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Regulation of Simian Virus 40 Early and Late Gene Transcription Without Viral DNA Replication

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Primary cultures of African green monkey kidney cells were infected with the simian virus 40 temperature-sensitive mutant *tsA58* at the nonpermissive temperature of 41°C for 12 to 20 h. Under these conditions, a defective T antigen was produced and no viral DNA replication was detected. Viral transcription complexes were extracted from infected nuclei using Sarkosyl and the nascent chains of RNA elongated in vitro. Sixty to 70% of the viral RNA synthesized in vitro hybridized to late gene sequences. In contrast, 80 to 90% of the nuclear viral RNA labeled in vivo during a 15-min pulse with [^{3}H]uridine hybridized to early gene sequences. This suggests that selective degradation of late gene transcripts occurs in vivo. The role of T antigen and viral DNA replication in regulation of simian virus 40 transcription is discussed.

A major unresolved problem concerning simian virus 40 (SV40) and other animal viruses that do not code for their own RNA polymerases is the mechanism whereby different classes of mRNA molecules are formed at early and late times in the infectious cycle.

SV40 produces two classes of mRNA molecules. One class, early mRNA, is found throughout the infectious cycle in permissive cells and is present in all transformed cells. The second class is late mRNA, which is detected only after initiation of viral DNA synthesis in permissive cells (for review, see reference 1).

Since stable late mRNA is not produced until viral DNA synthesis commences, it was hypothesized that late transcripts might be made on a template having a configuration similar to that of replicating DNA (7, 10) while DNA I from input virions would serve as the template for early transcripts made prior to DNA replication. This difference in template configuration could explain how SV40 produces two classes of mRNA. However, we have shown that SV40 DNA I, rather than replicating molecules, is the template for late transcription (3).

In previous studies we presented evidence that the nucleoprotein complexes that can synthesize viral RNA in vitro are the complexes that synthesize viral RNA in vivo (17). These transcription intermediates (TIs) have been characterized after the onset of viral DNA synthesis. The relative amounts of viral RNA synthesized in vitro at late times and its hybridization to the separated strands of viral DNA are similar to the viral RNA synthesized in vivo (2).

In this study, we characterized the nascent SV40 RNA synthesized in vitro by TIs isolated from infected cells in which viral DNA replication had not occurred. Cells were infected with the *A* gene temperature-sensitive mutant *tsA58* at a nonpermissive temperature to block initiation of viral DNA replication. TIs were then isolated by lysing infected nuclei with Sarkosyl. The unexpected results were that both early and late viral genes were transcribed in vitro and the major fraction of the viral RNA was transcribed from the late strand (14). However, when cells were examined in vivo at these same times, more than 85% of the pulse-labeled nuclear RNA hybridized to the coding strand of the early gene region. Therefore, there must be a rapid and selective degradation of the late transcripts. Based on the types of viral RNAs synthesized in vitro by TIs before and after initiation of viral DNA replication, a model is proposed for temporal control of transcription during the lytic cycle of infection.

MATERIALS AND METHODS

Tissue culture and virus. *tsA58* virus stocks were prepared by using African green monkey kidney (AGMK) BSC-1 cells that were infected at an input multiplicity of 0.01 to 0.10 PFU per cell. The stock cultures were maintained at 33°C until approximately

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80% of the cells were no longer attached to the cell monolayer. If the viral DNA was labeled, [^{3}H]thymidine (specific activity, 60.36 Ci/mmol; New England Nuclear) was added after 4 days to give a final concentration of 20 mCi/ml. Virus was then purified by centrifugation in CsCl density gradients as previously described (4).

Purified virus was used to infect primary cultures of AGMK cells (Microbiological Associates). Cultures were infected at 41°C with 200 to 2,000 PFU per cell and grown 12 to 20 h at 41°C before harvesting.

Extraction of transcription complexes. The procedure to prepare the Sarkosyl supernatant is the same as previously described (3), except the cells were trypsinized at 41°C. If a Sarkosyl lysate was used, the isolated nuclei were lysed and the entire lysate was added to an RNA polymerase reaction mixture. If a Sarkosyl pellet was used, the pellet obtained after centrifugation of a Sarkosyl lysate was scraped off the bottom of the centrifuge tube into lysis buffer and placed intact into an RNA polymerase reaction mixture.

