGEMENTS

Many colleagues contributed to the information in this paper. Special gratitude goes to Ms. A. Mellors who assembled information and helped collate it in a meaningful way. Original observations presented were carried out in studies supported, in part, under Contract No. 70-2024 within the Virus Cancer Program of the National Cancer Institute, National Institutes of Health, U.S. Public Health Service and by Public Health Service Research Grant No. CA-11647 from the National Cancer Institute.

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RECOGNITION OF VIRUS GENOMES IN CELLS BY MOLECULAR HYBRIDIZATION

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The application of molecular hybridization techniques to the detection of latent viruses, here in particular of potential human tumor viruses, appears to be a promising approach for several reasons:

1. Virus-specific nucleic acids can be detected in viral genome-harboring cells which do not synthesize viral particles.

2. The available techniques are sensitive enough to discover one genome equivalent per cell or even less.

3. Transcription of viral nucleic acids within tumor cells provides information on the genetic activity of the persisting genome.

4. <u>In situ</u> hybridizations permit the localization of viral genomes within tumor cells.

5. Tumors can be screened without applying biological procedures like virus isolation attempts in tissue culture cells or animals. These isolation procedures have been unsuccessfully tried with human materials since several decades.

Thus, nucleic acid homology studies would represent the method of choice for human tumor screening if the following questions had been resolved:

1. We do not have any indication which percentage of human tumors and which tumors in particular are indeed virus-induced.

2. We do not know the viruses which might be responsible for such cell proliferations.

3. We do not have permissive cell systems available which permit large scale production and purification of the suspected tumor viruses.

None of these prerequisites for a successful virus screening of human tumors is met at the moment. There exist additional obstacles which render the interpretation of hybridization results difficult, at least as far as the demonstration of viral genetic material in human tumors is concerned:

Even if virus-specific DNA is found consistently in human tumor biopsies, how do we know that this agent is responsible for the tumor? The tumor cells may offer favorable conditions for replication or even for persistence of viruses widely spread within the individual.

A second serious problem is posed by another facet. Even if DNA homologous to a known virus is found in human tumor cells, how do we know that this DNA originates from an identical agent? Our methods do not resolve homologies of more than 98% and most of them far less. One percent non-homology in base composition, however, could represent substantial differences in genetic terms.

In spite of these considerations, a number of useful indications and information for the role of viruses in the induction of human tumors have been obtained by applying nucleic acid homology studies. The following is an account of these data. Without discussing technical details it will be primarily concerned with the detection of Herpesvirus DNA in certain human malignancies. The demonstration of oncornavirus-specific information in tumors and some results obtained with human papilloma or wart virus will be briefly discussed.

The Epstein-Barr virus (EBV) represents the first agent which was found consistently within specific human tumor cells by nucleic acid hybridizations in 1970 (zur Hausen et al., 1970). At that time there existed already some evidence which linked EBV to a human malignancy: Burkitt's lymphoma, a tumor occurring predominantly in African children.

EBV was first detected electronmicroscopically by Epstein, Achong, and Barr in 1964 in lymphoblastoid tissue culture cells

derived from Burkitt's lymphoma (Epstein, Achong, and Barr, 1964). In indirect immunofluorescence test, developed by the Henles in Philadelphia, permitted the conclusion that EBV represented a hitherto unknown new member of the Herpesvirus group (Henle and Henle, 1965). In addition, large scale seroepidemiological studies became feasible. They resulted in the identification of EBV as an ubiquitous agent which causes infectious mononucleosis (Henle, Henle, and Diehl, 1968). In addition, they showed that antibodies against EBV-related antigens were present in virtually 100% of patients with Burkitt's lymphoma (BL) and another geographically clustered malignancy: nasopharyngeal carcinoma (NPC). The latter tumor is predominantly observed in southeast Asia. It comprises in certain parts of southern China more than 50% of all malignant tumors. The antibody titers in BL and NPC patients against EB viral antigens were unusually high in comparison to age-matched controls. Although a few other malignant diseases have been described which reveal elevated antibody titers against EB viral antigens, like the sarcomatous form of Hodgkin's disease, chronic lymphatic leukemia, and lymphocyte-lymphoblast lymphoma, the geometric mean of these titers is lower as compared to BL and NPC. In addition, sero-negative patients have been recorded in all of these groups.

Tissue culture experiments indicated that EBV transforms primary human lymphocytes and is responsible for their permanent growth *in vitro*. This was supported by the notion that spontaneous outgrowth of lymphoblastoid lines could only be obtained from EBV-sero-positive donors. Most of these lines contained a small percentage of cells which synthesized EBV-specific antigens, although some did not.

In order to assess the role of EBV for the permanent growth of lymphoblastoid tissue culture lines, first nucleic acid hybridization experiments were carried out with a non-virus-producing line derived from Burkitt lymphoma cells: Raji. By annealing ^{3}H-labeled EBV-DNA with DNA derived from Raji cells, increased hybridization was observed as compared to various controls of human cellular DNA preparations of non-Burkitt origin (zur Hausen and Schulte-Holthausen, 1970). Raji cells also synthesized an RNA which reacted specifically with EBV-DNA. Thus, despite the lack of viral particle and viral structural antigen synthesis, EB viral genomes persisted within Raji cells and exerted at the same time some genetic activity. This showed that EBV persisted in those cells analogous to other well-studied DNA tumor viruses.

By subjecting DNA derived from biopsy material of Burkitt lymphomas and nasopharyngeal carcinomas to the same test (zur Hausen *et al.*, 1970) both tumors annealed significantly above the background of DNA obtained from various other tumors of the head and neck region.

The hybridization of Burkitt lymphoma material seemed to exceed slightly that of nasopharyngeal carcinomas. It is worthwhile to note that multiple tumors of the same donor annealed approximately within the same range.

The extremely low yields of EB viral DNA from virus-producing lymphoblastoid lines was an obvious limitation for DNA-DNA hybridization studies. Therefore, EBV-DNA was transcribed *in vitro* into highly radioactive complementary RNA (cRNA) with the aid of *E. coli* RNA polymerase. Filter hybridizations with this cRNA basically confirmed the DNA-DNA annealing experiments (zur Hausen, 1972). In addition, a number of non-virus-producing lymphoblastoid lines, derived from various sources (Hodgkin's disease, nasopharyngeal carcinoma, healthy donors), were found to be positive for EBV-DNA. Most of these lines were subsequently shown to contain the complete viral information, since treatment of these cells with iododeoxyuridin (IUdR) or bromodeoxyuridine (BUdR) resulted in a few cells in synthesis of viral particles (Hampar *et al*., 1972; Gerber, 1972).

Burkitt lymphoma and nasopharyngeal carcinoma biopsies were also found positive in DNA-cRNA hybridizations for EBV-DNA. In view of the rather uniform histology of Burkitt's lymphoma it seems reasonable to assume that the tumor cells are indeed the ones which harbor the viral genomes. There exists now considerable evidence which links EBV to this malignancy:

(i) BL patients contain high antibody titers against EB viral antigens.

(ii) Apparently EBV-specific membrane antigens are found in fresh biopsy cells.

(iii) EBV-DNA is present in biopsy cells of these tumors.

(iv) Cells of tissue culture lines derived from BL contain EBV-DNA. A small percentage of them spontaneously produce virus or viral antigens.

(v) EB virus transforms human lymphocytes in tissue culture into established lines.

(vi) Shope, Dechairo, and Miller (1973) and Epstein, Hunt, and Rabin (1973) recently demonstrated EBV-induced lymphomas in marmosets and owl monkeys. Cell lines derived from these tumors contained EBV-DNA.

This shows that Koch's postulates are almost fulfilled as far as the etiology of EBV for Burkitt's lymphoma is concerned. A number of questions, however, still remain to be answered: the role of

cofactors, the pathogenesis of the disease, the time of appearance of tumors following the infection by EBV. As far as tumor induction in animals is concerned, neutralization tests should be of great importance. Despite these reservations, BL represents the first human tumor consistently associated with a tumor virus.

