JOURNAL OF VIROLOGY, Aug. 1979, p. 360-369
0022-538X/79/08-0360/10$02.00/0

Vol. 31, No. 2

Regulation of Simian Virus 40 Transcription: Sensitive Analysis of the RNA Species Present Early in Infections by Virus or Viral DNA

BARBARA A. PARKER AND GEORGE R. STARK*

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Received for publication 21 February 1979

We have examined the discrete species of simian virus 40 (SV40) RNA present very early in infection of monkey cells with wild-type virus, with mutant *tsA*58 virus, and with the corresponding DNAs to distinguish between two classes of models for control of late transcription: (i) positive control mediated by large-T antigen and (ii) negative control mediated by a repressor protein associated with viral DNA in the virion. Total cytoplasmic or nuclear polyadenylated RNAs from infected cells were denatured with glyoxal, separated by electrophoresis on agarose gels, and transferred to diazobenzyloxymethyl paper. The positions of specific early and late RNA species were determined with region-specific SV40 DNA probes. The technique can detect individual RNAs present at the level of less than one copy per cell. After 9.5 h at 37°C, appreciable amounts of two early RNAs (2.6 kilobases [kb] and 2.9 kb) were present in the cytoplasm of cells infected with wild-type virus or DNA, along with much smaller amounts of two late RNAs, 1.6 kb (16S) and 2.5 kb (19S). The amounts of the late RNAs were reduced, but they were still synthesized in the presence of cytosine arabinoside, an inhibitor of DNA synthesis. In comparable infections with *tsA*58 virus or DNA at nonpermissive temperature (41°C), substantial amounts of the two early RNAs were again present, but the two late RNAs could not be detected. However, small amounts of the late RNAs were found when infections with *tsA*58 virus or DNA were prolonged to 30 h at 41°C. These results are not consistent with negative control of late transcription through the action of a repressor and, taken together with other data, suggest that T antigen has an active role in late RNA synthesis. Specific early and late viral RNAs were also detected in the nuclear poly(A)$^+$ fractions and were similar in size to the RNA species found in the cytoplasmic polyadenylated fractions. The late nuclear RNAs (1.8 and 2.9 kb) were significantly larger than the late cytoplasmic species, possibly because they are precursors. The 2.6- and 2.9-kb early RNAs found in the cytoplasm are probably the messengers for large-T and small-t antigens, respectively.

Late transcription in cells lytically infected with simian virus 40 (SV40) occurs after expression of the *A* gene has begun (19, 26, 37). Late transcription could be delayed because the *A* gene product (large-T antigen) is required directly or because it is required indirectly; for example, templates appropriate for late transcription could arise only after SV40 DNA replication, a process that requires large-T antigen (40). Alternatively, late transcription could be delayed because a protein repressor associated with viral DNA in the virion blocks initiation or progress of RNA polymerase on the late DNA strand until early transcription or DNA replication generates a repressor-free template. Active participation of T antigen in late transcription seemed unlikely several years ago because Cowan et al. (10) showed that late transcription continued at a substantial rate after cells infected with the mutant *tsA*30 at low temperature were shifted to high temperature late in infection, whereas initiation of DNA replication, which requires the *A* gene product, ceased rapidly. Therefore, participation of a protein repressor in the control of late transcription seemed attractive.

Observations with temperature-sensitive *D* mutants provided a precedent and a model for the action of such a repressor. The phenotype of a *D* mutant can be explained by the presence in the virion of a protein repressor which must be removed before early transcription can begin. At

high temperature, *tsD* mutants fail to synthesize early RNA (4). However, protein-free DNA from a *D* mutant can initiate infection normally, presumably because the repressor protein has been removed (9, 36). An analogous late repressor could explain many observations concerning control of late transcription. For example, a *tsA* mutant would not be capable of initiating late transcription at nonpermissive temperature if DNA replication were required for removing the late repressor. However, in cells infected with a *tsA* mutant at permissive temperature and then shifted to nonpermissive temperature much later, late transcription could proceed, as observed by Cowan et al. (10), since templates free of repressor would then be present.

To distinguish between a repressor model for negative control of late transcription and the models for positive control involving large-T antigen, it was crucial to determine the extent of late transcription very early in infections by wild-type SV40, by a *tsA* mutant, and by the corresponding DNAs. Negative control by a repressor present in the virion should be abolished by infection with either wild-type or *tsA* DNA, since the repressor will be absent in either case. If large-T antigen is required for late transcription directly or indirectly, infection with either *tsA*58 virus or *tsA*58 DNA at high temperature should fail to give rise to synthesis of late RNA. The experiments required to distinguish between these two kinds of models are difficult. One must determine the specific RNA species present very early in infection, when only a few molecules of viral DNA are present in each cell and when transcription has barely begun. The problem of sensitivity is accentuated even further in DNA infections, where only a small fraction of the cells becomes infected. For these reasons, little information is currently available on the kinds of viral RNA present very early in infections by SV40. We recently developed a technique for transferring RNAs from agarose gels to diazobenzyloxymethyl paper, where they bind covalently. Specific RNAs are detected by hybridization with DNA probes of high specific radioactivity (1; J. C. Alwine, D. J. Kemp, B. A. Parker, J. Renart, G. R. Stark, and G. M. Wahl, Methods Enzymol., in press). As shown below, this technique is capable of detecting less than one molecule per cell of a particular RNA. Furthermore, recent improvements in a technique of infection with DNA (15) have allowed infection of a much higher proportion of the cells. The results we present now are fully consistent with a positive role for large-T antigen in late transcription and appear to rule out control of late transcription by a repressor protein associated with viral DNA in virions.