RNA synthesis conditions. The RNA polymerase reaction was incubated at 21°C for 60 min as previously described (3). When comparing Sarkosyl lysate, supernatant, and pellet fractions, each fraction was obtained after dividing the resuspended nuclei into three equal parts. Lysis buffer was added to each fraction to give equal final volumes before addition to the RNA polymerase reaction mixture.

Purification and hybridization of RNA. Twenty percent sodium dodecyl sulfate (SDS) was added to the reaction mixture to give a final concentration of 1% SDS. The samples were then digested with 20 μg of DNase (Worthington Biochemicals) per ml for 60 min at 21°C followed by digestion with 200 μg of proteinase K per ml for 90 min at 21°C. The RNA was extracted with a phenol-chloroform-isoamyl alcohol mixture, ethanol precipitated, DNase treated, and extracted again as described by Birkenmeier et al. (2). The pelleted and dried RNA was suspended in a solution of 1.0 ml of 10 mM Tris, 10 mM EDTA, 50 mM NaCl, and 0.5% SDS. The RNA was chromatographed in a Sepharose 4B column (24 by 2.5 cm) equilibrated with the same buffer to remove acid-soluble counts. The fractions containing ^{32}P-RNA were pooled and the RNA was precipitated by addition of 2 volumes of 95% ethanol at −20°C. The RNA was then hybridized, as previously described, to SV40 DNA and restriction enzyme fragments of SV40 DNA (11) or separated strands of SV40 DNA (2). Controls to insure DNA excess were performed by lowering the input counts of ^{32}P-RNA and the input multiplicity of infection or by rehybridizing unhybridizable ^{32}P-RNA to fresh filters. Under all conditions the patterns of hybridization remained constant.

Sedimentation of transcription complexes. Transcription complexes were sedimented in 5 to 20% (wt/wt) sucrose gradients after the RNA polymerase reaction as described by Birkenmeier et al. (3).

In vivo RNA synthesis. Infected cells were pulsed for 15 min at 41°C with 0.5 μCi of [5,6-^{3}H]uridine (specific activity, 49 Ci/mmol; New England Nuclear) per ml. The nuclei were isolated and the RNA was extracted for hybridization as described above. For the experiments in which the in vivo labeled RNA was hybridized to separated strands of SV40 DNA, the cells were pretreated with 20 mM glucosamine for 90 min before the pulse.

SV40 DNA replication. Cells were infected and maintained at 33 or 41°C. They were pulsed with 100 μCi of [^{3}H]thymidine per ml from 0 to 24 h postinfection. The viral DNA was selectively extracted as described by Hirt (8). The labeled DNA in the supernatant fluid was centrifuged in CsCl gradients containing ethidium bromide (16) or hybridized to SV40 DNA immobilized on nitrocellulose filters (5).

Radiography. Nitrocellulose filters containing the hybridized SV40 ^{32}P-RNA were exposed to Kodak XR-2 film with Cronex intensifying screens (DuPont) at −70°C. Fluorography of hybridized SV40 ^{3}H-RNA was performed under the same conditions except the nitrocellulose filters had been momentarily immersed in toluene containing 20% 2,5-diphenyloxazole and air dried. Densitometric tracings were made of each film, and the areas under the peaks were calculated as described by Birkenmeier et al. (2).