In nasopharyngeal carcinoma the available data are more difficult to interpret. These patients do reveal high antibody titers against EB viral antigen, and virus-specific DNA is found in biopsy material of these tumors. The tumor contains, however, a mixed cell population. Besides epithelial tumor cells it contains various admixtures of infiltrating lymphocytes and stroma cells. Since EBV up to now has only been detected within cells of lymphatic origin, it was tempting to speculate that the infiltrating lymphocytes would harbor the EB viral genomes demonstrated by nucleic acid hybridizations. In order to substantiate this assumption, we started to correlate the histology of frozen sections from NPC material with hybridization data obtained from the same tumor (Wolf, zur Hausen, and Becker, 1973). All tumor materials were obtained from Dr. George Klein, Stockholm. The histological examination was performed by Dr. Becker of the Department of Pathology of the University of Erlangen.

The result of these studies was rather surprising, since all tumors hybridizing in the highest range were of predominantly epithelial histology. Low hybridizing or negative tumors either consisted mainly of lymphatic cells or did not represent nasopharyngeal carcinomas. These data suggested that the epithelial tumor cells are indeed the ones which harbor EBV-DNA. Further studies provided additional evidence in this direction.

By exposing frozen sections of nasopharyngeal carcinomas to the anticomplementary fluorescence test (EBNA-test) recently described by Reedman and Klein (1973), cells of apparently epithelial morphology revealed a nuclear as well as cytoplasmic fluorescence (Wolf et al., 1973). This reaction depended on the presence of antibodies against EBV-antigens and was abolished following inactivation of the complement.

More direct proof for the presence of EBV-DNA in epithelial nasopharyngeal carcinoma cells resulted from in situ hybridization experiments performed according to the procedure of Gall and Pardue (1969). This procedure permitted in previous experiments to identify EB viral-DNA synthesizing cells and to localize EBV-DNA in Raji cell metaphase chromosomes (zur Hausen and Schulte-Holthausen, 1972). Frozen sections of nasopharyngeal carcinoma biopsies were denatured by alkali treatment, annealed with EBV-cRNA under a cover-slip, and after RNase treatment exposed to autoradiography.

By comparing undenatured sections of the same tumor with the *in situ* hybridization results, it became obvious that almost exclusively epithelial cells were labeled. Although alkali treatment of the slides considerably deteriorated the morphology, the label appeared to be restricted to the nuclear region. Control sections of non-nasopharyngeal carcinomas were completely unlabeled.

These data show that epithelial cells in nasopharyngeal carcinomas are at least predominantly EB viral genome carriers. Thus, this tumor represents the first example of EBV infection of non-lymphatic cells. The demonstration of EBV DNA within the tumor cells of NPC appears to contribute significantly to the circumstantial evidence for a causal role of EBV in this malignancy. Several problems, however, still have to be resolved: (i) NPC cells cannot be grown in long-term tissue culture. This prevents *in vitro* studies on the virus-host cell interrelationship. (ii) Nasopharyngeal carcinomas have not yet been induced in laboratory animals. (iii) The peculiar epidemiology of this tumor suggests either the existence of a cofactor or a genetically determined susceptibility. It is obvious that this tumor deserves more attention. The presented data show that it is also consistently linked with a tumor virus, and the available evidence renders it probable that EBV represents the causative agent of this carcinoma, too.

It is an obvious question whether the EB virus of infectious mononucleosis is identical with the ones demonstrated in BL and NPC biopsies. All available evidence suggests that these agents must at least be very closely related if not identical. Cot analysis revealed no differences in their reassociation kinetics (Nonoyama and Pagano, 1973) and EBV derived from infectious mononucleosis as well as from Burkitt lymphoma lines was shown to induce lymphomas in marmosets. Further studies have to reveal whether there exist minor biological differences which would escape the rather crude biochemical assays.

Since EBV has not yet been found consistently in any other human malignancy, BL and NPC remain the only tumors which regularly harbor tumor virus DNA within malignant cells.

Since a few years the role of Herpes simplex (HSV) type 2 in induction of human cervical carcinoma has been discussed. A significantly higher percentage of women with cervical cancer reveals antibodies against Herpes simplex type 2 viral antigens as compared to appropriately age-matched controls (Nahmias *et al*., 1970; Rawls, Tomkins, and Melnick, 1969). In addition, there have been reports on the presence of HSV-type 2-specified antigens in exfoliated cells derived from cervical carcinoma patients (Royston and Aurelian, 1970) and on the reactivation of HSV-2 from a continuous cell line which originated from cervical carcinoma (Aurelian *et al*., 1971).

HSV-2 as well as HSV-1 have been shown to transform hamster cells *in vitro* after partial inactivation by ultraviolet light (Duff and Rapp, 1971). These transformed cells produce malignant tumors following their inoculation into the appropriate host. Recently, Frenkel and Roizman reported the presence of a fragment of the HSV-2 genome within one cervical cancer biopsy by nucleic acid hybridization (Frenkel *et al.*, 1972). They showed that at least part of this DNA was covalently linked to host cell DNA. In addition, they could demonstrate a partial transcription of a small portion of the fragment.

In the meantime, additional nucleic acid hybridization studies were carried out: We subjected in our laboratory DNA from 23 different biopsy specimens to hybridization with HSV-2 and HSV-1 cRNA. In particular the last ten samples were carefully controlled to represent on their majority tumor cells. All 23 biopsies tested turned out to be negative. The sensitivity of the test was determined in reconstruction tests to be on the order of one genome equivalent per cell. Since cRNA derived from HSV-2 or HSV-1 annealed with the DNA of the heterologous virus almost to the same extent as HSV-2 DNA with HSV-1 DNA and vice versa, the assumption seems to be justified that the *in vitro* product is transcribed from representative parts of the genome. Although our results do not exclude the presence of small fragments of HSV-2-specific DNA in the biopsies tested, they demonstrate that the HSV-host cell relationship at least must be very different from EBV in Burkitt's lymphoma and nasopharyngeal carcinoma. If cervical cancer cells do contain any HSV DNA, it should amount to well below one genome equivalent per tumor cell.

Our results were supported by data of Pagano *et al.* (personal communication) who failed to find HSV DNA in DNA of six cervical cancer biopsies which was reassociated with *in vitro* labeled HSV-2 DNA. Thus, the role of HSV-2 in cervical cancer remains to be elucidated. Molecular hybridizations do not exclude at the moment the etiological role of HSV for this malignancy. In view of the seroepidemiological findings, they may hint, however, that a serologically related but not identical Herpesvirus might be associated with cervical cancer.

Since various reports have been published on a seriological cross-reactivity between Herpes simplex and varizella-zoster viral antigens (Kapsenberg, 1964; Ross, Subak-Sharpe, and Ferry, 1965; Schmidt, Lenette, and Magoffin, 1969), we also subjected DNA from cervical carcinoma patients to nucleic acid hybridizations with varizella-zoster viral cRNA. The results were, however, entirely negative.

Another member of the Herpesvirus group, human cytomegalovirus (CMV) has been repeatedly isolated from an African tumor, Kaposi's sarcoma (Giraldo et al., 1972), which shows a peculiar geographic clustering. Its occurrence coincides with regions of endemic Burkitt's lymphoma. Since cytomegalovirus-specific antigens were also reported in tissue culture cells derived from this malignancy, we initiated nucleic acid hybridization experiments with CMV-cRNA to establish the association of this virus with Kaposi's sarcoma. DNA of Kaposi biopsies, obtained from Dr. Deinhardt in Chicago and Dr. Giraldo in Paris, did neither hybridize with CMV- nor EBV-cRNA and was also found to be negative for HSV and varizella zoster DNA. All Kaposi derived cell lines were also found to be negative for CMV DNA. Again, these experiments do not exclude the presence of minute amounts of CMV DNA in this tumor. On the other hand, they do not support the available evidence for a role of this virus in the induction of this tumor.

A large part of this paper should be reserved for the discussion of hybridization experiments with RNA-tumor viruses. Since RNA-tumor virologists are well represented at this meeting, a brief comment to relevant experiments should be sufficient.

