Characterization of Simian Virus 40 *tsA58* Transcriptional Intermediates at Restrictive Temperatures: Relationship Between DNA Replication and Transcription

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When nuclei from simian virus 40 (SV40)-infected cells are lysed with Sarkosyl and the chromatin is pelleted, the supernatant fluid contains a nucleoprotein complex capable of synthesizing viral RNA (Laub and Aloni, *Virology* 75:346-354, 1976; Gariglio and Mousset, *FEBS Lett.* 56:149-155, 1975). The level of activity of the RNA polymerase in the complex increased during infection in parallel with the amount of viral DNA that had been synthesized. If cells infected at 33°C with the SV40 mutant *tsA58* were shifted to the nonpermissive temperature of 40°C at any time between 18 and 48 h postinfection, no viral DNA replication was detected after 45 min and no new rounds of synthesis were initiated after 20 to 30 min. However, after this shift, polymerase activity associated with the nucleoprotein complex did continue to increase for 5 h, at which time it reached a plateau. There was an increase of RNA synthesized from both the early (E) and late (L) SV40 DNA strands, and there was a threefold increase in the ratio of early-to-late RNA species after the shift. In comparable experiments with cells infected with wild-type virions, no increase in polymerase activity occurred because of the temperature change alone. At 33°C, the relative amount of RNA transcribed from the wild-type E-strand was less than *tsA58* at 33°C and did not increase after a shift to 40°C. The *tsA58* transcriptional complexes extracted from cells grown at 33°C sedimented heterogeneously in sucrose gradients, with a peak near 26S. There were no detectable alterations in the sedimentation properties of the complexes when *tsA58*-infected cells were shifted to 40°C for 2 h. We conclude that continued synthesis of viral DNA is not an obligatory prerequisite for maintenance of late viral transcription nor is the sedimentation of the transcriptional complex at 26S related to actively replicating DNA molecules serving as templates for transcription. Further, an increase in late transcription can occur under conditions where reinitiation of viral DNA synthesis is prevented. The increase in the synthesis of early and late RNA at the restrictive temperature without concurrent DNA synthesis is discussed in relationship to the function of the A gene product.

Lytic infection of African green monkey kidney cells by simian virus 40 (SV40) produces two classes of viral mRNA. The first is early mRNA, which is made throughout the infectious cycle and is complementary to approximately 50% of the early (E) strand of viral DNA. Early mRNA is translated into the A protein, T-antigen, and it has been proposed that its synthesis is regulated by the A protein through a negative-feedback mechanism (14, 20). The second class of viral mRNA is late mRNA, which appears after initiation of viral DNA replication and is complementary to ap-

proximately 41% of the late (L) strand of viral DNA (1, 11a). Late transcription and DNA replication are detected only if the early viral gene (gene A) is expressed (2). There may be a direct relationship between viral DNA replication and late transcription, since "replicating-like" viral DNA molecules have been reported to be associated with nascent viral RNA (4). However, once late transcription has begun, late mRNA synthesis continues when expression of the A gene is blocked (2). The hypothesis that replicating molecules serve as templates for late transcription is difficult to reconcile with the finding that maintenance of a pool of replicating molecules requires continued expression of gene A.

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Enzymatically active SV40 nucleoprotein complexes extracted from isolated nuclei by Sarkosyl are capable of synthesizing viral RNA *in vitro* and are believed to be derived from *in vivo* transcriptional intermediates of the virus (5, 9, 13). In this study we have characterized the transcriptional intermediates of the SV40 gene A mutant, *tsA58*, and measured the RNA polymerase activity contained in *tsA58* transcriptional intermediates extracted from infected cells grown at 33°C and subsequently shifted to the nonpermissive temperature of 40°C. These experiments provide further insight into regulation of viral transcription and how it is related to the gene A product and DNA replication.