RESULTS

Localization of viral DNA after infection. Primary cultures of AGMK cells were infected with [^{3}H]thymidine-labeled *tsA*58 virions at 41°C, and the cultures were maintained at that temperature. The nuclei were isolated 12 h postinfection and lysed with Sarkosyl, and the cellular DNA was pelleted. The number of trichloroacetic acid-insoluble ^{3}H counts in the supernatant fluid (Sarkosyl supernatant) and pellet was measured. They represented 8 to 10% of the total ^{3}H counts used to infect the cells. About 10% of the ^{3}H counts in the lysed nuclei were recovered in Sarkosyl supernatants, whereas the remaining 90% were in the pellet. Parallel experiments were done in which the procedure described by Hirt (8) was used to lyse the isolated nuclei with SDS. After centrifugation, 70 to 80% of the ^{3}H counts were recovered in the supernatant fluid, whereas the remainder were in the pelleted material that contained the cellular DNA. These data show that, in the presence of Sarkosyl, most of the low-molecular-weight ^{3}H-DNA remains associated with the cellular DNA due to entrapment. This is in contrast to late times of infection with *tsA*58 or wild-type virus where 80 to 90% of pulse-labeled viral DNA is found in the Sarkosyl supernatant (2, 17). This difference is probably due to the much larger quantities of viral DNA that exist at the late times.

Synthesis of SV40 RNA. Previous experiments have shown that extracts prepared from nuclei lysed with Sarkosyl are capable of incorporating radioactive nucleoside triphosphates into viral RNA during the late phase of lytic infection (6). When nuclei from AGMK cells

infected with *tsA*58 at 41°C for 12 h were lysed with Sarkosyl, SV40 RNA synthesis was detected. A *tsA* mutant has been used to infect cells at a restrictive temperature to insure that there is a block in viral DNA replication. Figure 1 shows the rate of incorporation of [α-^{32}P]UTP into viral RNA. The Sarkosyl supernatant and pellet synthesized viral RNA in nearly equal amounts after 60 min, but there were significant differences in the kinetics of the two reactions.

Since half of the viral transcriptional activity was contained in the Sarkosyl supernatant, and this fraction contains only 10% of the viral DNA in the nucleus, its specific activity was several-fold higher than the pellet fraction. However, as noted below, both nuclear fractions produced a similar population of viral transcripts.

It was determined which regions of the SV40 DNA were transcribed in the absence of DNA replication by hybridizing the in vitro-synthesized RNA to restriction enzyme fragments of the SV40 genome. Digestion of SV40 with *Bam*I and *Hpa*II produces two fragments, A (0.16 to 0.735 map unit) and B (0.735 to 0.16 map unit). Fragment A (early region) contains the genes for T antigens, and fragment B (late region) contains the genes for late viral proteins VP1, VP2, and VP3. The RNA synthesized in vitro during a 15-min reaction was hybridized to the A and B fragments contained on nitrocellulose filters.

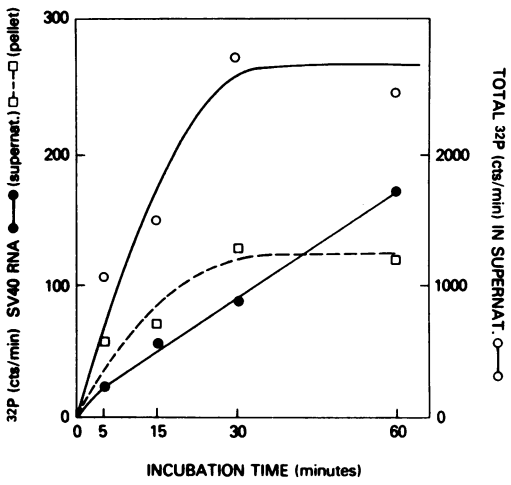

FIG. 1. *Rate of incorporation of [α-^{32}P]UTP into viral RNA and total RNA. Supernatant and pellet fractions prepared from tsA58-infected nuclei lysed with Sarkosyl were incubated for various times at 21°C in an RNA polymerase reaction mixture. Portions were removed from the supernatant reaction to measure total acid-insoluble counts and the number of counts incorporated into SV40 RNA. The four pellets were added to reaction mixtures for varying times of incubation to measure the number of counts incorporated into SV40 RNA.*

Fifty-five percent of the SV40 RNA synthesized by the Sarkosyl supernatant hybridized to the B fragment. Seventy-one percent of the SV40 RNA synthesized by the pellet also hybridized to the B fragment.

