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Patterns of Simian Virus 40 DNA Transcription after Acute Infection of Permissive and Nonpermissive Cells*

(SV40/tissue culture/monkey/mouse/plus and minus strands/RNA synthesis)

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ABSTRACT **Small amounts of fractionated, denatured, ^{32}P-labeled DNA from SV40 virus were incubated with a large excess of the complementary RNA of SV40 prepared *in vitro* with *Escherichia coli* RNA polymerase; the viral DNA strands were separated on hydroxyapatite columns. The RNA present in green monkey cells late in the lytic cycle reacted with 40–42% of the strand complementary to the *in vitro* complementary RNA (minus strand), and 60–64% of the opposite (plus) strand. "Early lytic" RNA failed to significantly interact with the plus strand, but formed stable duplex molecules with 35–39% of the minus strand. The RNA prepared from mouse embryo cells 24 hr after infection with SV40 combined with 35–38% of the minus strand and 60–62% of the plus strand. In all cases, the same regions of either the plus or minus strand appear to be transcribed in permissive and nonpermissive infections.**

A study of the control factors that regulate Simian Virus 40 (SV40) DNA transcription is of considerable interest, since the viral genome may exist in at least two different physical states in mammalian cells. In cells undergoing productive infection, viral DNA can be detected as free, supercoiled molecules at a time when viral capsid proteins are being synthesized. The SV40 genome appears to be covalently linked to the animal cell genetic material in virus-free lines transformed by this agent (1). In transformed cells, the virus-specific RNA represents, in most cases, a portion of the SV40 RNA present late in lytic infection (2, 3). The factors that modulate the transcription of SV40 DNA have been difficult to analyze because viral gene products have only been superficially characterized. In transformed cells, for example, the synthesis of virus-specific RNA may be regulated by both host-cell and viral control mechanisms. On the other hand, during the latter stages of productive infection, free SV40 DNA most likely codes for capsid proteins, as well as for other products involved in virus assembly.

SV40 DNA can also be transcribed *in vitro* by DNA-dependent RNA polymerase prepared from *Escherichia coli*. When supercoiled SV40 DNA is used as template for the enzyme, the viral RNA produced is asymmetric (transcribed from one DNA strand), does not self-anneal to any appreciable extent, and can be used to separate small amounts of the intact SV40 DNA strands (20). We have recently corroborated these findings (4) by showing that 50% of intact SV40 DNA interacts with SV40 RNA (cRNA) made *in vitro*, when the reaction products are analyzed on hydroxyapatite. When the viral DNA is mechanically sheared to 10% of the genome size, 50% of the DNA continues to react with the SV40 cRNA. If, however, intact SV40 DNA is incubated with RNA prepared from infected monkey cells late in the lytic cycle, 98–99% of the DNA binds to hydroxyapatite columns. This value falls to 53–57% when the viral DNA is sheared to pieces 3×10^5 daltons in size (4). These results imply that one strand of SV40 DNA is transcribed *in vitro*, and that regions of both DNA strands are expressed late in the lytic cycle, although, in both situations, about 50% of the viral DNA is transcribed.

In this communication, we attempt to quantitate the proportion of each viral DNA strand transcribed in green monkey or mouse-embryo cells after exposure to the virus. Our results indicate that: (*a*) 38–42% of the strand complementary to SV40 cRNA made *in vitro* (minus strand) and 60–64% of the plus strand are transcribed during the later stages of productive infection; (*b*) 35–39% of the minus strand, and little or none of the plus strand, are expressed early in the lytic cycle; and (*c*) 35–38% of the minus strand and 60–62% of the plus strand are complementary to the RNA present in mouse (nonpermissive) cells 24 hr after infection with SV40.

MATERIALS AND METHODS

Tissue Culture and Virus. Primary African green monkey kidney cells (AGMK), Vero cells (5), and primary mouse embryo cells were propagated in Eagle's minimal essential medium supplemented with 0.03% glutamine and 5% fetal-calf serum. Small-plaque purified SV40 was a gift of Dr. Kenneth Takemoto and was used in all studies.