MATERIALS AND METHODS

Tissue culture and virus. SV40 mutant virus *tsA58*, provided by Peter Tegtmeyer, and SV40 wild-type stocks were propagated in African green monkey kidney BSC-1 cells grown in plastic tissue culture flasks (150 cm²; Costar) and infected at input multiplicities of 0.01 PFU/cell. All experiments used BSC-1 cells grown in plastic petri dishes (60 mm for DNA replication studies and 150 mm for transcription studies; Falcon Plastics). Confluent monolayers grown in Eagle medium with 2 mM glutamine, 1.35 mg of streptomycin per ml, 0.62 mg of penicillin per ml, 0.20 mg of auromycin per ml, 500 U of mycostatin per ml, and 10% fetal calf serum were trypsinized and replated. After 24 h at 37°C, the cells, now 70 to 90% confluent, were infected with SV40 *tsA58* or SV40 wild-type virus at an input multiplicity of 1 to 5 or 20 PFU/cell, respectively, at 33°C. Infected cells were then grown in the same medium except that it contained 2% fetal calf serum. Cells were shifted from 33 to 40°C by floating the petri dishes on water in a 40°C incubator.

Extraction of transcriptional complexes. The procedure for extraction of transcriptional complexes is the same as previously described (13), with certain modifications. The 150-mm petri dishes were removed from the incubator and immediately placed onto an ice bath. The cells, extracts, and reagents were kept at 4°C during the isolation procedure. The medium was removed, and 2.0 ml of calcium- and magnesium-free phosphate-buffered saline with trypsin (1:250; Difco Laboratories), 0.5 mg/ml, and EDTA, 0.2 mg/ml, were added to each petri dish. The cells were then gently scraped from the petri dish with a rubber policeman and pelleted at 500 × *g* for 5 min. The nuclei were isolated as previously described (16) and resuspended in 0.15 ml of 0.05 M Tris-hydrochloride (pH 7.9), 0.05 M KCl, and 0.0005 M dithiothreitol (Re buffer) per petri dish. Then, 1 volume of 0.5% Sarkosyl NL 97 (recrystallized in 95% ethanol) in 0.8 M NaCl was added, and the resultant nuclear lysate was centrifuged in polyallomer centrifuge tubes in a Beckman SW50.1 rotor at 21,000 rpm for 30 min to pellet the chromatin. The resulting supernatant fluid was poured out of the centrifuge tube and is referred to as the Sarkosyl

supernatant. The number of petri dishes used to prepare each Sarkosyl supernatant varied from 2 to 6.

Assay of RNA polymerase activity. The assay of RNA polymerase activity was performed as previously described (18), with several modifications. The standard assay mixture contained, in 0.125 ml: 0.05 μmol of ATP, GTP, and CTP; varying amounts of UTP and [³H]UTP (specific activity, 27.6 to 41.5 Ci/mmol) or [α -³²P]UTP (specific activity, 200 Ci/mmol) as described below; 5 μmol of Tris-hydrochloride, pH 7.9; 5 μmol of (NH₄)₂SO₄; 0.2 μmol of MnCl₂; 0.05 μmol of EDTA; 0.5 μmol of dithiothreitol; and 0.075 ml of Sarkosyl supernatant. The reactions were run at low UTP concentrations (0.12 to 0.43 nmol per standard assay mixture), where the UTP concentration was rate limiting, or at high UTP concentration (4.20 nmol per standard assay mixture), where the enzyme velocity approached *V*_{max}. Several experiments were run in parallel using high and low UTP concentrations, and similar data were observed except for the expected increase in enzyme velocity at high UTP concentrations. For each experiment presented, the UTP concentration in the assay is stated. The reaction mixture was incubated at 21°C for 1 h unless otherwise stated, and the reaction was terminated by adding 2 to 3 ml of 5% trichloroacetic acid in 0.01 M sodium pyrophosphate at 4°C unless RNA hybridization was done, in which case 10% sodium dodecyl sulfate was added to give a final concentration of 1%. To measure the amount of [³H]UMP incorporated, the [³H]UMP-labeled RNA was collected on Whatman GF/C glass-fiber filters, washed under suction with 100 ml of 2.5% trichloroacetic acid in 0.01 M sodium pyrophosphate, rinsed with 5 ml of 95% ethanol, dried under an infrared heat lamp, and counted in 7 ml of toluene-based scintillant {toluene with 4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter}. Background counts were determined by adding the 5% trichloroacetic acid in 0.01 M sodium pyrophosphate immediately after addition of the Sarkosyl supernatant to the reaction mixtures and were subtracted from the counts measured after incubation at 21°C. For enzyme activity expressed as [³H]UMP counts per microgram of protein, the protein concentration in the Sarkosyl supernatant was determined as described by Lowry et al. (10).