To confirm that the late region of SV40 (fragment B) was also transcribed in vivo in the absence of DNA synthesis, a 15-min in vivo pulse was done with [^{3}H]uridine just before isolating the nuclei. The nuclear ^{3}H-RNA was then hybridized to the A and B fragments. Thirty-two percent of the SV40 RNA (0.1% of the total ^{3}H-RNA) hybridized to the B fragment.

Strand selection of transcription. RNA transcripts that hybridized to the late region were detected in vitro and in vivo. However, the interpretation of these findings required that we determine which strand of SV40 DNA was being transcribed. The early genes in fragment A are transcribed from the early, or E, strand of DNA, whereas the late genes in fragment B are transcribed from the late, or L, strand of DNA (9). A DNA mixture of fragments A and B was denatured in alkali; the strands of each fragment were separated by electrophoresis in an agarose gel; and the DNA was transferred to nitrocellulose filters by the method of Southern (18).

Figure 2 shows the hybridization of RNA synthesized in vitro by *tsA*58-infected nuclei lysed with Sarkosyl. One-half of the nuclei lysed with Sarkosyl (Sarkosyl lysate) was assayed directly for RNA polymerase activity (Fig. 2a), and the other half was lysed and then centrifuged to give a Sarkosyl supernatant (Fig. 2b) and pellet (Fig. 2c) that were also assayed. Figure 3 shows a densitometric tracing of the hybridized ^{32}P-RNA from a Sarkosyl supernatant reaction. These data support several conclusions. First, the total amount of SV40 transcription in the Sarkosyl lysate is divided nearly equally between the supernatant and pellet. Second, the relative amounts of the SV40 RNA species synthesized by the lysate, supernatant, and pellet are the same. Third, there are L strand transcripts, and most of these are from the late region (fragment B). Fourth, the E strand in both fragments A and B is transcribed in approximately equal amounts.

Figure 3 also shows that a majority of the primary transcripts are transcribed from the L strand. In vivo experiments by Rosenthal and Brown (13) showed that the stable cytoplasmic viral mRNA of *tsA*58-infected cells at 41°C was exclusively transcribed from the E strand. To understand this apparent discrepancy, we looked for evidence in vivo of nuclear RNAs that could represent species intermediate between the RNA synthesized by TIs and stable cytoplasmic early mRNA. Cells infected with *tsA*58

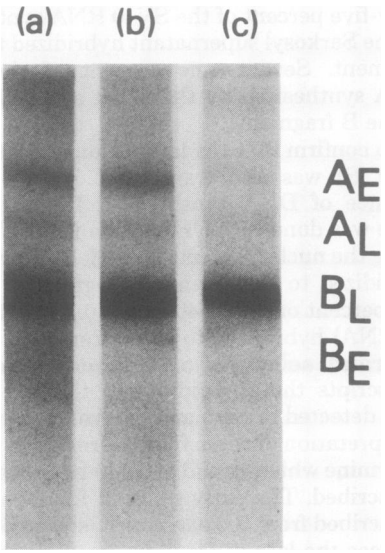

FIG. 2. *Autoradiogram of RNA hybridized to separated strands of SV40 DNA. Isolated nuclei were suspended in buffer and divided in half. One-half was lysed with Sarkosyl to give a nuclear lysate. The other half was lysed with Sarkosyl and centrifuged to give a pellet and a supernatant fluid. The lysate, supernatant, and pellet were then assayed for RNA polymerase activity and incorporated 150,000 cpm, 31,000 cpm, and 68,000 cpm, respectively, into RNA. The ^{32}P-RNA synthesized was purified and hybridized to separated strands of SV40 DNA: A_E, A fragment—early strand; A_L, A fragment—late strand; B_L, B fragment—late strand; B_E, B fragment—early strand. (a) Lysate RNA; (b) supernatant RNA; (c) pellet RNA.*