SV40 [^{32}P]DNA. Confluent monolayers of Vero cells were infected with SV40 at a multiplicity of 1–3 plaque-forming units (PFU)/cell in phosphate-free Eagle's medium containing 2% dialyzed fetal-calf serum. Carrier-free [^{32}P]orthophosphate (Tracerlab) (100 μCi/ml), as well as fresh phosphate-free Eagle's medium, were added 24–30 hr after infection. Virus was prepared (6) from cells and medium 7–8 days later. SV40 DNA I was isolated from purified virus after it was treated with 1% sodium dodecyl sulfate for 30 min at 50° and centrifuged isopycnically in CsCl in the presence of ethidium bromide (200 μg/ml) (7, 3). Labeled DNA preparations had specific activities from 0.7 to 2.6 $\times$ 10^6 cpm/μg. The SV40 [^{32}P]DNA I was mechanically sheared (50,000 psi) in a Ribi Cell Fractionator to a molecular size of about 3.1×10^5 daltons (4).

Abbreviation: cRNA, complementary RNA.

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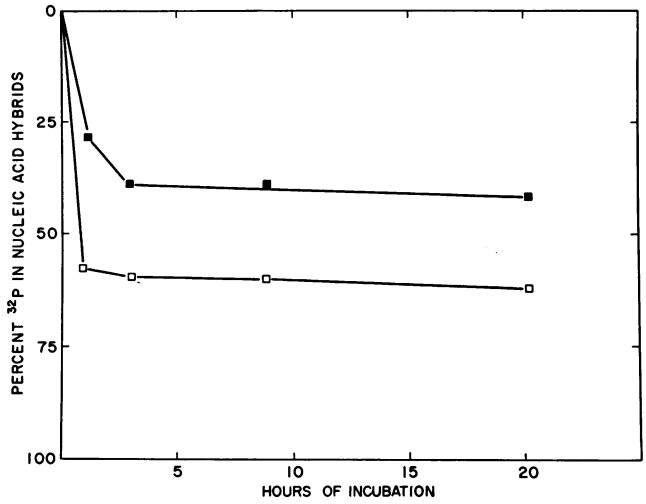

FIG. 1. Hybridization of "late lytic" RNA to the plus and minus strands of ^{32}P-labeled, fragmented SV40 DNA. Infected cellular RNA (0.5 mg/ml) obtained 55 hr after SV40 inoculation was incubated with plus (□——□) or minus (■——■) strands of SV40 DNA (3.5 ng/ml) in 1.0 M NaCl at 68°. Aliquots were taken at the indicated times, and the percentage of ^{32}P-labeled DNA in DNA–RNA hybrid molecules was determined.

Cell RNA. "Early lytic" RNA was prepared by infection of African green monkey kidney cells with SV40 (multiplicity of 40–80 PFU/cell) for 12 hr. "Early lytic" RNA was also purified from green monkey cells infected in the presence of arabinosyl cytosine (20 μg/ml), 30–36 hr after exposure to the virus. The infected cells were washed with phosphate-buffered saline (pH 7.6), suspended in RNA Lysing Solution [0.1 M NaCl–0.01 M sodium acetate, pH 5.3–bentonite (1 mg/ml)–0.35% sodium dodecyl sulfate], and extracted twice with phenol at 60°. The RNA was treated with electrophoretically purified pancreatic DNase (Worthington, 100 μg/ml) for 1 hr at 20°, extracted a final time with phenol, applied to Sephadex G-100 (Pharmacia), and collected in the void volume. "Late lytic" RNA was prepared from cells in a similar fashion 22, 55, or 72 hr after infection.

The RNA associated with SV40 abortive infection was prepared from a confluent monolayer of mouse embryo cells 24 hr after exposure to virus (multiplicity of 80–100 PFU/cell), and purified as described above.

In Vitro cRNA. DNA-dependent RNA polymerase containing sigma factor from *E. coli* was a gift of Dr. Walter Keller. Reaction mixtures (2.0 ml) consisting of 0.14 M KCl, 0.04 M Tris·HCl (pH 7.9), 0.1 mM dithiothreitol, 0.01 M $MgCl_2$, 4 mM each CTP, UTP, GTP, and ATP, 125 μg of SV40 DNA I, and 60 μg of RNA polymerase were incubated at 37° for 1 hr, extracted twice with phenol, digested with electrophoretically purified pancreatic DNase (20 μg/ml) for 1 hr at room temperature, applied to a Sephadex G-50 column equilibrated with 0.1 M NaCl after a final phenol extraction, and collected in the void volume.