RNA extraction and hybridization. The RNA polymerase reaction mixture after 1 h of incubation was diluted with 10 volumes of 0.01 M Tris-hydrochloride, pH 7.4, containing 0.05 M NaCl, 0.006 M EDTA, 1% sodium dodecyl sulfate, and 200 μg of carrier RNA. An equal volume of a 25:25:1 mixture of phenol, chloroform, and isoamyl alcohol was then added. This extraction mixture was shaken mechanically at room temperature for 5 min and centrifuged at 5,000 rpm for 5 min in a Sorvall HB-4 rotor. The RNA and DNA in the aqueous phase were precipitated overnight with 2 volumes of 95% ethanol at -20°C and then centrifuged at 10,000 rpm for 30 min in a Sorvall HB-4 rotor. The pellet was dried under vacuum, dissolved in 1.0 ml of 0.01 M Tris-hydrochloride, pH 7.6, containing 0.01 M NaCl and 0.01 M MgCl₂, and digested with 50 μg of DNase I per ml

(RNase free; Worthington Biochemicals Corp.) for 30 min at room temperature. After DNase treatment, the mixture was again extracted with the phenol, chloroform, and isoamyl alcohol solution, and the RNA was precipitated by ethanol as described above.

To determine what fraction of the RNA synthesized was SV40 specific, [^3H]UMP-labeled RNA was hybridized to SV40 DNA immobilized on filters as previously described (12). To determine the relative amounts of RNA synthesized from early and late strands, [^{32}P]UMP-labeled RNA was hybridized to separated strands of SV40 DNA immobilized on strips of nitrocellulose as described by Michael Botchan (personal communication). Separated strands of SV40 DNA were prepared by denaturing 5 to 10 μg of the two DNA fragments produced by *Bam*I and *Hpa*II restriction enzyme cleavage in 0.17 M NaOH for 10 min at room temperature. The denatured fragments were then subjected to electrophoresis in a 1.4% agarose slab gel 128 mm wide, 150 mm long, and 3 mm thick. Electrophoresis was at 20 mA for 20 h at 21°C using the buffer system of Hayward (6). The separated strands of each fragment were then transferred to sheets of nitrocellulose as described by Southern (17), and the dried sheets were cut into strips 5 mm wide and 70 mm long. [^{32}P]UMP-labeled RNA was hybridized to the SV40 DNA on these strips in 0.8 ml of $6\times\text{SSC}$ (0.15 M NaCl plus 0.15 M sodium citrate) at 68°C for 18 h. The strips were treated with RNase, washed, and dried as previously described (12). Autoradiography was performed using Kodak Royal X-Omat film. Autoradiograms were scanned in a Gilford spectrophotometer at 560 nm to obtain a densitometric tracing. The areas under the peaks were determined and used to calculate the percentage of RNA hybridizing to the separated strands of each DNA fragment. ^3H -labeled complementary RNA transcribed from the early strand by *Escherichia coli* RNA polymerase was also hybridized under the same conditions, except the nitrocellulose strip was momentarily immersed in toluene containing 2,5-diphenyloxazole (20%, wt/vol) and air-dried before autoradiography.

Sedimentation of transcriptional intermediates. Samples (0.2 ml) of Sarkosyl supernatant were layered onto 3.6-ml linear 5 to 20% (wt/wt) sucrose gradients in Re buffer, 0.3 M NaCl, and 0.25% Sarkosyl. ^{14}C -labeled SV40, form I, isolated from virions propagated in BSC-1 cells and purified by isopycnic banding in cesium chloride-ethidium bromide (3, 15), was added as a marker. The gradients were centrifuged in a Beckman SW60 Ti rotor at 54,000 rpm for 100 min at 2°C. Tubes were punctured at the bottom and 0.16-ml fractions were collected. Samples (0.075 ml) from each fraction were assayed for RNA polymerase activity as described above. The standard reaction mixture contained 0.18 nmol of [^3H]UTP (specific activity, 41.5 Ci/mmol). The reaction was terminated after 2 h at 21°C, and the number of [^3H]UMP counts incorporated was determined as described above.