at 41°C for 12 h were pretreated with glucosamine to lower the intracellular UTP concentration (15) and then were pulsed for 15 min with [^{3}H]uridine. Nuclei were isolated, and the RNA was extracted. Figure 4 shows the hybridization of the nuclear ^{3}H-RNA to the separated strands of fragments A and B. The ^{3}H-RNA hybridizes mainly to the E strand of the A fragment and most likely represents mature early mRNA or its precursor. There is a detectable amount of L strand transcripts, but they represent only a small fraction of the pulse-labeled SV40 nuclear RNA. In summary, the data with in vitro transcription intermediates strongly suggest that most of the E strand and at least a portion of the L strand are transcribed during *ts*A58 infection at 41°C. Based on in vivo studies, it is seen that the primary transcripts must be rapidly processed in the nucleus so that only transcripts of the E strand of fragment A are found in stable cytoplasmic mRNA.

Characterization of the *ts*A58 virus infection. The assumption is made with a *ts*A58 virus mutant that there is a complete block in viral DNA replication at 41°C. To verify this, AGMK cells were infected with *ts*A58 or were mock infected at either 41 or 33°C and then grown 24 h at the same temperature with [^{3}H]thymidine in the medium. The low-molecular-weight DNA was extracted from whole cells by the procedure described by Hirt (8). Isopycnic banding of this DNA in cesium chloride gradients containing ethidium bromide demonstrated that labeled SV40 DNA I could only be found in the *ts*A58-infected cells grown at 33°C. The DNA was also hybridized to SV40 DNA immobilized on nitrocellulose filters. DNA prepared from cells in-

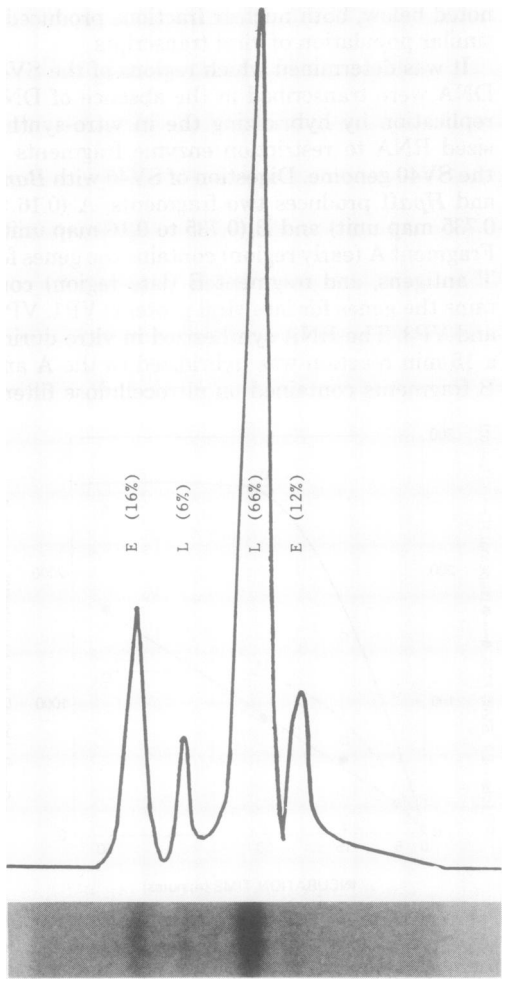

FIG. 3. *Densitometric tracing of RNA hybridized to separated strands of SV40 DNA. The ^{32}P-RNA synthesized in vitro by the Sarkosyl supernatant was hybridized to the early (E) and late (L) strands of SV40 DNA. A densitometric tracing of the autoradiogram shown in the figure was made, and the relative area under each peak was calculated.*