MATERIALS AND METHODS

Cells and virus. CV-1 cells were grown as described previously (33). CV-1P cells were obtained from Paul Berg and grown by the method of Mertz and Berg (31). Wild-type SV40 virus strain SVS (39) and the mutant *tsA*58 (41) were obtained from Paul Berg and grown by the method of Mertz and Berg (31) or Estes et al. (12).

SV40 DNA. DNA for infection was prepared by infecting semiconfluent monolayers of CV-1 cells with SVS virus at 37°C or with *tsA*58 virus at 32°C at multiplicities of 0.01 PFU/cell. The viral DNAs were isolated by the method of Hirt (18) 8 to 10 days after infection by wild-type virus or 12 to 14 days after infection by *tsA*58. The Hirt supernatants were digested with proteinase K (E. Merck, Darmstadt, Germany) (100 μg/ml at 42°C for 2 h). The digests were extracted by shaking them with an equal volume of phenol equilibrated with 0.1 M Tris-hydrochloride buffer (pH 8), then with the same volume of chloroform. The aqueous phase was then extracted twice more with chloroform. This procedure minimizes formation of precipitates at the interfaces. The DNA was precipitated with ethanol and purified (33). Alternatively, SV40 DNA was purified directly from the Hirt supernatant, followed by dialysis into 10 mM Tris-hydrochloride buffer (pH 7.5) containing 0.2 M NaCl and 2 mM EDTA. After precipitation with ethanol, the DNA was digested with proteinase K (20 μg/ml at 42°C for 30 min), extracted with phenol and chloroform as described above, and precipitated with ethanol.

The SV40 DNA used for making early and late probes was prepared from SVS virions rather than from infected cells, since it was essential to avoid even minor contamination by host DNA, which can contribute significantly to the background in hybridizations (unpublished data). Virions which had been banded in CsCl were digested with proteinase K (100 μg/ml at 42°C for 1 h) in 50 mM Tris-hydrochloride buffer (pH 7.5), containing 0.2 M NaCl, 1 mM EDTA, and 0.6% sodium dodecyl sulfate (SDS). The digest was extracted with phenol and chloroform, and the DNA was precipitated with ethanol and purified (33).

Infections with DNA. Semiconfluent monolayers of CV-1 cells were infected with SV40 DNA by the calcium phosphate technique used for adenovirus DNA (16) or by its recent modification (15). Briefly, wild-type viral DNA was diluted in isotonic saline buffered with HEPES (*N*-2-hydroxyethyl piperazine-*N'*-ethanesulfonic acid) and containing phosphate (16) (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose, 21 mM HEPES, pH 7.1). Salmon sperm DNA was added to a final total DNA concentration of 50 μg/ml. After the addition of 2 M $CaCl_2$ to 125 mM, the DNA coprecipitated with calcium phosphate during 20 to 30 min at room temperature, with formation of a visible fine precipitate. The supernatant fluids were removed from each monolayer, and 0.5 ml of the DNA suspension was added for 20 min at room temperature. Fresh medium containing 5% fetal bovine serum was added, and the monolayers were incubated at 37°C for 4 h. At that time, the supernatant fluids were removed and the monolayers were washed once with fresh medium. For DNA infections by the un-

modified calcium phosphate technique, fresh medium was added for continued incubation at 37°C. For DNA infections by the modified technique, monolayers were incubated for 3 min at 37°C with 2 ml/plate of 15% glycerol in isotonic saline buffered with HEPES. The monolayers were then washed once with fresh medium and incubated with fresh medium at 37°C until harvest. Infections with *tsA*58 DNA were done with the modified technique as described above, but at 41°C.

Infective center assay. After infection with wild-type DNA, monolayers were incubated for 1 h at 37°C. The cells were trypsinized, suspended in fresh medium, and counted. Portions of 10-fold dilutions of the suspended cells were added to the medium above fresh CV-1P monolayers. After 8 to 10 h at 37°C, the medium was removed gently, and the monolayers were overlaid as in a standard plaque assay (31).