Viral DNA replication. To measure the amount of viral DNA replication at 33°C, [^3H]thymidine (1 mCi/ml) in sterile water (specific activity, 40 to 60 Ci/mmol) was added to each 60-mm petri dish con-

taining 3 ml of medium to give a final concentration of 5 $\mu\text{Ci/ml}$ for 1 h. To measure the amount of viral DNA replication at various times after shifting cells to 40°C, [^3H]thymidine was added to a final concentration of 10 $\mu\text{Ci/ml}$ for 15 min. At the end of each pulse-labeling period, viral DNA was selectively extracted by the 0.6% sodium dodecyl sulfate-1.0 M NaCl method of Hirt (7). Aliquots from the resulting Hirt supernatant fluid were spotted on Whatman GF/C filters, washed in 5% trichloroacetic acid at 4°C, dried, and counted in 7 ml of the toluene-based scintillant described above. Mock-infected cells were used in parallel as a control for each measurement.

RESULTS

Characterization of infection by *tsA58*. When *tsA58* was grown in BSC-1 cells at 33°C, viral DNA synthesis was first detected at 18 to 24 h after infection, and the rate of synthesis increased in a linear fashion until a maximal rate was achieved at about 48 h after infection (data not shown). After a rapid temperature shift from 33 to 40°C at 33 h after infection, replicating DNA molecules were chased into form I within 45 min. From this, it was calculated that no new rounds of DNA synthesis were initiated after 20 to 30 min. These properties are characteristic of complementation group A mutants (19).

Characterization of the *tsA58* Sarkosyl supernatant and RNA polymerase reaction. The Sarkosyl extraction procedure used in this study yielded greater than 90% of the RNA polymerase activity synthesizing SV40 RNA in the Sarkosyl supernatant (M. Shani, E. H. Birkenmeier, E. May, and N. P. Salzman, J. Virol., in press), in agreement with previous findings of Mousset and Gariglio (13). In the RNA polymerase reaction the kinetics of incorporation of [^3H]UMP during 1 h of incubation is shown in Fig. 1 and is identical to that seen with wild-type virus (5; Shani et al., in press). After shifting *tsA58*-infected cells at 40°C, RNA continued to be synthesized by the Sarkosyl supernatant. Because of variations in the number of nuclei in each Sarkosyl extraction, the protein concentration in the Sarkosyl supernatant varied from 2.3 to 4.8 mg/ml, but showed no apparent relationship to time after infection or time at 40°C. For this reason enzyme activity in Sarkosyl supernatants was measured as [^3H]UMP counts per minute incorporated per microgram of protein during a 1-h incubation at 21°C unless stated otherwise. The relative amount of [^3H]UMP incorporated into SV40-specific RNA varied from 40 to 80% for each experiment, presumably due to varying amounts of sheared host chromatin remaining in the Sarkosyl supernatant. In several experiments the labeled RNA was hybridized to SV40 DNA immobilized on filters, and only

FIG. 1. Pattern of incorporation of $[^3\text{H}]\text{UMP}$ by transcriptional intermediates. At 42 h after infection with *tsA58* at 33°C , cells were either shifted to 40°C or allowed to remain at 33°C for 2 h. Sarkosyl supernatants were then prepared from cells at each temperature, and the RNA polymerase activity was assayed as described in the text. The reaction was terminated after 15, 30, or 60 min of incubation at 21°C , and the $[^3\text{H}]\text{UMP}$ counts incorporated at each time were determined. Each standard reaction mixture contained 0.18 nmol of $[^3\text{H}]\text{UTP}$ (specific activity, 27.6 Ci/mmol) and 0.25 nmol of unlabeled UTP.

hybridizable counts were used in calculating enzyme activity. Since labeled SV40 RNA is the major species of RNA synthesized in the extracts, the results obtained by this procedure were not significantly different from the overall pattern obtained when activity was expressed as $[^3\text{H}]\text{UMP}$ counts incorporated.