Spiegelman and his associates reported the hybridization of a fraction of polysomal RNA derived from human mammary tumors with DNA synthesized in vitro on a mouse mammary tumor virus template with the aid of viral reverse transcriptase (Hehlmann, Kufe, and Spiegelman, 1972). Similarly, annealing of polysomal RNA from cells of various human leukemias, lymphomas, and nasopharyngeal carcinomas with DNA synthesized on a mouse Rauscher leukemia virus template has been observed (Axel, Gulati, and Spiegelman, 1972). The same group devised a simultaneous detection test which enables them to detect a complex of 70s RNA associated with reverse transcriptase activity (Baxt, Hehlmann, and Spiegelman, 1972). This activity is only found in cells of the human malignancies mentioned above. Normal cells were regularly negative in the simultaneous detection test.

Although demonstration of biological activity of such "C-type-like" human particles and their detailed characterization are still missing, they might be of considerable significance. An interaction of such possibly endogenous RNA-tumor viruses with viruses of the Herpes group has recently been proposed (Peters et al., 1973). This is based on synergistic effects in tumor induction by infecting chicken simultaneously with RAV-oncornavirus and the Herpesvirus of Marek's disease. In this experiment neither agent produced tumors by itself. Only their combination was effective.

As far as human tumors are concerned, however, the available data are not yet conclusive.

By returning to DNA viruses, the role of human papilloma or wart virus in certain human benign and malignant tumors should be given some attention. Since the beginning of this century, this virus has been known to induce papillomas in men, but still it belongs to the most neglected human pathogens. Since a number of investigators could induce warts with cell-free extracts of laryngeal papillomas and condylomata acuminata, it was of particular interest to study the role of human wart virus in these tumors. For this reason, wart virus was purified from human plantar warts, its DNA was extracted and component I of this DNA transcribed into cRNA with the aid of E. coli RNA polymerase. The resulting cRNA which did not cross-react with human cellular DNA was used in hybridization tests. Surprisingly, DNA from 11 condylomata acuminata and 6 laryngeal papillomas did not anneal with wart viral cRNA. In contrast, DNA from human plantar warts bound a considerable percentage of the input cRNA. DNA derived from verrucae vulgares annealed to a lower extent with wart viral cRNA. Four of these preparations were found to be negative under these conditions. This result shows that if laryngeal papillomas and condylomata acuminata are indeed induced by human wart virus, their viral genome content must be very small and seems to be not measurable with the technique used. It is possible, however, that the failure to demonstrate wart viral DNA within these tumors is due to the presence of another papova virus in these tumor preparations which shows no significant base homology with the agent commonly called "human papilloma virus."

In conclusion, virus-specific DNA was demonstrated consistently in two human malignancies by molecular hybridization. Within the tumor cells of Burkitt's lymphoma and nasopharyngeal carcinoma, Epstein-Barr viral nucleic acid is present usually in large quantities. Thus, these tumors represent the first human malignancies regularly associated with a tumor virus.

Promising results, obtained by hybridization methods, seem to point to the presence of oncornavirus nucleic acid in various human leukemias and lymphomas. It appears that these data need some substantiation as far as the viral nature of the measured activity is concerned. In addition, their biological significance remains to be elucidated.

The association of Herpes type-2 with human cervical cancer and human cytomegalovirus with Kaposi's sarcoma has not been regularly confirmed by molecular hybridization methods. If these viruses are involved in the etiology of the respective malignancies, their interaction must be different from EB virus in Burkitt's lymphoma and nasopharyngeal carcinoma.

Human wart viral DNA has up to now only been demonstrated in DNA of plantar warts and verrucae vulgares, but not in condylomata

acuminata and laryngeal papillomas. The etiology of the latter remains to be clarified.

ACKNOWLEDGEMENT

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg.

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THE CHARACTERIZATION OF AN *IN VITRO* ISOLATED NUCLEI SYSTEM FOR THE INVESTIGATION OF THE MECHANISM OF HERPESVIRUS DNA REPLICATION IN INFECTED HUMAN EMBRYONIC LUNG CELLS

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SUMMARY

Infection of human embryonic lung (HEL) cells by Herpes simplex type II virus stimulates labeled thymidine incorporation into an acid-precipitable product as much as twentyfold higher than by mock-infection. The rates of overall *in vivo* and *in vitro* DNA synthesis and the relative fractions of Herpes and cell DNA synthesized *in vivo* and *in vitro* by nuclei isolated from the infected cells are the same at various times after infection. All four rNTPs strikingly stimulate ^{3}H-TTP incorporation in nuclei from Herpes-infected but not mock-infected cells. The thermal lability of *in vitro* DNA synthesis is different for Herpes-infected than for mock-infected cells, although the relative fractions of cell and viral DNA made at 42°C and at 34°C are the same. Results of variable time pulse label experiments with the isolated nuclei system suggest a discontinuous mode of DNA replication.

Herpes simplex type II virus has become the object of considerable study recently because of its implication in genital cancer (Klein, 1972), and the recent demonstration of the ability of this virus to transform cells in tissue culture (Duff and Rapp, 1971). Unlike the small oncogenic viruses, Herpesvirus DNA has a molecular weight of about 10^{8} daltons (Becker, Dym, and Sarov, 1968) and has a buoyant density of 1.729 gms/cm^{3}, equivalent to a C + G content of 68%. The Herpes genome codes for about 60 structural proteins (Roizman, 1969). The biophysical properties of Herpes DNA have

been studied in some detail (Graham et al., 1972), and it is known that the virus codes for several enzymes involved in DNA synthesis (Hay, Moss, and Halliburton, 1971), including a Herpes-specific DNA polymerase (Weissbach et al., 1973). In this communication I would like to report the results of experiments on Herpes DNA replication in an in vitro isolated nuclei system.

MATERIALS AND METHODS

Herpes simplex type II virus, isolated from a genital infection site, was plaque-purified 3 times and passaged in HeLa cells. The cells used in these experiments were human embryonic lung cells isolated from a biopsy specimen, cloned from a single cell and grown in Dulbecco's modified Eagle's medium containing 3x amino acids, 5% fetal calf serum and penicillin. Viral stocks were prepared by freeze-thawing infected monolayers at 24 hours after infection, centrifuging the cell debris and storing the supernatant at -70°C. Virus was titered by plaque-assay. The cells and virus were the generous gift of Dr. G. Darai of the Virologie Institut, Universität Heidelberg. In the experiments reported here, cells were used during the 15-25 passage at a cell density of 3-8 x 10^4 cells/cm^2. Cells were subcultured at 5 x 10^5 cells per Roux flask of 200 cm^2 surface area containing 60 ml of medium. Infection was performed by incubating the cell monolayer with 1-2 PFU/cell Herpesvirus in 10 ml medium, at 37°C with gentle rocking, replacing the medium with 60 ml fresh medium and continuing the incubation at 37°C. Cells were harvested and nuclei prepared essentially as described by Winnacker, Magnusson, and Reichard (1971). The monolayer was washed once, the medium replaced by 20 ml 20 mM Hepes pH 8.1 buffer (Good et al., 1966) containing 5 mM $MgCl_2$, and incubated for about 10 min at 0°C. The swelling buffer was discarded and the cells scraped into 5 ml buffer and nuclei prepared by hand homogenization with a glass Dounce homogenizer. Homogenization was continued until direct observation with a phase microscope showed complete cell breakage. Because this Herpesvirus is a syncytium-former, the nuclei are relatively easy to prepare without adding a detergent. Nuclei from mock-infected cells are only slightly more difficult to prepare than from Herpes-infected cells. The nuclei are separated from the cytoplasm by centrifugation at 500 x g for 10 minutes at 0°C, and are resuspended at 0°C in Hepes buffer containing 110 mM NaCl. Nuclei lose only 10-20% of their in vitro DNA synthesizing capability after 10 hours storage at 0°C.