Preparation of DNA probes for hybridization. SV40 DNA isolated from virions was digested with *BglI* (New England Biolabs) and *Hinc*II (Bethesda Research Laboratories, Inc.), followed by digestion with *TaqI* (a generous gift of Stephen Goff) (Fig. 1A). The resulting DNA fragments were separated by electrophoresis in horizontal 1.5% agarose gels (29), isolated by binding them to hydroxylapatite (38), and labeled with ^{32}P by nick translation (35). The resulting A fragment from the triple digest (map coordinates 0.758 to 0.053 [14]) contains sequences complementary only to late mRNA's (7, 14, 22) and was used as the late probe. The B and C fragments (map coordinates 0.169 to 0.373 and 0.373 to 0.566 [14]) contain sequences complementary only to early cytoplasmic RNAs (7, 14, 22). These two fragments were used together as the early probe. The hybridization of these probes to cytoplasmic RNAs isolated at 41 h, late in an infection by wild-type virus, is shown in Fig. 1B. Duplicate samples of RNA were bound covalently to paper after separation by electrophoresis in agarose gels. In lane b, the early probe hybridizes to polyadenylated [poly(A)$^+$] RNAs in the size range of 2.6 to 2.9 kb. Two discrete 2.6- and 2.9-kb early RNAs are seen early in infection (Fig. 2 through 5). In addition, two faint bands of about 5 kb probably represent full-length transcripts which have leaked from the nucleus. In lane d the late probe hybridizes to much more abundant 1.6-kb (16S) and 2.5-kb (19S) RNAs. These results demonstrate that the early and late probes hybridize to RNA species of the expected size and relative abundance (7, 20, 22, 24).

Preparation of SV40 RNAs. Subconfluent CV-1 monolayers (20 plates) were infected with SVS virus (50 PFU/cell) for 1 h at 37°C or with *tsA*58 virus (10 PFU/cell) for 1 h at 37°C, followed by 1 h at 41°C. Fresh medium containing 2% serum was then added, and incubations were continued at 37 or 41°C. In experiments with cytosine arabinoside, the drug (20 μg/ml) was added 1 h after infection. Twenty plates of cells were infected by wild-type DNA (10 μg/plate) or *tsA*58 DNA (5 μg/plate) at 37 or 41°C, respectively, using the modified calcium phosphate technique.

After the stated time of infection, cells were washed twice with cold saline buffered with Tris and once, briefly, with a cold solution of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM NaCl and 5 mM $MgCl_2$ (TNM). TNM (1 ml/plate) was then added,

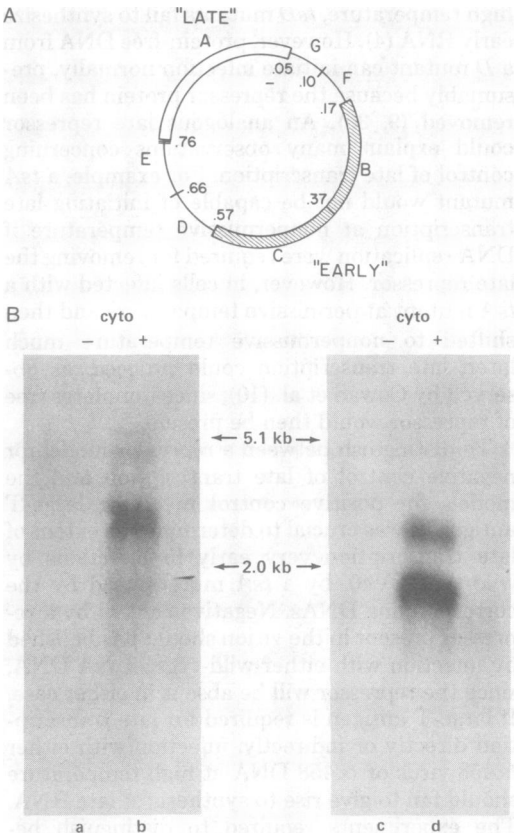

FIG. 1. *Construction and specificity of region-specific early and late DNA probes. (A) SV40 DNA isolated from virions was digested with HincII, BglI, and TaqI. The resulting A fragment was labeled with ^{32}P and used as the late probe, and B and C fragments, labeled with ^{32}P, were used as the early probe. (B) Cytoplasmic RNA was isolated 41 h after infection by wild-type virus. Poly(A)$^-$ (lanes a and c) and poly(A)$^+$ (lanes b and d) RNAs were denatured with glyoxal and duplicate samples were separated by electrophoresis on 1.5% agarose gels and transferred to sheets of diazobenzyloxymethyl paper. One sheet (lanes a and b) was incubated with ^{32}P-labeled early DNA (2×10^6 cpm/track; 4.5×10^7 cpm/μg), and the other (lanes c and d) was incubated with ^{32}P-labeled late DNA (6×10^6 cpm/track; 9×10^7 cpm/μg).*

and the cells were scraped off with a rubber policeman and allowed to swell for 10 min at 0°C. The cells were disrupted with a Dounce homogenizer, and nuclei were separated by pelleting at 2,000 rpm for 2 min at 4°C. Immediately, 0.05 volume of 1 M Tris-hydrochloride buffer (pH 8), 0.05 volume of 4 M NaCl, 0.03 volume of 0.25 M EDTA, and 0.1 volume of 10% sodium lauroyl sarcosinate were added to the supernatant solution, which was then extracted with phenol and chloroform as described above. The nuclear pellet was washed by gentle agitation twice with 10 ml of TNM containing 0.4% Nonidet P-40 (NP40) and 0.05% deoxycholate. The nuclei were then pelleted and sus-