Velocity sedimentation of transcriptional intermediates in sucrose gradients. *tsA58*-infected cells were grown at 33°C for 46 h. At that time, one-half of the cells were shifted to 40°C while the remainder stayed at 33°C . After 2 h (48 h after infection), cells at each temperature were harvested and Sarkosyl supernatants were prepared. The transcriptional intermediates in each supernatant were sedimented in a sucrose gradient, and the fractions from the gradients were assayed for RNA polymerase activity. Figure 2 shows that a peak of enzyme activity is obtained from both the 33 and 40°C supernatants, and the profiles for each temper-

ature are identical. The activity from each supernatant sedimented in a broad band with a peak near 26S. This sedimentation pattern is identical to that obtained with wild-type virus (5, 9; Shani et al., in press). Enzyme activities determined with the remainder of the original 33 and 40°C supernatants were 7.7 and 9.0 hybridizable $[^3\text{H}]\text{UMP}$ counts incorporated per μg of protein, respectively. In both supernatants 64% of the total counts incorporated hybridized to SV40 DNA on filters. We conclude that there is no change in the velocity sedimentation of *tsA58* transcriptional intermediates after shifting to the nonpermissive temperature for 2 h and that sedimentation near 26S is not related to actively replicating molecules serving as templates for transcription.

Maintenance of RNA polymerase activity after shift to 40°C . At different times during infection with *tsA58* at 33°C , cells were shifted to 40°C for 2 h; then a Sarkosyl supernatant was prepared and assayed for enzyme activity. A Sarkosyl supernatant was also prepared from cells at 33°C before the 2-h shift. Table 1 shows that transcriptional activity continues for at

FIG. 2. Sucrose sedimentation analysis of transcriptional intermediates. Sarkosyl supernatants were prepared from cells infected with *tsA58* for 46 h at 33°C and then either shifted to 40°C or allowed to remain at 33°C for 2 h. Each supernatant was centrifuged in a linear 5 to 20% (wt/wt) sucrose gradient, fractions were collected, and each fraction was assayed for RNA polymerase activity as described in the text. Each standard reaction mixture contained 0.18 nmol of $[^3\text{H}]\text{UTP}$ (specific activity, 41.5 Ci/mmol) and was incubated for 2 h at 21°C . ^{14}C -labeled SV40 DNA I isolated from virions was added to each gradient as a 21S marker. Since the gradients were centrifuged simultaneously and the marker was at the same position in each gradient, the RNA polymerase activities in both gradients were plotted together. The sedimentation of the DNA I marker in the gradient with the 33°C supernatant is also presented. Sedimentation is from right to left.

least 2 h after shifting the cells to the nonpermissive temperature, irrespective of the time after infection or level of transcriptional activity before the shift. In fact, the RNA polymerase activity can increase by twofold during the 2-h shift. We conclude that viral transcription in *tsA58*-infected cells is maintained at 40°C at a level greater than that observed at 33°C before the shift, even though no actively replicating viral DNA molecules are detectable in the cells.

Increase of RNA polymerase activity after shift to 40°C. To further examine the increase in RNA polymerase activity that occurred during the 2-h shift to 40°C (see Table 1), wild-type SV40-infected cells grown at 33°C for 24 h were shifted to 40°C for varying times, and the enzyme activity in the Sarkosyl supernatants was compared to activity in Sarkosyl supernatants from cells remaining at 33°C. Figure 3 shows that for 2.5 h after the shift only a slight increase of enzyme activity occurs at 40°C, and the activity at 40°C is less than the activity at 33°C for at least 5 h after the shift. The lower activity at 40°C for the first 5 h may be a result of the cells responding to rapid temperature change. Thermal perturbation of cellular processes has been reported in relation to protein synthesis (11). However, by 11 h at 40°C, the enzyme activity was greater than the activity measured in Sarkosyl supernatants from cells at 33°C. This delayed increase may be related to an increase of SV40 DNA synthesis in the cell at 40°C.