In vitro DNA synthesis was studied by incubating the isolated nuclei in a reaction mixture of the following composition: 40 mM Hepes pH 8.1 buffer, 100 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM dithiothreitol, 400 μM each dATP, dCTP, dGTP, 2.5 mM rATP and radioactively labeled dTTP (^{3}H-TTP, 5500 cpm/pMole, 28 Ci/mMole,

Amersham Radiochemicals, England; ^{32}P TTP 10 Ci/mMole, the gift of Mr. H. L. Lauppe, Max Planck Institut für Medicinische Forschung, Heidelberg). rNTPs are added, when indicated, at 400 μM. The total volume of the reaction mixture, and the number of nuclei added are given in the figure legends. For kinetic (labeled TTP incorporation) studies, 25 μl of reaction mixture was transferred at various time intervals to 2 ml 0°C acid-precipitation mixture containing 5% trichloroacetic acid and 1% RNA hydrolysate (TCA). The precipitate is filtered onto 2.5 cm nitrocellulose millipore filters, washed by filtration with 20 ml 0°C TCA, the filters dried at 80°C for one hour, placed in scintillation vials, 5 ml PPO-POPOP-toluene scintillation fluid added and the cpm assayed in a Packard liquid scintillation counter. When required, DNA was extracted from the reaction mixtures by adding a lysis buffer containing 40 mM Hepes buffer, 100 mM NaCl, 2 mM EDTA and 2% sodium dodecyl sulfate (SDS) to 20% of the lysate volume. The clear lysate was brought to 2 ml with Hepes buffer containing 150 mM NaCl (high-salt buffer) and extracted twice with buffer-saturated phenol, the phases separated by centrifugation. Phenol was removed by extraction with 2 ml diethylether and the ether removed by blowing nitrogen through the extract. The extract was normally dialyzed against 2000 volumes of an appropriate buffer prior to further analytical procedures. Caesium chloride equilbrium gradients were prepared by diluting the dialyzed extracts to 5 ml with buffer, adding solid $CsCl_2$ to 1.710 gms/cm^3, overlaying the solution with paraffin oil and centrifuging 65 hours at 20°C at 35,000 rpm in a Spinco 50 Ti anglehead rotor. Fractions were collected dropwise from a hole punctured in the bottom of the tube and each fraction processed for assay of radioactivity as described above. The density of selected fractions was determined by refractive index measurement. Marker Herpesvirus DNA was prepared by radioisotopic labeling of HEL cells 18 to 24 hours after infection by Herpesvirus, lysis of the washed monolayers with 5 to 10 ml of SDS lysis buffer, dilution to 10 ml with high-salt buffer, phenol extraction, and dialysis as described above. The Herpes DNA was separated from contaminating cell DNA by consecutive $CsCl_2$ equilibrium gradient centrifugations. DNA concentration was measured by optical absorbance at 254 nMeters.

RESULTS AND DISCUSSION

(i) <u>In Vitro</u> Incorporation of ^{3}H-TTP by Nuclei Isolated from Herpes- and Mock-Infected HEL Cells

Infection by Herpesvirus strikingly stimulates overall thymidine incorporation by infected cells (Olshevsky, Levitt, and Becker, 1967; Figure 4), and this seems to be the case also for nuclei isolated from HEL cells isolated 10 hours after infection. Figure

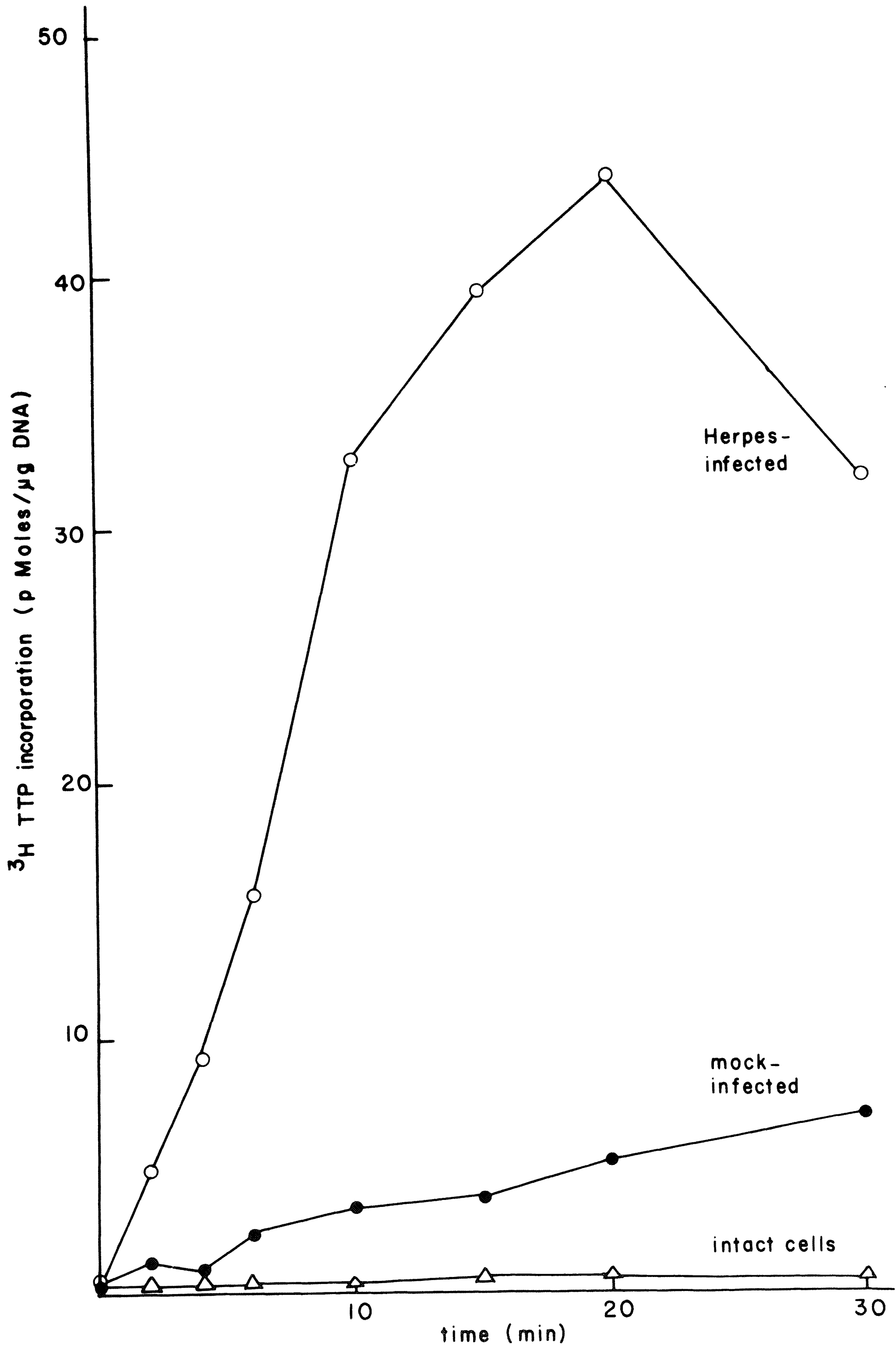

50
40
30
20
10
^{3}H TTP incorporation (p Moles/μg DNA)
Herpes-
infected
mock-
infected
intact cells
10
20
30
time (min)

FIG. 1. Kinetics of ^{3}H-TTP incorporation by nuclei isolated from HEL cells mock-infected and infected with Herpes type II virus.

1 Roux flask of HEL cells, cell density 7 x 10^4 cells/cm^2, was infected with 1 PFU/cell Herpes type II virus in 10 ml medium. After one hr. gentle rocking for virus adsorbtion, the medium was discarded and replaced with 60 ml fresh medium at 37°C. The cells were incubated for an additional 15 hr. at 37°C and nuclei prepared as described in the Methods, and resuspended in isotonic Hepes buffer at 10^8 cells/ml. 25% of the cells scraped off the monolayer were resuspended in isotonic buffer as intact cells, without further treatment.