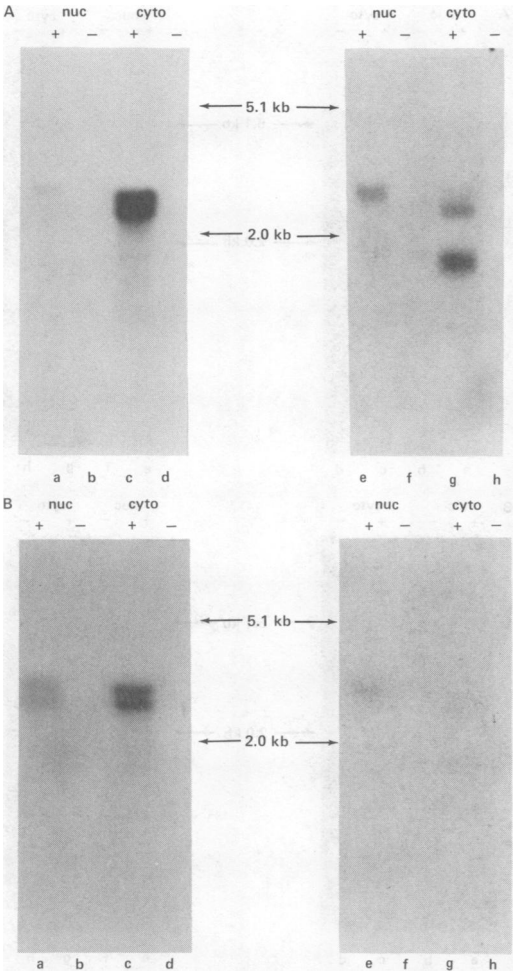

FIG. 2. *Analysis of viral transcripts isolated 9.5 h after infection by wild-type virus or wild-type DNA. (A) Nuclear poly(A)$^+$ (lanes a and e) and poly(A)$^-$ (lanes b and f), cytoplasmic poly(A)$^+$ (lanes c and g) and poly(A)$^-$ (lanes d and h) RNAs from infections by virus were denatured with glyoxal. Duplicate samples were separated by electrophoresis, transferred to diazobenzyloxymethyl paper and hybridized with ^{32}P-labeled early probe (2×10^6 cpm/track; 4.5×10^7 cpm/μg) (lanes a through d) or ^{32}P-labeled late probe (6×10^6 cpm/track; 9×10^7 cpm/μg) (lanes e through h). Autoradiograms were exposed for 48 h. (B) Nuclear poly(A)$^+$ (lanes a and e) and poly(A)$^-$ (lanes b and f), cytoplasmic poly(A)$^+$ (lanes c and g), and poly(A)$^-$ (lanes d and h) RNAs isolated from a DNA infection were analyzed, hybridized, and autoradiographed as in (A).*

pended in 20 mM Tris-hydrochloride buffer (pH 7.5) containing 0.2 M NaCl, 10 mM EDTA, and 1% sodium lauroyl sarcosinate (0.3 ml/plate), shaken, and extracted with phenol and chloroform as described above. The nuclear and cytoplasmic RNAs were precipitated with 2 volumes of ethanol, and the precipitates were washed with 75% ethanol, dried briefly, and dissolved in 20 mM Tris-hydrochloride buffer (pH 7.4) containing 10 mM NaCl and 10 mM $MgCl_2$. RNase-free DNase I (45) was added (30 μg/mg of nucleic acid), and the mixture was incubated at room temperature for 2 h. After the addition of EDTA to 15 mM and of NaCl to 0.2 M, the mixture was extracted with phenol and chloroform and the RNA was precipitated with ethanol. The RNA pellets were dried briefly and then suspended in 2 M LiCl, and precipitates containing RNA larger than 5S were allowed to form overnight at 42°C. Oligodeoxynucleotides remain in the supernatant solution under these conditions. The precipitates were pelleted and suspended in water, and 0.1 volume of 100 mM Tris-hydrochloride buffer (pH 7.5) containing 2 M NaCl and 20 mM EDTA was added. The RNA was precipitated with ethanol, washed once with 70% ethanol, and suspended for passage over oligo (dT) cellulose (5) to select poly(A)$^+$ RNAs.

Analysis of RNAs. Poly(A)$^+$ and poly(A)$^-$ RNAs were separated by electrophoresis as reported previously (1, 44; Alwine et al., in press). Briefly, the RNAs were denatured with glyoxal (30) and separated in horizontal 1.5% agarose gels (29) (20 μg/gel track), using 10 mM sodium phosphate buffer (pH 7.0). ^{32}P-labeled 18S (2.0-kb) and 28S (5.1-kb) ribosomal RNAs from CV-1 cells were included in each gel as transfer standards and molecular weight markers. After electrophoresis, the gel was immersed in 50 mM NaOH containing ethidium bromide (1 μg/ml) for 1 h. After neutralization, the RNAs were transferred from the gel to freshly prepared diazobenzyloxymethyl paper (1; Alwine et al., in press), treated with prehybridization buffer, and hybridized for 60 h at 42°C with denatured, ^{32}P-labeled DNA probes. The probes had specific radioactivities of 0.4×10^8 to 1.0×10^8 cpm/