The level of transcriptional activity in wild-type virus-infected cells at 24.5 h after infection at 33°C is 2.21 cpm/μg of protein (Fig. 3) and is comparable to the level of transcriptional activ-

FIG. 3. Effect of temperature on transcription of wild-type virus. Cells infected with wild-type virus for 24 h at 33°C were either shifted to 40°C or allowed to remain at 33°C for 0.5, 2.5, 5, 11, and 24 h. Sarkosyl supernatants were prepared from cells at each temperature for each time period, and RNA polymerase activity was measured as [³H]UMP counts incorporated per microgram of protein as described in the text. Each standard reaction mixture contained 0.12 nmol of [³H]UTP (specific activity, 41.7 Ci/mmol).

ity of 2.52 cpm/μg of protein in *tsA58*-infected cells at 36 h after infection (Table 1). Therefore, *tsA58*-infected cells grown at 33°C were shifted to 40°C at 36 h after infection, and Sarkosyl supernatants were prepared at various times after the shift. An increase of RNA polymerase activity synthesizing SV40-specific RNA in the Sarkosyl supernatants occurred between 0.5 and 5 h after the shift (Fig. 4). After 5 h, the enzyme activity remained constant for at least another 6 h at 40°C. Also, it is important to note that cells infected with *tsA58* and maintained at 33°C took approximately 50% longer to reach the maximum enzyme activity achieved by the cells at 40°C in 5 h, even though the total amount of viral DNA in the cells at 33°C continued to increase. Results similar to these were also obtained when the temperature shift was carried out at 24 and 48 h postinfection, and polymerase activity remained constant even if cells were at 40°C for 24 h.

RNA synthesized by transcriptional complexes. To characterize the RNA synthesized by *tsA58* transcriptional complexes, *tsA58*-infected cells were grown at 33°C for 36 h, and then one half were shifted to 40°C for 5 h. The remainder

TABLE 1. Transcriptional activity after shift to 40°C^a

Time of shift (h after infection)	Enzyme activity	
	Before shift	After shift
18	0.09	0.43
21	0.24	0.54
24	0.53	1.00
36	2.52	5.07
48	8.75	10.51

^a Cells were infected with *tsA58* and grown at 33°C. At various times after infection, cells were either shifted to 40°C for 2 h or harvested without shifting. Sarkosyl supernatants were prepared from cells before and after the shift, and the RNA polymerase activity was measured as [³H]UMP counts incorporated per microgram of protein, as described in the text. Each standard reaction mixture contained 0.12 nmol of [³H]UTP (specific activity, 41.5 Ci/mmol).

Fig. 4. Rate of increase of RNA polymerase activity after the shift to 40°C. Cells infected with *tsA58* for 36 h at 33°C were either shifted to 40°C or allowed to remain at 33°C for 0.5, 2.5, 5, and 11 h. Sarkosyl supernatants were prepared from cells at each temperature for each time period, and RNA polymerase activity was assayed as described in the text. Each standard reaction mixture contained 0.45 nmol of [³H]UTP (specific activity, 41.4 Ci/mmol) and 3.75 nmol of unlabeled UTP, which caused the velocity of the enzyme to approach V_{max} . After 1 h of incubation, a sample from each reaction mixture was removed, and the number of [³H]UMP counts incorporated in SV40 RNA was determined by hybridizing the newly synthesized RNA to SV40 DNA on filters, as described in the text. The enzyme activity is measured as hybridizable [³H]UMP counts incorporated per microgram of protein. The number of counts hybridized after a 0.5-h shift were 229 cpm at 33°C and 359 cpm at 40°C. At 11 h after the shift the counts hybridized were 1,668 cpm at 33°C and 756 cpm at 40°C.

stayed at 33°C for 5 h. In a parallel experiment, cells infected with wild-type virus at an identical input multiplicity were shifted to 40°C or remained at 33°C for 5 h. Sarkosyl extracts were prepared and RNA was synthesized in the standard reaction mixture containing [α -³²P]UTP to label the RNA. The RNA was then isolated, and an equal number of counts from each sample was hybridized to separated strands of the early and late regions of the SV40 genome immobilized on strips of nitrocellulose. Figure 5 shows an autoradiogram of the strips from which a densitometric tracing was made and the relative amounts of RNA in each band quantitated (Table 2). Seventy-eight percent or

more of the RNA synthesized by the wild-type or *tsA58* transcriptional complexes before or after the shift to 40°C was transcribed from the L-strand, and 24 to 34% of this late RNA was from the early region of the genome, i.e., was antisense early RNA. The RNA transcribed from the E-strand was almost entirely from the early region of the genome; i.e., no significant amount of antisense late RNA was detected. With wild-type infection either at 33°C or shifted to 40°C, no more than 2 to 4% of the newly synthesized RNA was complementary to the E-strand. However, with *tsA58* the relative amount of early transcription at 33°C was twice that seen with wild type, and shifting to 40°C caused an additional two- to threefold increase in the percentage of RNA transcribed from the E-strand. Thus, whereas the ratio of early to late RNA synthesis was lower in wild-type-infected cells and did not increase after the shift, the ratio of early to late RNA synthesis with *tsA58* increased approximately threefold after the shift to 40°C.