100 µl of nuclei preparation (or intact cells) were added to 300 µl of in vitro reaction mixture, producing the following final concentrations: 400 µM dATP, dCTP, dGTP; 2.5 mM ATP, 8 mM $MgCl_2$, 0.5 mM $CaCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 40 mM Hepes buffer pH 8.1, and 7 µCi ^{3}H dTTP, 23 Ci/mMole. The 300 µl reaction mixture was preincubated to 34°C, and 100 µl nuclei or intact cell preparation added. At the indicated times, 25 µl of reaction mixture was removed from the reaction mixture with a capillary micropipette and mixed with 2 ml 0°C 5% TCA-RNA hydrolysate. The precipitation mixture was incubated for 10-15 min. at 0°C and filtered through cellulose nitrate millipore filters. The filters were washed with 20 ml 0°C TCA-RNA hydrolysate, dried at 80°C for 1 hr., the dry filters placed in scintillation vials containing 7 ml toluene, PPO, POPOP scintillation mixture, and the radioactivity measured with a Packard liquid scintillation counter. DNA concentration was estimated by the optical density at 254 mµ of a phenol extracted lysate of the reaction mixture.

^{3}H-TTP incorporation by nuclei isolated from mock-infected cells was determined exactly as described above for Herpes-infected cells. 1 Roux flask of HEL cells of the same cell density was mock-infected by addition of 10 ml medium to the monolayer for a 1 hr. "adsorbtion" period at 37°C.

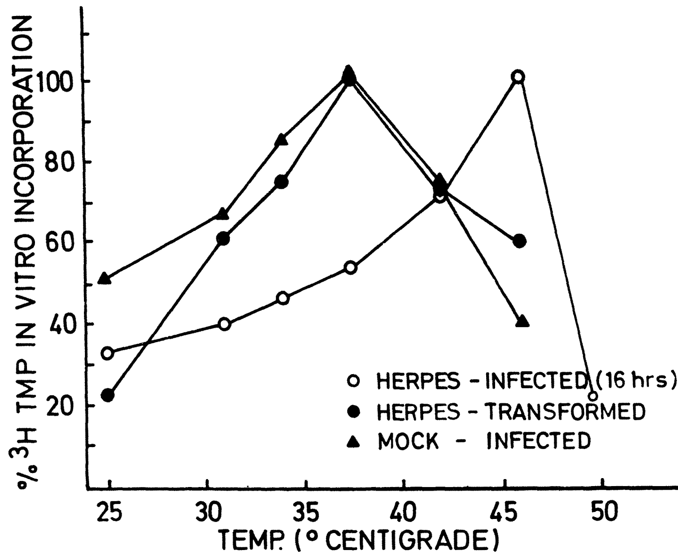


FIG. 2. Temperature optima for DNA synthesis in nuclei isolated from mock-, Herpes-infected and Herpes-transformed human embryonic lung cells.

Nuclei were isolated from mock-, Herpes-infected and Herpes-abortively infected (Becker *et al.*, 1968) human embryonic lung cells as described in the legend to Figure 2. The kinetics of ^{3}H-TTP uptake in the *in vitro* reaction mixture (described in *Methods*) were determined for each temperature tested, as shown in Figure 5. The initial velocity for the incorporation into acid-precipitable counts per unit time at the maximum incorporation for the range of temperatures tested was expressed as 100%. Maximum incorporation rate was about equal for mock-infected and Herpes-abortively infected cells, and about tenfold higher for the Herpes-infected cells. o——o, Herpes-infected (16 hr.); ●——●, Herpes-abortively infected; ▲——▲, mock-infected.

1 illustrates the dramatic stimulation of ^{3}H-TTP incorporation by nuclei from Herpes-infected HEL cells compared to mock-infection, and also demonstrates that intact Herpes-infected cells do not incorporate label. The reaction at 34°C is linear for about 20 minutes for Herpes-infected cells, but nuclei from mock-infected cells can linearly incorporate ^{3}H-TTP under these conditions for 120 minutes or longer.

(ii) Effect of Temperature on In Vitro DNA Synthesis by Isolated Nuclei

Overall ^{3}H-TTP incorporation by nuclei isolated from mock-infected cells is more temperature-sensitive than incorporation by Herpes-infected cells, as illustrated by Figure 2. Each point in Figure 2 is calculated from the initial velocity of ^{3}H-TTP incorporation by isolated nuclei determined from a 15 minute total incorporation time. The percent incorporation given on the vertical axis is the fraction of the maximum incorporation rate for each of the three nuclei preparations shown. For mock- and abortively-infected cells, the maximum rate was about the same, 500 cpm/min; for Herpes-infected cells, 4,000 cpm/min. The cells abortively infected by Herpesvirus show some properties of transformed cells (Darai and Munk, 1973). The temperature inactivation curve for these cells seems to be the same as for mock-infected cells, even though these abortively-infected cells were selected for by their ability to grow at 42°C, a temperature at which Herpesvirus cannot normally replicate (Stevens, 1966) and at which normal cells will die after a few days. In vitro incorporation by nuclei from Herpes-infected cells demonstrates a remarkable temperature optimum. It is possible that Herpes-infection induces factors which render the nuclei resistant to high temperature. (The Herpes-induced DNA polymerase has a higher thermal stability than either of the three known cellular DNA polymerases [Weissbach et al., 1973]). At 50°C, the in vitro incorporation rate by nuclei isolated from Herpes-infected cells falls sharply. Figure 3 demonstrates that the relative fractions of Herpes and cell DNA synthesized at 34°C and 42°C by nuclei isolated from Herpes-infected HEL 10 hours after infection are identical. Therefore, the ability of the nuclei isolated from Herpes-infected cells to synthesize DNA at high temperature does not represent an increase in some random, or non-specific mode of DNA replication; the cellular or viral-induced regulation seems to be retained at 42°C.

(iii) Herpes and Cell DNA Synthesized in Vitro and in Vivo at Various Times after Infection

Figure 4 illustrates a one-step viral DNA growth curve. The

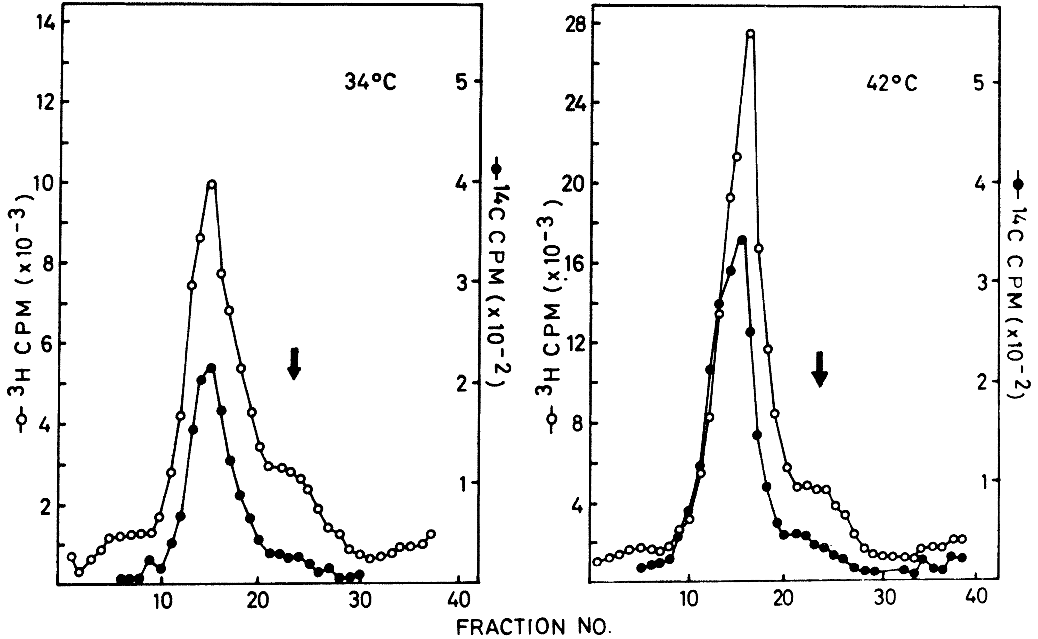


FIG. 3. Host and viral DNA synthesized in vitro at 34° and 42°C by nuclei isolated from Herpes-infected human embryonic lung cells.