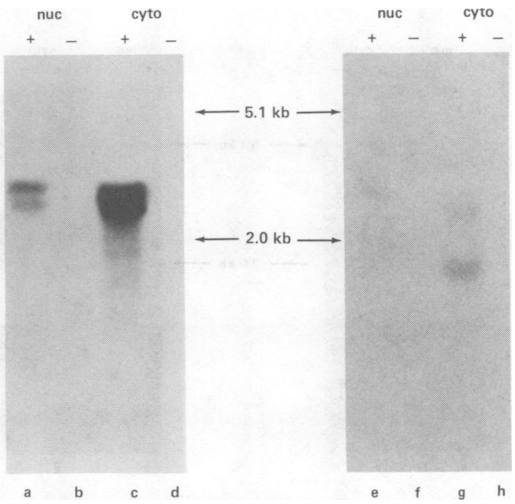

FIG. 3. *Analysis of RNAs isolated 9.5 h after infection by wild-type virus in the presence of cytosine arabinoside (20 μg/ml). The order of nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs in lanes a through h is the same as in Fig. 2A and B. The RNAs were analyzed, hybridized, and autoradiographed as described for Fig. 2A.*

μg, and 2×10^6 to 6×10^6 cpm/gel track were used. As a precaution against any cross-contamination of the probes, each hybridization mixture with ^{32}P-labeled DNA contained an equal concentration of denatured, unlabeled DNA from the opposite region. The RNA papers were washed for 5 h at 42°C with five changes of 20 mM sodium phosphate buffer (pH 6.8), containing 50% formamide, 2.5× SSC (1.0× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), and 0.15% SDS. The sheets were washed for an additional 90 min with 3 changes of 20 mM sodium phosphate buffer (pH 6.8) containing 50% formamide, 0.5× SSC, and 0.15% SDS. The sheets were blotted dry and autoradiographed by using Kodak XR-5 film at −80°C with a Dupont Cronex Lightning Plus intensifying screen.

RESULTS

Use of the modified calcium phosphate technique for efficient SV40 DNA infections.

Analysis of transcripts in infections by SV40 DNA requires an efficient method for DNA infection, since the SV40-specific RNA present early in a virus infection at high multiplicity is only about 0.01% of the total cytoplasmic RNA (3). Reasonably efficient SV40 DNA infections can be obtained by a modification (15) of the calcium phosphate technique used for adenovirus DNA (16). The modification includes treating the infected monolayer with 15% glycerol (our procedure) or 20% glycerol (15) 4 h

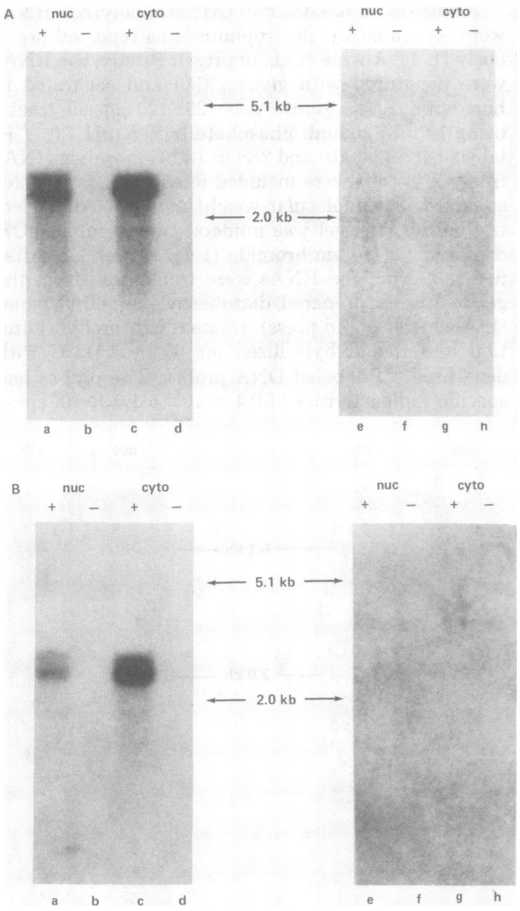

FIG. 4. *Analysis of RNAs isolated 9.5 h after infection by tsA58 virus or tsA58 DNA at 41°C. Nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs isolated from infections with tsA58 virus (A) or tsA58 DNA (B) are organized (lanes a through h), analyzed, and hybridized as described in Fig. 2A and B. The autoradiograms were exposed for 10 days.*