Separation and Purification of SV40 DNA Strands. To separate the SV40 DNA strands, 110 μg of SV40 RNA (cRNA), prepared *in vitro* with *E. coli* RNA polymerase, was incubated with 0.03 μg of denatured SV40 [^{32}P]DNA fragments in a reaction mixture (50 ml) containing 0.5 M NaCl–0.01 M Tris·HCl, pH 7.5, for 1 hr at 68°. The reaction mixture was applied to a hydroxyapatite column. Single-stranded DNA was eluted in a small volume of 0.14 M phosphate buffer at 60°, and the DNA associated with the cRNA was recovered by washing the column with 0.4 M phosphate buffer at 60° (8). SV40 cRNA present in this eluate was eliminated by treatment of this fraction with 0.5 N NaOH for 6 hr at 37°. Both fractions were allowed to "self-associate" at a sodium ion concentration of 1.0 M for 24–48 hr at 60°, equivalent to a DNA C_0t of 6×10^{-2} in 0.14 M phosphate buffer. In our laboratory, SV40 [^{32}P]DNA fragments reassociate with a $C_0t_{1/2}$ of 2×10^{-3} in 0.14 M phosphate buffer. Any residual DNA reassociation products (never more than 10%) were removed by passage through hydroxyapatite columns.

DNA–RNA Hybridization. The proportion of each DNA strand capable of reacting with cellular RNA was determined by reaction of a large excess of infected cellular RNA with small amounts of fragmented viral [^{32}P]DNA (4). In most cases, separated strands of labeled SV40 DNA, at a concentration of 2–5 ng/ml, were incubated with cell RNA (0.1–2.0 mg/ml) in 1.0–1.2 M NaCl and 0.01 M Tris·HCl, pH 7.5, at 68°. Aliquots of the reaction mixture containing 300–400 cpm were removed at the indicated times and analyzed on hydroxyapatite.

RESULTS

We showed (4) that 50% of either intact or fragmented SV40 DNA reacts with SV40 cRNA. The *in vitro* cRNA almost certainly forms stable duplex structures with the *same* regions of the two DNA preparations, and fractionation into plus and minus strands involves the same polynucleotide sequences in both cases. Our earlier results indicate that only 1.5% of the plus strand reannealed under incubation conditions where 72% of unfractionated, sheared, SV40 DNA reassociated.

In addition, only 4% of the plus strand reacted with SV40 cRNA, as compared to 54% of an unfractionated SV40 DNA preparation run simultaneously under identical conditions (4). The minus strand of SV40 DNA has now been purified; it self-anneals 1–3% when incubated at a concentration of 0.05–0.10 μg/ml in 1.0 M NaCl for 24 hr at 68°. More than 90% of unfractionated SV40 DNA fragments reassociate under these conditions. In addition, when the minus strand of SV40 DNA (3.5 ng/ml) is incubated with SV40 cRNA (5 μg/ml) for 2 hr at 68° in 1.0 M NaCl, over 95% forms stable duplex molecules. A mixture of the plus and minus strands of SV40 [^{32}P]DNA in 1.0 M NaCl readily reanneals, with a $C_0t_{1/2}$ of 3.9×10^{-4}, as compared to a value of 3.3×10^{-4} for unfractionated viral DNA fragments.

Reaction of SV40 DNA with cellular RNA from productively infected monkey cells

SV40 undergoes a typical lytic cycle in African green monkey kidney cells (permissive). Productive infection of such monkey cells has been arbitrarily divided into "early" and "late" phases, with the temporal separation occurring at the time of viral DNA synthesis. In Vero cells, DNA synthesis can be detected 24–26 hr after infection (L. Gelb and M. Martin, unpublished observation). The "early lytic" RNA used in these studies was prepared from cells 12 hr after virus inoculation, or from cells infected in the presence of arabinosyl cytosine, a potent inhibitor of DNA synthesis. "Late lytic"

RNA was isolated from monkey cells 55–72 hr after exposure to SV40.

"Late lytic" RNA (0.5 mg/ml), prepared from Vero cells 55 hr after infection with SV40, was incubated separately with the plus and minus strands of viral DNA. Fig. 1 indicates that 42% of the minus strand and 62% of the plus strand react with the preparation of "late" RNA. This pattern of RNA synthesis could be detected as early as 22 hr after infection, in agreement with the findings of Sauer (9).