DISCUSSION

When cells are infected and maintained at a restrictive temperature with the A gene temperature-sensitive mutant *tsA30*, viral DNA synthesis and late viral mRNA synthesis do not occur. When these cells are infected at a permissive temperature and then shifted to a restrictive temperature after DNA and late mRNA synthesis have begun, late mRNA synthesis continues for at least 20 h, but DNA synthesis is inhibited within 1 h (2).

We have examined cells infected with the A gene temperature-sensitive mutant *tsA58* and have determined the level of viral transcriptional activity after Sarkosyl extraction. The Sarkosyl extraction procedure yields SV40 transcriptional complexes that contain bound RNA polymerase B and a small number of other proteins. When cells infected with *tsA58* virions are shifted to the restrictive temperature at any time after viral DNA synthesis has begun, DNA synthesis is completely inhibited in 45 min. The behavior of *tsA58* transcriptional intermediates after a shift in temperature to 40°C is similar to the behavior of infected cells in vivo; i.e., transcriptional activity is maintained in extracts prepared from cells that were held for an extended time period at the restrictive temperature. This is true irrespective of the time after initiation of viral DNA synthesis that the cells are shifted to 40°C or the level of transcriptional activity before the shift. These findings strongly support the idea that transcriptional complexes contained in a Sarkosyl extract are derived from the complexes that generate viral RNA in vivo. When

FIG. 5. Autoradiogram of RNA hybridized to separated strands of the early and late regions of the SV40 genome. Cells infected with wild-type (WT) or *tsA58* virus for 36 h at 33°C were either shifted to 40°C or allowed to remain at 33°C for 5 h. Sarkosyl extracts were then prepared, and RNA was synthesized in a standard reaction mixture containing 0.28 nmol of [α - 32 P]UTP (specific activity, 200 Ci/mmol). The RNA synthesized was hybridized to separated strands of the early (A) and late (B) regions of the SV40 genome immobilized on strips of nitrocellulose. 3 H-labeled complementary RNA was also hybridized on a separate strip to locate the positions of the E-strand indicated in the figure. Although it is difficult to discern the E-strand, early region bands in this photograph, they could be detected by visual observation of the original autoradiogram and gave distinct peaks in the densitometric tracings. The sample order is (a) WT at 33°C; (b) WT at 40°C; (c) *tsA58* at 33°C; (d) *tsA58* at 40°C. The bands are: EA—early strand, early region; LA—late strand, early region; LB—late strand, late region; and EB—early strand, late region.

the rate of viral mRNA synthesis is measured after a shift to 40°C, not only is it maintained, but there is an increase in the *in vitro* transcriptional activity, which reaches a level that is two- to threefold higher than the level that existed prior to the temperature shift. This increase in transcriptional activity occurs over a 5-h period, and then the activity remains at this high level for at least an additional 20 h. The increase occurs at an accelerated rate over that seen at permissive temperatures. A comparison of *tsA58* and wild-type transcriptional activi-

ties after a temperature shift demonstrates that the increase in activity is related to the temperature sensitivity of the mutant A gene and is not the result of the temperature shift alone. Wild-type-infected cells shifted to 40°C initially show no stimulation of transcriptional activity under conditions where viral DNA synthesis is not blocked.

Since no [3 H]thymidine is incorporated into SV40 replicating molecules after *tsA58*-infected cells are at 40°C for 45 min and transcriptional complexes continue to produce late RNA for at least 5 h at 40°C, actively replicating molecules cannot be serving as templates for late transcription at 40°C. The identical sedimentation of transcriptional complexes from cells at 33 and 40°C argues that they may have similar, if not identical, templates. We conclude that continued synthesis of viral DNA is not an obligatory prerequisite for maintenance of late viral transcription nor is the sedimentation of the transcriptional complexes at 26S related to actively replicating viral DNA molecules serving as templates for late transcription.