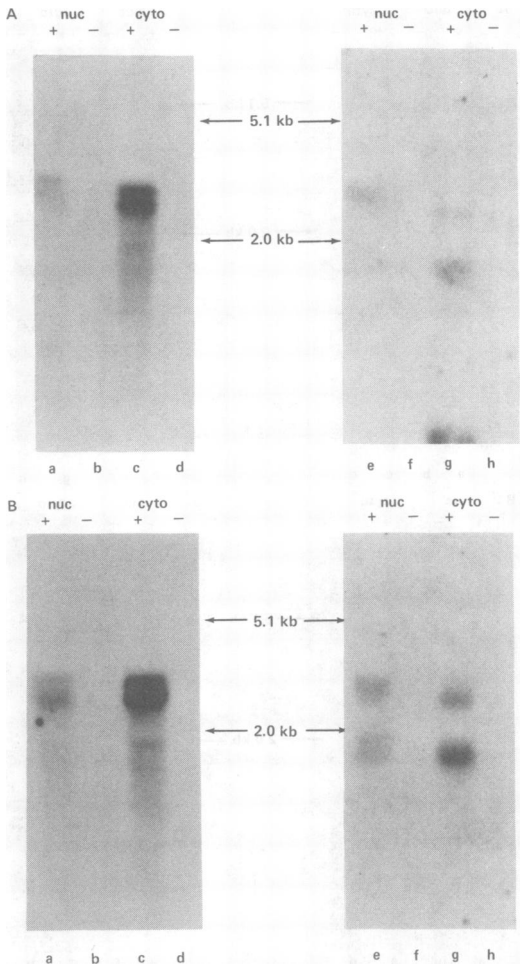

FIG. 5. *Analysis of RNAs isolated 30 h after infection by tsA58 virus (A) or tsA58 DNA (B) at 41°C. Nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs are organized (lanes a through h) as described for Fig. 2A and B. Lanes a through d were hybridized with ^{32}P-labeled early DNA (4.5×10^6 cpm/track; 9×10^7 cpm/μg) and autoradiographed for 5 h. Lanes e through h were hybridized with ^{32}P-labeled late DNA (3×10^6 cpm/track; 1×10^8 cpm/μg) and autoradiographed for 24 h.*

after initiating the infection. Comparison of the modified and unmodified calcium phosphate techniques (Table 1) indicates that the modified technique is about 100 times more efficient in forming infectious centers at very high concentrations of SV40 DNA (28 μg/plate). Analysis of the effect of DNA concentration on the efficiency of infection by the modified technique (Table 1) indicated that approximately 6% of the cells could be infected at an optimal concentration of 10 μg of DNA per plate.

Sensitivity of the RNA transfer method. In an infection with SV40 virions, the amount of early SV40 RNA present at 9.5 h after infection is about 0.01% of the total cytoplasmic RNA (3) or about 0.5% of the total cytoplasmic poly(A)$^+$ RNA. Assuming that these values hold for the 2.6- and 2.9-kb RNAs shown in Fig. 2A, and knowing that a DNA infection is about 20 times less efficient than a virion infection under the conditions used, we can estimate that there is about 2 ng of SV40-specific poly(A)$^+$ RNA in the cytoplasmic track (lane c) and about 200 pg in the nuclear track (lane a) of Fig. 2B. Therefore, 100 pg/band of RNA is visible after 2 days of autoradiographic exposure, using a high concentration (25 ng/ml) of DNA probe of high specific activity (>4 × 10^7 cpm/μg). Since backgrounds are so low with this technique, it is possible to detect additional RNAs of lower abundance (10 to 50 pg of specific RNA in a band) during 1 week of autoradiography. For SV40 RNA of approximate molecular weight 1 × 10^6, 1 pg of RNA corresponds to 6 × 10^5 copies. Starting with poly(A)$^+$ cytoplasmic RNA from 5 × 10^7 cells, 10 to 50 pg of specific RNA corresponds to 0.6 × 10^7 to 3 × 10^7 copies, or 0.1 to 0.6 copy per cell.

Comparison of SV40 transcripts in infections by wild-type virus or wild-type DNA. Nuclear and cytoplasmic poly(A)$^+$ and poly(A)$^-$ RNAs were isolated 9.5 h after infections by wild-type virus or wild-type DNA. Duplicate samples were separated by electrophoresis in agarose gels and transferred to paper. Parallel sets of transfers were hybridized with ^{32}P-labeled early or late probes. The results with RNA isolated from the infection with wild-type virus are shown in Fig. 2A. In the left panel, lane c, the early probe hybridizes to two poly(A)$^+$ cytoplasmic RNAs of sizes 2.6 and 2.9 kb. In the right panel, lane g, the late probe hybridizes to smaller amounts of 1.6-kb (16S) and 2.6-kb (19S) poly(A)$^+$ cytoplasmic RNAs, sizes identical with those of authentic late 16S and 19S mRNAs (see Fig. 1B).

Figure 2B displays the results of the hybridization to RNAs isolated from infections with wild-type DNA. In the left panel, lane c, early probe hybridizes to the 2.6- and 2.9-kb poly(A)$^+$ RNAs. In the right panel, lane g, late probe hybridizes to less abundant 16S and 19S poly(A)$^+$ RNAs. Both early and late RNAs in the DNA infection are about 10 times less abundant than in the virus infection. Their decreased abundance probably reflects the smaller proportion of cells infected by SV40 DNA, about 6% under the conditions used (Table 1). We estimate that there is 20 to 100 times more early RNA present than late RNA 9.5 h after infection by wild-type virus or wild-type DNA, based on the relative intensities of the autoradiographic signals. The early nuclear RNAs are approximately the same size as the cytoplasmic species, but the late nuclear RNAs are considerably larger (1.8 and 2.9 kb) than the late cytoplasmic RNAs (1.6 and 2.5 kb). The larger late nuclear RNAs may represent intermediates in the synthesis of late cytoplasmic RNAs (22).

Analysis of RNAs isolated after infection by wild-type virus in the presence of cytosine arabinoside. In an experiment done in parallel with those shown in Fig. 2A and 2B, RNA was isolated 9.5 h after infection by wild-type virus in the presence of cytosine arabinoside, an inhibitor of SV40 DNA synthesis at elongation (27, 28) (Fig. 3). In the left panel, lane c, the 2.6- and 2.9-kb early RNAs can be seen. In the right panel, lane g, the less abundant 16S and 19S late RNAs are also detected, but the amounts are less than those seen in Fig. 2A. Again, the nuclear poly(A)$^+$ late RNAs (lane e) are larger than their cytoplasmic counterparts.