200 µl lysate from 15 min. incubation of 4 x 10^6 nuclei isolated from HEL cells 16 hr. after infection by Herpesvirus (2 PFU/cell) in the standard in vitro reaction mixtures described in Methods were diluted and the DNA extracted as described in Methods. The extracts were prepared for CsCl equilibrium density gradients as described in Methods and centrifuged 60 hr. at 35,000 rpm at 20°C in a 50 Ti Beckman angle head rotor. Fractions were collected dropwise through a hole punctured in the bottom of the tubes and the DNA in each fraction precipitated with 2 ml cold TCA-RNA hydrolysate, filtered and the radioactivity assayed as described in the legend to Figure 2. Open circles represent the in vitro (^{3}H-TTP), synthesized DNA; closed circles, in vivo DNA synthesized in a 2 hr. pulse just prior to isolation of nuclei. The arrow marks the density position of cell DNA (1.700 gm/cm^3). Panel a, in vitro reaction at 34°C; panel b, in vitro reaction at 42°C.

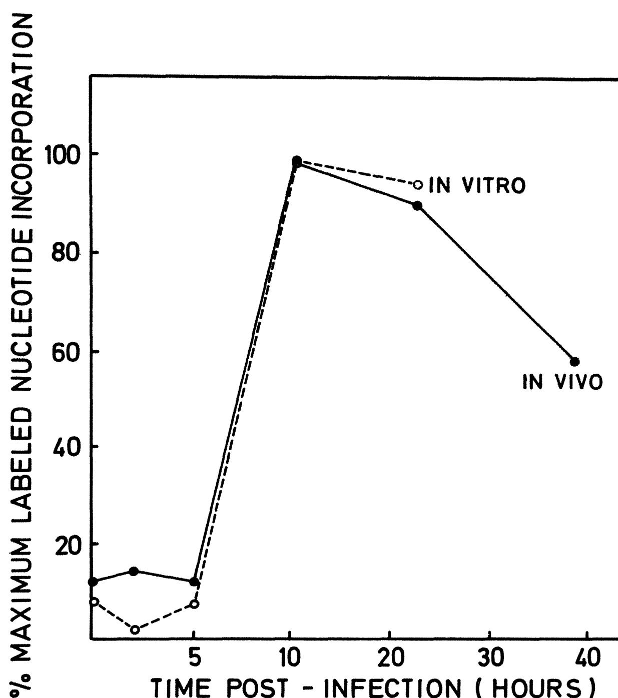


FIG. 4. Comparison of _in vivo_ and _in vitro_ DNA synthesis after infection of human embryonic lung cells by Herpesvirus.

Human embryonic lung cells cultured in 6 cm petri dishes were infected with 2 PFU/cell Herpes simplex type II virus prepared as described in the _Methods_. One hour after each infection fresh medium containing 10% calf serum was added and at each point indicated (solid line) 0.5 μCi ^{14}C thymidine (59 mCi/mMole) was added to 8 ml medium and incubated for 2 hr. at 37°C. The medium was discarded and 2 ml of a lysis buffer containing 5 mM EDTA, 0.5% sodium dodecyl sulfate and 20 mM Tris buffer pH 8.1 was added. The viscosity of the lysate was reduced by passage through a number 21 needle, aliquots of the lysate were precipitated with cold trichloroacetic acid (TCA), the precipitate collected on filters and the radioactivity assayed in a liquid scintillation counter. The rate of DNA synthesis for the _in vitro_, isolated nuclei system was assayed by incubating nuclei isolated at the indicated times from HEL cells infected with Herpesvirus as described above with the _in vitro_ reaction mixture described in the _Methods_ for 15 min. at 37°C. An aliquot of the nuclear reaction mixture was transferred to 3 ml of cold TCA, filtered and the radioactivity assayed as described above. The maximum incorporation rate was expressed as 100 percent and all other values are calculated as a fraction of the maximum. The actual maximum rates were 6000 cpm for the _in vivo_ reaction, 2 hr. labeling, and 90,000 cpm for the isolated nuclei (10^6 nuclei), dashed line.

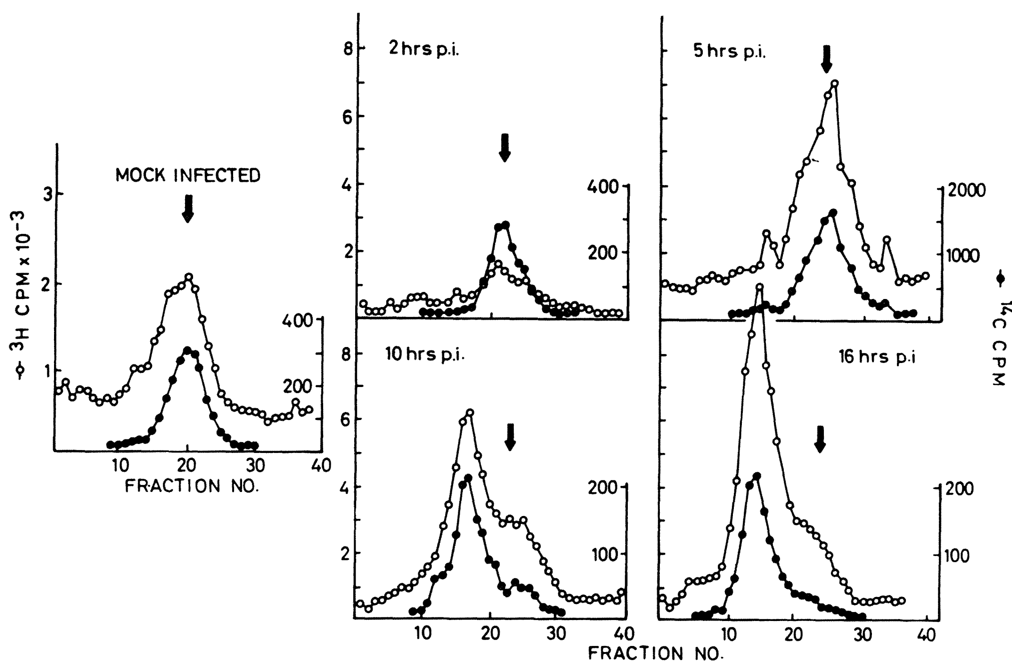


FIG. 5. CsCl equilibrium gradients of viral and host DNA synthesized in vitro and in vivo at various times after infection of HEL cells by Herpesvirus.