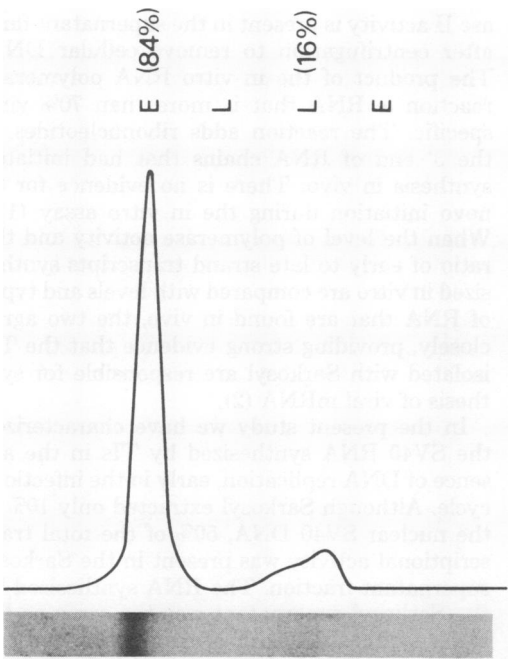

FIG. 4. *Densitometric tracing of RNA hybridized to separated strands of SV40 DNA. Infected cells at 41°C were pulsed 15 min with [^{3}H]uridine. The nuclei were isolated and the RNA was extracted. The ^{3}H-RNA was hybridized to the early (E) and late (L) strands of SV40 DNA. A densitometric tracing of the fluorogram was made, and the relative area under each peak was calculated.*

fected with *tsA*58 at 41°C gave low levels of hybridization identical to levels observed with mock-infected cells (data not shown). Thus, there was no evidence of any *tsA*58 DNA replication in AGMK cells maintained at 41°C.

Another important variable in these studies is the number of PFU used to infect the cells. The multiplicity of infection varied in experiments in which different preparations of purified labeled virus were used to infect the cells. Cells were infected with ^{3}H-labeled virus at a high number of PFU per cell to provide enough nuclear counts to allow characterization of the DNA and also allow easy detection of viral transcription. To determine whether our results were a function of the input multiplicity, cells were infected with different numbers of PFU per cell, and the nuclei were isolated after growth for 12 h at 41°C. A Sarkosyl lysate was prepared, and the ^{32}P-RNA synthesized in vitro was hybridized as shown in Fig. 5. As the input multiplicity decreased, there was a corresponding decrease in the amount of SV40 RNA synthesized. The overall pattern of hybridization did not change, and the L strand of fragment B was always the predominant band.

Characterization of the transcription complexes. A Sarkosyl supernatant was prepared 12 h postinfection from cells infected with *tsA*58 at 41°C, and SV40 RNA was synthesized in vitro in the presence of [α-^{32}P]UTP. The reaction mixture was then sedimented in a sucrose gradient, and the acid-insoluble counts were measured in each fraction as shown in Fig. 6. The ^{32}P-RNA sedimenting faster than 21S (fractions 1 to 12) and the more slowly sedimenting ^{32}P-RNA (fractions 13 to 26) were isolated and hybridized to separated strands of SV40 DNA as shown in Fig. 7. Most of the SV40 ^{32}P-RNA sedimented faster than 21S since it is presumably still associated with the DNA template. These results are similar to those found after initiation of viral DNA replication. *tsA*58 transcription complexes have been isolated at late times during infection at 33°C or after a 2-h shift to 41°C. In both cases they had a sedimentation velocity greater than 21S (2).

α-Amanitin, at a concentration of 0.4 μg/ml, totally inhibited synthesis of all SV40 RNA in vitro (data not shown). RNA polymerase II, therefore, is the enzyme transcribing the SV40 DNA prior to initiation of viral DNA synthesis.

DISCUSSION

We have previously characterized TIs that are extracted from the nuclei of infected cells by lysing the nuclei with Sarkosyl. RNA polymer-