Comparison of transcripts in infections by *tsA*58 virus or *tsA*58 DNA at a nonpermissive temperature. Infections by *tsA*58 virus or *tsA*58 DNA were for 9.5 h (Fig. 4A and B)

TABLE 1. *Comparison of DNA infection techniques*[a]

Technique	% of cells forming infectious centers
Mock infection	0
Virus infection, 20 PFU/cell	75
DNA, calcium phosphate, 28 μg/plate	0.02
DNA, calcium phosphate + glycerol, 28 μg/plate	3.4
DNA, calcium phosphate + glycerol, 10 μg/plate	6
DNA, calcium phosphate + glycerol, 1 μg/plate	0.5
DNA, calcium phosphate + glycerol, 0.1 μg/plate	0.02

[a] Wild-type virus or DNA were used. Average percentage is based on plaque counts from 5 CV-1P plates for each 10-fold dilution of infected cells.

or 30 h (Fig. 5A and B) at 41°C. The results of hybridization of early and late probes to duplicate sets of RNAs isolated after infection by *tsA*58 virus for 9.5 h are shown in Fig. 4A. In the left panel, lane c, early probe detects the 2.6- and 2.9-kb early RNAs. In the right panel, no hybridization of the late probe is observed. The same pattern is seen in Fig. 4B, which shows the hybridization pattern of early (left panel) and late (right panel) probes to duplicate sets of RNA isolated from infections by *tsA*58 DNA at 41°C for 9.5 h.

The RNAs found in an infection by *tsA*58 virus for 30 h at 41°C are shown in Fig. 5A. In the left panel, lane c, early poly(A)$^+$ RNAs of sizes 2.6 and 2.9 kb are seen. The right panel, lane g, shows hybridization of the late probe to much less abundant 16S and 19S late RNAs. Analysis of the RNAs isolated from an infection with *tsA*58 DNA for 30 h at 41°C is shown in Fig. 5B. In the left panel, lane c, the 2.6- and 2.9-kb early RNAs are again seen, and in the right panel, lane g, 1.6- and 2.5-kb late RNAs are detected. There is much less late RNA in Fig. 5A, lanes e through f, than in the corresponding lanes of Fig. 5B, reflecting a decreased efficiency of transfer from the agarose gel to the diazobenzyloxymethyl paper in this case only, determined by transfer in parallel of ^{32}P-labeled marker RNAs. Lanes a through d of Fig. 5A were transferred with our more usual efficiency (about 90%). In these prolonged infections by *tsA*58 virus or *tsA*58 DNA, the early RNAs are 20 to 100 times more abundant than the late RNAs. Late nuclear RNAs (1.8 and 2.9 kb) larger than the late cytoplasmic RNAs are again observed.

DISCUSSION

Infections with wild-type virus or DNA. Duplicate transfers of RNAs from infections with wild-type virus or DNA were hybridized with ^{32}P-labeled DNA derived from the early or late regions of the SV40 genome. A strong signal is obtained with 100 copies per cell of RNA of discrete size in 10 h of autoradiography, and less than one copy per cell can be detected in 7 days of autoradiography, starting with poly(A)$^+$ RNA from 5×10^7 cells. This technique would not have detected small amounts of rapidly degraded or randomly nicked, newly synthesized RNA. Region-specific probes are appropriate for this kind of analysis, since the major cytoplasmic early and late RNAs correspond almost completely to opposite halves of the SV40 genome (7, 14, 22). A small region of approximately 150 nucleotides where the early and late RNAs overlap is not present in the probes we have used (Fig. 1A). Since the early and late RNAs are completely resolved from each other in agarose gels, each experiment has an internal control which shows that the early and late probes are free of detectable contamination with probe from the other region; the early probe does not hybridize detectably with the late RNAs present in the transfer shown in Fig. 1B, and the late probe does not hybridize detectably with the early RNAs present in the transfer shown in Fig. 2, 3, and 5. It is very unlikely that the double-stranded, region-specific probes hybridize with antisense RNA, since all the SV40 RNAs we detected were polyadenylated, and since they are all detected as discrete species of stable RNA which correspond closely to the known sizes of viral mRNAs. Antisense RNA would not be expected to have these properties.

Analysis of SV40 transcripts in cells infected by wild-type virus or wild-type DNA reveals no qualitative or major quantitative differences (Fig. 2). In both cases, there are two cytoplasmic RNAs of sizes 2.6 and 2.9 kb which hybridize with early probe. The amount of these early RNAs in the virus infection is approximately 10 times greater than in the DNA infection, where about 6% of the cells are infected (Table 1). Infection with wild-type virus or DNA for 9.5 h also gives rise to a small amount of RNA which hybridizes to the late probe. In the cytoplasmic fractions, 1.6-kb (16S) and 2.5-kb (19S) late RNAs are present in both cases, whereas 1.8- and 2.9-kb late RNAs are present in the nuclear RNA pools. The same small proportion of late RNA is present relative to the amount of early RNA in each type of infection (about 1 to 5%).