There are three ways to explain the increase of enzyme activity observed after the temperature shift to 40°C. First, the increase in polymerase activity may result from more RNA polymerase molecules on each template at 40°C. We believe this to be unlikely since the sedimentation pattern of transcriptional complexes does not change after the shift, and addition of only one RNA polymerase molecule per template with its associated nascent RNA would change the molecular weight of the complex by over 0.5×10^6 (8). In addition, no comparable increase is seen when wild-type transcriptional complexes are compared at 33 and 40°C.

TABLE 2. Hybridization of 32 P-labeled RNA synthesized by wild-type (WT) and *tsA58* transcriptional complexes at 33°C and after a 5-h shift to 40°C^a

DNA fragment	Hybridized RNA (%) complementary to each DNA fragment			
	WT, 33°C	WT, 40°C	<i>tsA58</i> , 33°C	<i>tsA58</i> , 40°C
E-strand, early region (0.16 to 0.735 map unit)	4	2	9	22
L-strand, early region (0.16 to 0.735 map unit)	34	34	24	29
L-strand, late region (0.735 to 0.16 map unit)	62	64	67	49
E-strand, late region (0.735 to 0.16 map unit)	— ^b	—	—	—

^a The autoradiogram in Fig. 5 was scanned and a densitometric tracing was obtained. The percentage of the total area under each peak was calculated and is presented in the table.

^b No measurable amount was transcribed.

The increase in RNA polymerase activity may also reflect an increase in the number of templates. These additional templates can come from (i) the pool of replicating molecules present in the cell prior to the shift up, (ii) DNA I molecules that are still able to enter the replicative pool during the first 20 to 30 min after the shift up before the block in initiation of DNA synthesis was complete, or (iii) DNA I molecules present in the cell prior to the shift to 40°C, which must undergo processing before they can be used as templates for transcription. The replicating molecules would complete replication within 40 min after the shift up. Since the newly synthesized DNA I molecules cannot initiate new rounds of DNA synthesis, they could preferentially be shunted into the pool of molecules being transcribed and cause an accelerated increase in transcriptional activity.

A third explanation is that additional smaller-molecular-weight proteins or even the A gene product itself have either been added to or removed from the complexes after the shift and thus affect the rate of movement of RNA polymerase along the DNA template *in vitro* or the initiation of transcription *in vivo* and the species of mRNA that are made. Reed et al. (14) have reported that after shifting *tsA58*-infected CV-1 cells from 32 to 41°C at 72 h postinfection, the rate of synthesis of RNA complementary to the E-strand is about 15 times greater than in cells infected with wild-type virus. These findings are consistent with Tegtmeier's suggestion (20) that T-antigen regulates its own synthesis, and the rate of synthesis increases at the restrictive temperature. Similarly, in this study we have observed that in *tsA58*-infected BSC-1 cells, the ratio of early to late RNA synthesized by transcriptional complexes increases 3-fold after the shift and is about 12-fold higher than seen in wild-type infection shifted to 40°C.

Since DNA synthesis and late viral mRNA synthesis are both inhibited when *tsA30*-infected cells are infected and maintained at a restrictive temperature, it has been suggested that the A gene has a direct role in the regulation of both of these functions. The first event in the synthesis of viral DNA involves the synthesis of an RNA primer, and so DNA synthesis and transcription share a common mechanism of initiation. Further, the DNA initiation site (0.67 map unit) may be close to the promoter site for early and late viral mRNA. In this study we have observed that although there is a large increase in the relative amount of early RNA synthesized by *tsA58* transcriptional complexes at 40°C, 70% of the increase in the total transcriptional activity measured *in vitro* dur-

ing a 5-h shift is late mRNA. Thus, the two events, late transcription and replication, can, in a temporal way, be separated. Viral DNA synthesis is blocked completely within 45 min of the time cells are shifted to the restrictive temperature. However, an increase in transcriptional activity occurs over a 5-h period. We conclude that the one known function of the A gene product is indirectly required for late transcription because of its role in initiation of viral DNA synthesis, which allows a pool of replicating and newly synthesized DNA I molecules to be maintained.

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