The results obtained with "early lytic" RNA were quite different. Fig. 2 shows that RNA, prepared from cells 32 hr after virus infection in the presence of arabinosyl cytosine, reacted with about 39% of the minus strand. The plus strand of SV40 DNA failed to form any significant amount of stable duplex molecules with "early lytic" RNA under these conditions. When "early lytic" and "late lytic" RNA preparations were mixed and incubated with the minus strand, about 43% of the DNA reacted. This result is virtually identical to the value obtained for "late lytic" RNA alone, in the experiment shown in Fig. 1. Experiments with the RNA prepared from green monkey cells grown in the absence of arabinosyl cytosine, 12 hr after infection, gave similar results.

Reaction of SV40 DNA with cellular RNA prepared from SV40-infected mouse embryo cells

SV40 is not able to replicate in mouse cells (nonpermissive). Attempts to detect viral DNA synthesis and SV40 capsid proteins have been unsuccessful (10–12). Some "early" functions, presumably specified by the viral genome, such as tumor antigen synthesis and the induction of cellular DNA synthesis, do occur after infection. Cellular RNA was prepared from mouse-embryo cells 24 hr after exposure to SV40 and incubated with the plus and minus strand of viral DNA. Fig. 3 shows that the RNA present during "abortive infection" reacts with 38% of the minus strand and 62% of the plus strand. If mixtures of "late lytic" and "abortively infected" RNA are added to the fragmented, separated strands of SV40 DNA, about 40 and 64% of the minus and plus strands, respectively, react (Fig. 3). These values are indistinguishable from those obtained when "late lytic" RNA, alone, was added to the separated strands (Fig. 1). In another experiment, a mixture of "early lytic" and "abortively infected" RNA reacted with about 42% of the minus strand.

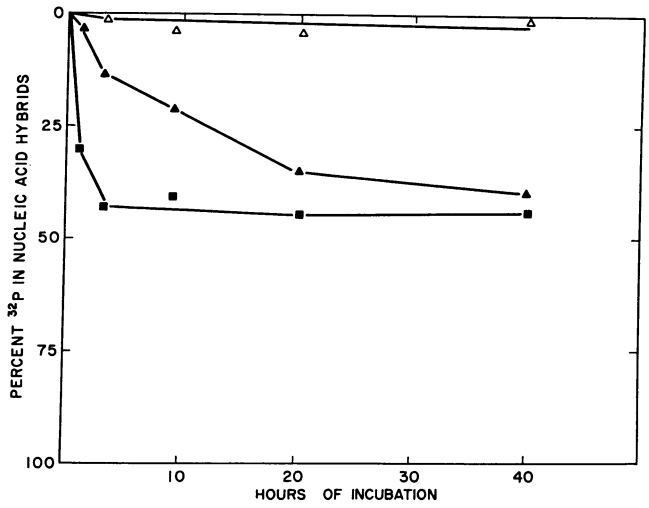

FIG. 2. Hybridization of "early lytic" RNA to the plus and minus strands of ^{32}P-labeled fragmented SV40 DNA. Infected cellular RNA (1.9 mg/ml) obtained 34 hr after SV40 infection in the presence of arabinosyl cytosine was incubated with plus (△——△) or minus (▲——▲) strands of SV40 DNA (3.5 ng/ml) in 1.0 M NaCl at 68°. Under identical conditions, "early lytic" RNA (1.5 mg/ml) plus "late lytic" RNA (0.5 mg/ml) were incubated with the minus strand of SV40 DNA (■——■). Aliquots taken at the indicated times were analyzed on hydroxyapatite.