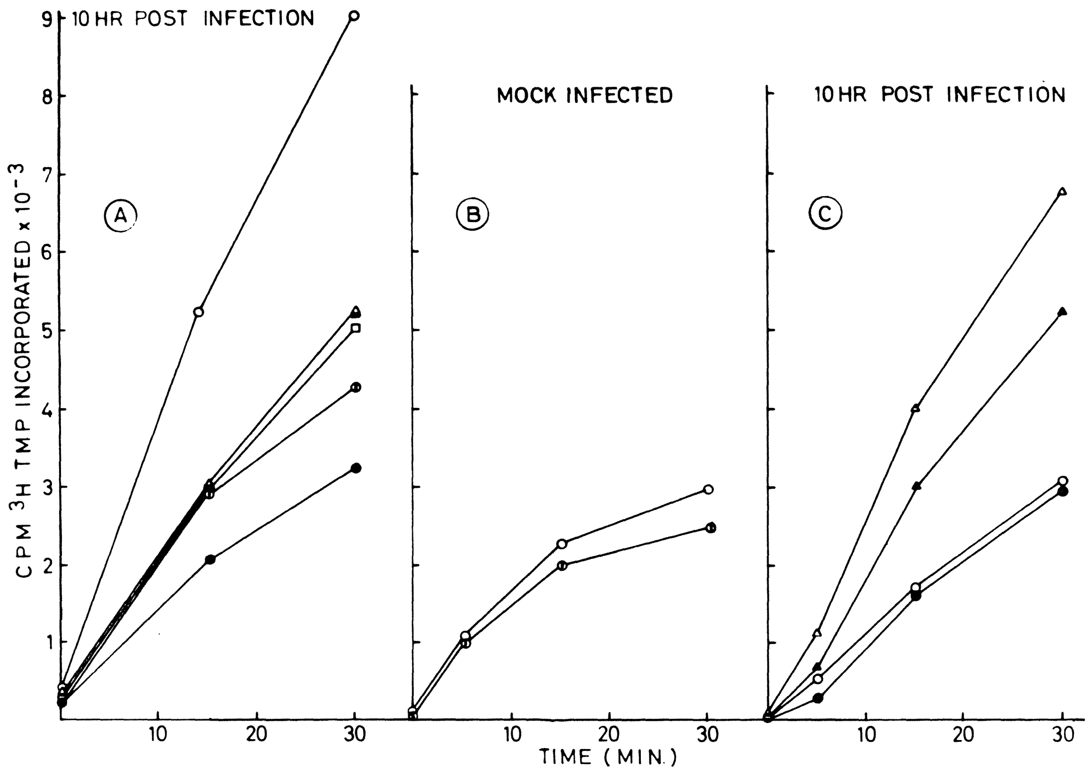
2×10^7 HEL cells were infected at 2 PFU/cell and at one hr. after infection fresh medium containing 10% calf serum was added. Two hr. prior to the times indicated in the panels 1 μCi/ml ^{14}C thymidine (59 mCi/mMole) was added and the cells were labeled for 2 hr. The medium was discarded and nuclei isolated from the infected cultures as described in the Methods. 4×10^6 nuclei in a volume of 200 μl were incubated in the in vitro reaction mixture containing ^3H-TTP for 15 min at 34°C and the reaction stopped by the addition of 50 μl of a lysis buffer described in the legend to Figure 1. The DNA was extracted from the lysates as described in the Methods, diluted to 5 ml, solid CsCl added to a density of 1.700 gm/cm^3, and the gradients centrifuged to equilibrium (60 hr. at 20°C, 35K rpm, Beckman 50 Ti angle head rotor). Fractions were collected dropwise through a hole punctured in the bottom of the tube. Each fraction was diluted with 2 ml 4°C TCA and the precipitate collected on filters and assayed for radioactivity as described in the legend to Figure 1. In each panel the open circles represent the acid-precipitable ^3H cpm (in vitro reaction), the closed circles acid-precipitable ^{14}C cpm (in vivo prelabeling). The arrow indicates the position of cell DNA marker (d. 1.700 gm/cm^3). Refractive index measurements of selected fractions of each gradient (not shown) place the density of the Herpes peak, when extant, at 1.726-1.729 gm/cm^3. Panel a, mock-infected cells.

in vivo rate of DNA synthesis was measured as cpm ^{14}C thymidine incorporated in a 60 minute pulse period just before nuclei preparation. The vertical axis is the percent maximum incorporation observed. The *in vitro* incorporation rate represents the cpm ^{3}H-TTP incorporated in a 15 minute reaction. The maximum was 3,000 cpm *in vivo*; 85,000 cpm *in vitro*. It can be seen that the stimulation of overall DNA synthesis *in vivo* and *in vitro* is the same. It was not possible to obtain a 36 hour *in vitro* time point because nuclei isolated from cells this late after infection are too fragile to isolate intact. Figure 5 demonstrates that the relative fractions of cell and viral DNA synthesized at any time after infection are the same *in vitro* and *in vivo*. The DNA was labeled for 90 minutes *in vivo* with ^{14}C thymidine just before nuclei isolation, and ^{3}H-TTP incorporation carried out in the *in vitro* reaction for 15 minutes, the nuclei lysed and the DNA extracted and centrifuged to equilibrium in $CsCl_2$ gradients as described above. Therefore, each gradient contained an internal control for the distribution of cell and viral DNA synthesized at each time after infection at the time the nuclei were isolated. Thus, it can be concluded that the nuclei of cells infected by Herpesvirus retain the control mechanisms regulating the expression of viral and host DNA synthesis without the influence of the cytoplasm, and with the results presented in Figure 4, suggest that the isolated nuclei system can serve as a useful *in vitro* model system for Herpesvirus DNA replication.

(iv) The Effect of Added rNTPs on DNA Synthesis in Isolated Nuclei

The difficulty of denaturing Herpesvirus DNA (Becker *et al.*, 1968) and its unusual sedimentation behavior in alkaline sucrose gradients (Frenkel and Roizman, 1972; Gordin *et al.*, 1973) has led to the speculation that the mature Herpes genome may contain ribonucleotides. In addition, the involvement of ribonucleotides has been demonstrated as primers for the initiation of Okazaki fragments in discontinuous synthesis of *E. coli* DNA (Sugino, Hirose, and Okazaki, 1972) and ribonucleotides have been demonstrated in the DNA of mitochondria (Grossman, Watson, and Vinograd, 1973), bacteriophage T4 (Speyer, Chao, and Chao, 1972), and polyoma virus (Franke and Hunter, 1974). Figure 6 demonstrates that DNA synthesis of Herpesvirus DNA in isolated nuclei is strikingly stimulated by the addition of all four rNTPs. Any one or combination of two or three rNTPs are not effective in producing this stimulation. DNA synthesis by nuclei isolated from mock-infected is very slightly, if at all, stimulated by the addition of rNTPs.

At 800 μMoles dNTPs in the *in vitro* reaction, the triphosphate pool is most certainly saturated (panel c, Figure 6). But 400 μM



10 HR POST INFECTION
MOCK INFECTED
10 HR POST INFECTION
A
B
C
CPM 3H TMP INCORPORATED x 10-3
9
8
7
6
5
4
3
2
1
10
20
30
TIME (MIN.)

FIG. 6. The effect of added rNTPs on the incorporation of labeled dNTPs by nuclei isolated from mock- and Herpes-infected HEL cells.

Panel a. 2 Roux flasks of HEL cells subcultured and grown as described in Methods were infected with 2 PFU/cell Herpes simplex type II virus at a cell density of 7 x 10^4 cell/cm^2. After one hour incubation at 37°C, the medium was replaced with 60 ml fresh medium and incubation continued for 10 hours at 37°C. Nuclei were prepared as described in Methods and resuspended in isotonic buffer at 5.2 x 10^7 nuclei/ml. The in vitro reaction mixture was as described in Methods. rNTPs, when added, were 130 μM final concentration. dNTPs were added to 400 μM. Nuclei in the in vitro reaction were incubated at 31°C, and at the times indicated 25 μl of reaction mixture, containing 2.2 x 10^5 nuclei, were transferred to 2 ml 0°C TCA-RNA hydrolysate and processed for assay of acid-precipitable radioactivity as described in Methods. ◐——◐, no added rNTPs; ○——○, rGTP, rCTP, rUTP (rATP is present in all reaction mixtures at 2.5 mM); △——△, rUTP; ■——■, rGTP; ●——●, rUTP, rGTP; □——□, rCTP. 25 μl of reaction mixture contained 3.5 x 10^5 cpm ^{3}H-TTP total added labeled triphosphate.

Panel b. 2 Roux flasks of HEL cells were subcultured and grown as described in Methods to 7 x 10^4 cells/cm^2 and mock-infected by replacing the growth medium with 10 ml fresh medium and incubating for 1 hour at 37°C. The medium was replaced with 60 ml fresh medium and incubation continued for 10 hours at 37°C. Nuclei were prepared as described in Methods and resuspended in isotonic buffer at a concentration of 6 x 10^7 nuclei/ml. The in vitro reaction mixture was as described in Methods. rNTPs, when added, were 400 μM final concentration. Nuclei in in vitro reaction were incubated at 31°C, and, at indicated times 25 μl, containing 2.5 x 10^5 nuclei and 3.5 x 10^5 cpm ^{3}H-TTP were transferred to 2 ml 0°C TCA-RNA hydrolysate and processed for assay of acid-precipitable radioactivity as described in Methods. ◐——◐, no added rNTPs; ○——○, 400 μM rNTPs added. 25 μl reaction mixture contained 5 x 10^5 cpm ^{3}H-TTP.