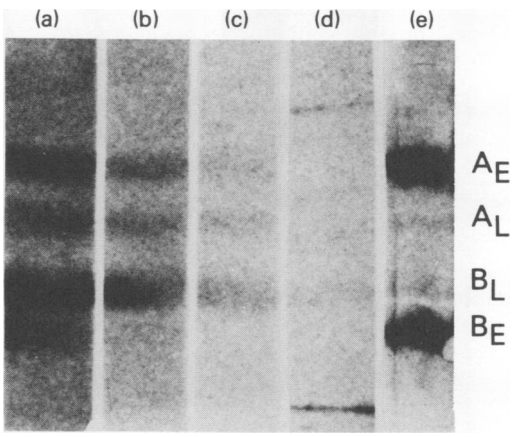

FIG. 5. *Autoradiogram of RNA hybridized to separated strands of SV40 DNA. Cells were infected with different numbers of PFU. The nuclei were isolated and lysed with Sarkosyl, and the lysate was assayed for RNA polymerase activity. The RNA synthesized was hybridized to the separated strands of SV40 DNA: A_E, A_L, B_L, and B_E as in Fig. 2. (a) 2,000 PFU/cell; (b) 200 PFU/cell; (c) 20 PFU/cell; (d) 2 PFU/cell; (e) hybridization of SV40 early strand complementary RNA transcribed by Escherichia coli RNA polymerase.*

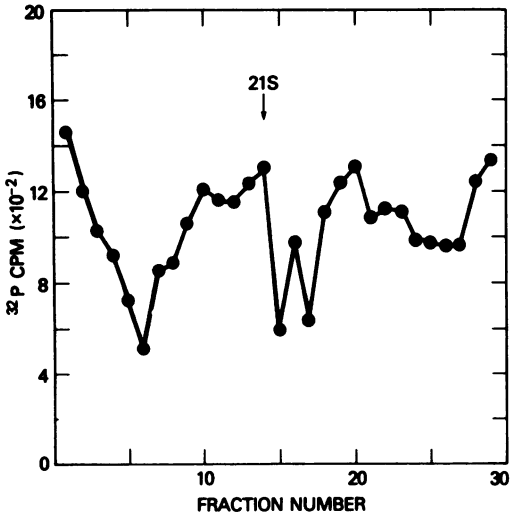

FIG. 6. *Sedimentation analysis of reaction products. Cells were infected at 41°C with tsA58 virus. A Sarkosyl supernatant was prepared and incubated in an RNA polymerase reaction mixture containing [α-^{32}P]UTP. The reaction products were immediately sedimented in a 5 to 20% (wt/wt) sucrose gradient. The acid-insoluble ^{32}P counts were measured in each fraction. Sedimentation is from right to left.*

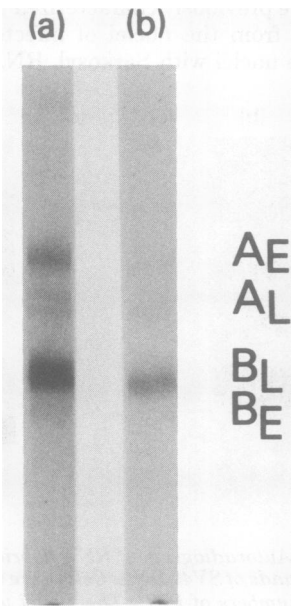

FIG. 7. *Autoradiogram of RNA hybridized to separated strands of SV40 DNA. Sucrose gradient fractions 1 to 12 and 13 to 26 in Fig. 6 containing 176,000 cpm and 185,000 cpm, respectively, were pooled, and the ^{32}P-RNA was hybridized to the separated strands of SV40 DNA: A_E, A_L, B_L, and B_E as in Fig. 2. (a) RNA in fractions 1 to 12; (b) RNA in fractions 13 to 26.*

ase II activity is present in the supernatant fluid after centrifugation to remove cellular DNA. The product of the in vitro RNA polymerase reaction is RNA that is more than 70% viral specific. The reaction adds ribonucleotides to the 3′ end of RNA chains that had initiated synthesis in vivo. There is no evidence for de novo initiation during the in vitro assay (17). When the level of polymerase activity and the ratio of early to late strand transcripts synthesized in vitro are compared with levels and types of RNA that are found in vivo, the two agree closely, providing strong evidence that the TIs isolated with Sarkosyl are responsible for synthesis of viral mRNA (2).