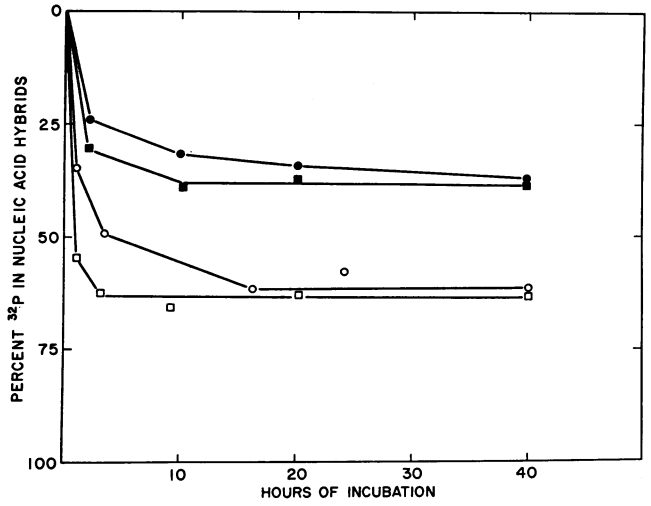

FIG. 3. Hybridization of "abortively infected" mouse 3T3 RNA to the plus and minus strands of ^{32}P-labeled, fragmented SV40 DNA. Abortively infected 3T3 RNA (0.45 mg/ml) obtained 24 hr after inoculation with SV40 was incubated with plus (○——○) or minus (●——●) strands of SV40 DNA (3.5 ng/ml) in 1.0 M NaCl at 68°. Also shown in this figure is the hybridization of "abortive" RNA (0.40 mg/ml) plus "late lytic" RNA (0.5 mg/ml), to the plus (□——□) or minus (■——■) strands of SV40 DNA. The conditions of incubation were identical to those described above. Aliquots were taken at the indicated times and analyzed on hydroxyapatite.

One can calculate the relative amounts of virus-specific RNA present early and late in the lytic cycle, as well as in cells abortively infected with SV40, from the plateau values shown in Figs. 1–3. The "late lytic" RNA contains about 30- to 50-fold more SV40-specific RNA than the "early lytic" preparation; it also has about 5 times more virus-specific RNA than the sample prepared from the abortively infected mouse cells.

DISCUSSION

These studies clearly indicate that the SV40 gene products present during the early and late phases of productive infection are transcribed from different DNA strands. Early in the lytic cycle, only 40% of the minus strand is expressed; at later times, this region continues to be transcribed, but is accompanied by viral gene activity involving about 60–64% of the plus strand (Figs. 1 and 2). These results are in agreement with the data of D. Lindstrom and R. Dulbecco, who have recently shown that "early lytic" RNA has the same polarity as cRNA, while a significant portion of "late lytic" RNA is complementary to cRNA (13). Hydroxyapatite does not discriminate between partially reannealed and completely reassociated nucleic acids (4). We found, for example, that as the SV40 DNA probe becomes smaller in size, the proportion capable of reacting with infected cellular DNA falls from 98%

to about 55%, obviously reflecting the elimination of regions from large molecules that do not interact. Consequently, our values may also represent an "overestimate", even though we used labeled viral DNA equivalent in size to 10% of the SV40 genome.

One can only speculate about those modulating factors that bring about a switch to the plus strand after the initiation of viral DNA synthesis. One possibility would be the appearance of a new virus-specified RNA polymerase late in productive infection that can transcribe polynucleotide sequences on the plus strand. Such a new RNA polymerase has been reported after infection of *E. coli* with T7 phage (14, 15). A second possibility may involve the physical state of the viral genome. There have been both direct and indirect experiments suggesting that integration of SV40 occurs during productive infection (16–19). If the viral genome becomes covalently associated with mammalian chromosomal DNA early in the lytic cycle, cellular control factors may be the sole determinant of those regions of the SV40 DNA that will be expressed. At later times, however, when large numbers of "free" viral DNA molecules abound in the nuclei of infected cells, regions of the SV40 genome may then become "accessible" to the RNA-polymerizing apparatus, leading to the appearance of new ("late") species of RNA.

The pattern of SV40 DNA transcription in (nonpermissive) mouse embryo cells was most unexpected. Detectable amounts of newly synthesized viral DNA or viral structural proteins have not been found after infection of murine cells (10–12). Yet, the results shown in Fig. 3 clearly indicate that the plus and minus SV40 DNA strands are expressed to extents similar to that seen late in productive infection. It is not surprising that similar regions of the minus strand are transcribed in abortively infected mouse cells and, at early times, in monkey cells, since both cell types share many biological and immunological properties. However, the presence of a a transcript of the plus strand, indistinguishable from that found in permissive monkey cells, seems unusual. This result suggests that regulation of SV40 gene activity in abortively infected mouse cells occurs at a step subsequent to transcription. Whether such modulation occurs at the level of transport of RNA from the nucleus to the cytoplasm, or involves control at the translational level, remains to be determined.

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