Panel c. 2 Roux flasks HEL cells were subcultured as described in Methods. Infection with 2 PFU/cell Herpesvirus was performed as described in panel a above, at a cell density of 3 x 10^4 cells/cm^2 The infected cells were incubated 10 hours at 37°C and nuclei prepared as described in Methods, and resuspended in isotonic buffer at 5 x 10^7 nuclei/ml. The in vitro reaction mixture was as described in Methods; the nuclei were incubated in the reaction mixture at 31°C and at indicated times 25 μl, containing 10^5 nuclei and 1.25 x 10^6 cpm ^{3}H-TTP was transferred to 2 ml TCA-RNA hydrolysate at 0°C and the samples processed for assay of acid-precipitable radioactivity as described in Methods. ○——○, 400 μM dNTPs, no rNTPs; ●——●, 800 μM dNTPs, no rNTPs; △——△, 400 μM dNTPs, 400 μM rNTPs; ▲——▲, 800 μM dNTPs, 400 μM rNTPs.

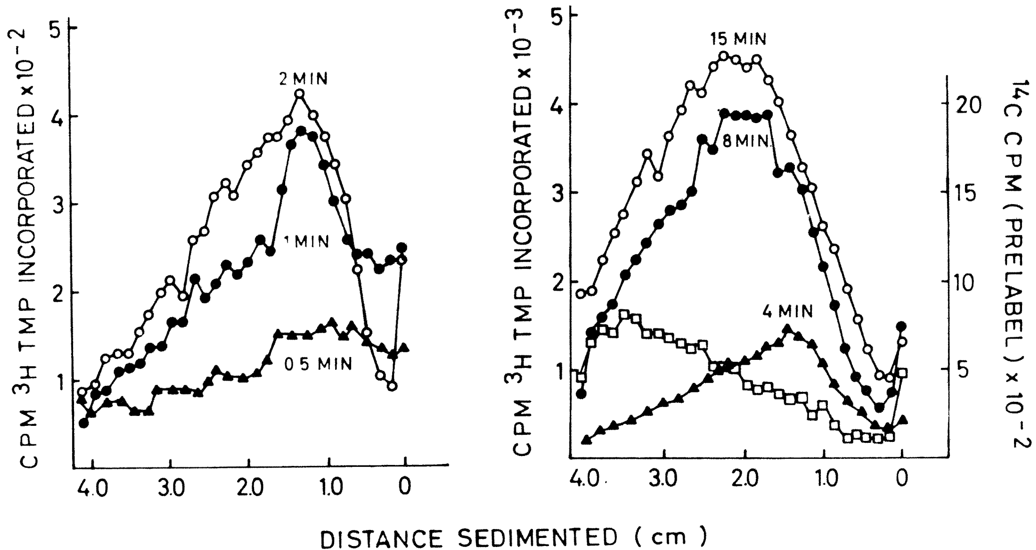


FIG. 7. Sucrose velocity gradient centrifugation of lysates of Herpes-infected HEL cells pulse-labeled *in vitro* for various lengths of time with ^{3}H-TTP.

4 Roux flasks of HEL cells were subcultured and infected with Herpes simplex type II virus as described in Methods and Figure 1, panel a, at a cell density of 7 x 10^4 cells/cm^2. Incubation of the infected cells was continued for 8 hours. The medium was discarded and 20 μCi ^{14}C thymidine added with 20 ml new medium (at 37°C) and the incubation at 37°C continued for 2 hours. Nuclei prepared as described in Methods were resuspended in isotonic buffer at 0°C at 5.6 x 10^7 nuclei/ml. *In vitro* reaction mixtures, containing 400 μM dNTPs and 400 μM rNTPs were prepared as described in Methods. Total volume of each reaction mixture was 350 μl, containing 2.8 x 10^6 nuclei and 60 μCi ^{3}H-TTP (28 Ci/mMole, Amersham Radiochemicals, England). After the indicated incubation times at 31°C, 50 μl of SDS-EDTA-NaCl lysis buffer and 50 μl 1 N NaOH were added to the reaction mixture. After 30 seconds, 120 μl of the clear viscous lysate was carefully pipetted from a pipette with a 3 mm orifice onto a 4.4 ml 5 to 20% linear sucrose gradient prepared in 0.3 M NaOH as described in Methods, and centrifuged 15 hours at 35,000 rpm at 10°C in a Beckman SW 50.1 rotor. Fractions were collected and processed for assay of radioactivity as described in Methods. Left panel: ▲—▲, 0.5 minute pulse, 3740 ^{3}H cpm applied to gradient; ●—●, 1 minute pulse, 3300 ^{3}H cpm applied; ○—○, 2 minute pulse, 6240 ^{3}H cpm applied. Right panel: ▲—▲, 4 minute pulse, 12390 ^{3}H cpm applied; ●—●, 8 minute pulse, 29200 ^{3}H cpm applied; ○—○, 15 minute pulse, 65100 ^{3}H cpm applied; □-□, ^{14}C cpm (from *in vivo* label), about 5500 cpm ^{14}C cpm were applied to each gradient and the ^{14}C cpm profile for only one of the gradients is plotted here (1 minute pulse label).

rNTPs added to the in vitro reaction already saturated with dNTPs (800 μM dNTPs) stimulates ^{3}H-TTP incorporation as much as addition of 400 μM rNTPs to the reaction mixture containing 400 μM rNTPs. It can be concluded from these results that the stimulatory effect of added rNTPs on DNA synthesis by nuclei isolated from Herpes-infected cells is due to a process other than augmentation of the dNTP pool through metabolic pathways of nucleotide precursor synthesis.

(v) Evidence for Discontinuous Synthesis of Herpes DNA *in Vitro*

Okazaki *et al*. (1970) demonstrated that at least one strand of *E. coli* DNA was synthesized in a discontinuous manner; 6-8S pieces of DNA were observed in alkaline velocity gradients when extremely short pulse periods (6-15 seconds) were used to label the newly synthesized DNA. After longer pulse periods, the short pieces ceased to appear and longer DNA pieces were observed. Polynucleotide ligase mutants of T4 phage were unable to synthesize long phage DNA pieces, or complete phage DNA molecules (Sugimoto, Okazaki, and Okazaki, 1968), and it was concluded that normal replication of DNA in both *E. coli* and phage T4 involved the synthesis of short DNA single strands which were subsequently annealed to a long single-strand complementary newly-synthesized daughter strand, and the 3' to 5' nick remaining sealed by ligase. Since rNTPs markedly stimulated Herpesvirus DNA replication *in vitro*, I followed the experimental protocol of Okazaki (Sugimoto *et al*., 1968) to look for discontinuous synthesis of Herpesvirus DNA. The results of this experiment are shown in Figure 7. In order to rule out the generation of small pieces of Herpesvirus DNA by nuclease activity induced by Herpes infection, the infected cells were prelabeled for 90 minutes prior to nuclei isolation with ^{14}C thymidine. Following the *in vitro* ^{3}H-TTP incorporation for various pulse periods from 30 seconds to 15 minutes, lysates of nuclei were layered onto alkaline sucrose velocity gradients and centrifuged. The right-hand panel of Figure 7 illustrates the ^{14}C cpm (*in vivo* labeled) gradient profile. The peak material sediments to the bottom of the gradient but there is a tailing of smaller DNA fragments through the gradient. The DNA synthesized *in vitro* during the pulse periods illustrated varies from 8-10S to 22S, the shorter pieces being synthesized during the shorter pulse periods. If nuclease activity were responsible for the generation of the short DNA pieces observed during the short pulse-periods, it would be expected that the size of the ^{14}C DNA labeled for a long period *in vivo* would also be small. This was not the case. Figure 7 demonstrates that, in the gradients of nuclear lysates from the short *in vitro* pulse periods, the ^{14}C-labeled DNA sediments much faster than the ^{3}H *in vitro* labeled DNA. These experiments thus provide evidence for discontinuous synthesis of Herpesvirus DNA, and further emphasize the

usefulness of the *in vitro* isolated nuclei system in the investigation of the mechanism of Herpesvirus DNA replication.

ACKNOWLEDGEMENTS

I would like to thank Ms. Klutsch, Einoder, and Knopfle for their competent technical assistance, and Drs. G. Darai, W. Doerfler, and E. Winnacker for helpful discussions.

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APPENDIX

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