In the present study we have characterized the SV40 RNA synthesized by TIs in the absence of DNA replication, early in the infectious cycle. Although Sarkosyl extracted only 10% of the nuclear SV40 DNA, 50% of the total transcriptional activity was present in the Sarkosyl supernatant fraction. The RNA synthesized by the Sarkosyl supernatant was the same as the RNA synthesized with an unfractionated nuclear lysate (Fig. 2). In contrast with the findings at late times, at these early times there is a striking difference between the RNA synthesized in vitro (Fig. 3) and the pulse-labeled nuclear RNA that is found in vivo (Fig. 4). Almost all of the in vivo-labeled viral nuclear RNA hybridizes to the half of the early DNA strand that codes for T antigens. In contrast, the majority of the RNA transcribed in vitro hybridizes to the late DNA strand, and only about 30% hybridizes to the early strand.

Late in the infectious cycle, when cells infected at a permissive temperature are shifted to a restrictive temperature for 5 h, the RNA synthesized in vitro by transcription complexes is qualitatively similar to that observed at the early time; namely, about 70% of the transcripts come from the late strand, and 30% of the RNA comes from the early strand. If the infected cells at the late time are allowed to remain at the permissive temperature, greater than 90% of RNA synthesized in vitro is complementary to the late strand (2).

Based on the types of RNA synthesized by transcription complexes at early and late times, we can propose a tentative model as to how temporal regulation of SV40 RNA synthesis is affected (Fig. 8). We propose that when input virus becomes available as a template for transcription prior to the start of DNA synthesis, there are promotors available on both the early and late strands of the DNA. The greater abundance of late strand transcripts synthesized in vitro at the early time may reflect a higher affinity of late strand promotors for the RNA

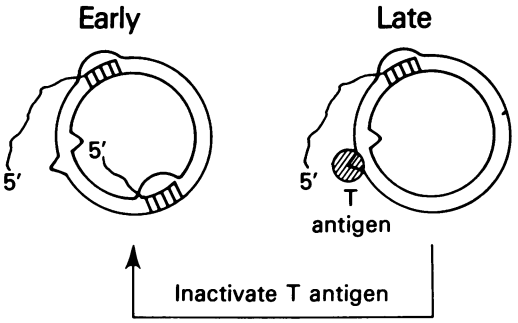

FIG. 8. *Regulation of transcription at early and late times in the SV40 lytic cycle. At the start of the infectious cycle, as input virions become available as templates for transcription, promotors are available for the synthesis of both early and late viral transcripts. After T antigen is made, it binds to the early promotor and blocks early transcription. When T antigen is inactivated (in cells infected with tsA58 virions which are shifted to a restrictive temperature), TIs again regain the capacity to synthesize both early and late viral transcripts.*

polymerase. The presence of in vivo RNA complementary to the late strand is difficult to demonstrate at these early times. When in vivo pulse-labeled nuclear RNA is examined, more than 85% is early SV40 RNA. Therefore, there must be a rapid and selective degradation of the late transcripts in the nucleus. The early transcripts give rise to mRNA molecules that code for the synthesis of T antigens.

When viral DNA synthesis begins, the newly synthesized DNA molecules are also used as templates for transcription. However, the binding of T antigen to these DNA molecules blocks the promotor on the early strand and so, at late times, late strand transcripts predominate. If T antigen is inactivated by shifting cells infected with a *tsA58* mutant to a restrictive temperature, then the promotor on the early strand is again available (Fig. 8). The pattern of transcription changes and becomes identical to the pattern we observe with input virus at the early time. This is consistent with the idea that the newly synthesized DNA molecules that are responsible for the late transcription are also competent templates for early transcription provided T antigen is inactivated.

The present study suggests that temporal regulation of transcription occurs at two levels. There is a selection for promotors which is regulated by T antigen. There is also a selective degradation of late transcripts at the early time. However, at late times stable cytoplasmic late mRNA is formed. The synthesis of late mRNA continues in vivo when *tsA58* cells are shifted to restrictive temperatures at late times (12). One basis for the synthesis of stable late mRNA at late times may be the 50- to 100-fold more rapid rate of transcription at the late times as compared with the early time. The pool of newly synthesized late RNA may be so large that transport of late RNA sequences to the cytoplasm can occur at late times even if late RNA is degraded at the same rate as occurs early in the infectious cycle. Alternatively, the late strand transcripts or the cellular processing of RNA may be different after viral DNA synthesis is initiated.

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