The presence of significant amounts of 16S and 19S late RNAs 9.5 h after infection by wild-type virus or DNA (Fig. 2) is of special interest. Late transcripts have not been detected early in SV40 infection, before the onset of DNA replication, but less-sensitive techniques have been used (19, 26, 37). However, P. Piper (J. Mol. Biol., in press) has recently found late transcripts 12 h after polyoma infection, when no viral DNA synthesis is detected. In our experiments, it is conceivable but unlikely that the late transcription detected 9.5 h after infection is linked to a small amount of SV40 DNA replication. We have also detected late transcripts 9.5 h after infection even when cytosine arabinoside was present continuously (Fig. 3). In this case, DNA replication may still be initiated but there should be no complete rounds. From this result it seems unlikely that new, fully replicated SV40 DNA is the only template that can be used for late RNA synthesis. Irrespective of whether a small amount of replication has occurred in the infections without cytosine arabinoside, the relative amounts of RNA synthesized are about the same in infections by wild-type virus or wild-type

DNA. This observation is not consistent with repressor control of late transcription unless the repressor is resistant to rigorous deproteination or is a host molecule that binds to SV40 DNA after uncoating of the virions.

Infections by *tsA*58 virus or DNA. The most crucial observation for ruling out a repressor model for control of late transcription is that no late RNA is found in cells infected by *tsA*58 DNA for 9.5 h at 41°C, where initiation of DNA synthesis is blocked (Fig. 4B). Since the *tsA*58 mutation maps in the early region far from the origin (23), there is no reason why infection with *tsA*58 DNA should fail to give rise to late RNA unless prior expression of an early function is required. The same result is obtained in an infection by *tsA*58 virus for 9.5 h at 41°C (Fig. 4A). In both cases, the abundant formation of early RNA serves as an internal control for efficient infection. In contrast to the relative inefficiency of infections with wild-type DNA compared to infections with wild-type virus (Table 1 and Fig. 2), the amount of early RNA synthesized in the infection by *tsA*58 DNA is about the same as in the parallel infection with *tsA*58 virus. Part of the effect may be due to a general increase in the efficiency of DNA infections at 41°C (our unpublished data).

When infection with *tsA*58 virus or *tsA*58 DNA was prolonged for 30 h at nonpermissive temperature, using the same stocks of virus and DNA as before, 16S and 19S late RNAs were detected (Fig. 5). Since neither *tsA*58 virus nor *tsA*58 DNA stocks formed plaques at 41°C (data not shown), it is unlikely that these late RNAs could have arisen from a few wild-type revertants in each stock. Khoury and May (21) have also detected small amounts of late RNA in prolonged infections by *tsA*58 virus at 37 or 41°C. It is possible that a small amount of large-T antigen could accumulate gradually at the nonpermissive temperature and stimulate late RNA synthesis in the absence of viral DNA replication. This possibility is discussed more fully below.

Nature of the SV40-specific RNAs. The early (2.6- and 2.9-kb) and late (1.6- and 2.5-kb) cytoplasmic RNAs we detect are larger than the early and late cytoplasmic RNAs reported recently (7, 22), analyzed by the method of Berk and Sharp (6). Part of the discrepancy is due to removal of poly(A) tails from the 3′ ends in the method of Berk and Sharp, which involves digestion with nuclease S1. The ends of the protected DNA fragments may also be shortened somewhat in the Berk and Sharp method. The 2.6- and 2.9-kb early RNAs are very likely to be messengers for large-T and small-t antigens, respectively (7, 11, 32). To our knowledge, this is the first time that these intact RNAs have been observed with such high resolution, although Paucha et al. (32) did show a small separation of the translational activities for large-T and small-t antigens on sucrose gradients. The late nuclear RNAs, considerably larger than the corresponding cytoplasmic species, may be intermediates in the processing of the initial transcripts (22). Pulse-chase experiments would be necessary to prove this point conclusively.

Alternative models for control of late transcription. Our data are inconsistent with negative control of late transcription by a repressor present in the virion. Furthermore, the experiments with *tsA*58 (Fig. 4 and 5) suggest that large-T antigen may have a positive role in late RNA synthesis. Large-T antigen could be required to synthesize some special form of DNA (for example, a replicative intermediate) which would be an obligatory template for late transcription. However, evidence from the laboratories of Ferdinand et al. (13), Green and Brooks (17), and Birkenmeier et al. (8) indicates that replicative intermediates are not templates for late transcription and that form I DNA molecules probably are. Alternatively, large-T antigen could be required more directly for late RNA synthesis. If this is so, we must account for the distinction between the functions of large-T antigen in DNA replication and late transcription revealed by the temperature-shift experiments of Cowan et al. (10) with *tsA*30, confirmed more recently by Reed et al. (34) and Khoury and May (21), using the less leaky mutant *tsA*58. We must also reconcile the persistence at nonpermissive temperature of a T-antigen activity required for late RNA synthesis (Fig. 5A and B) (21) with the observation that most of the antigenic reactivity of T antigen is lost rapidly at 41°C (2, 42). It is possible that initiation of DNA replication requires large-T antigen at a much higher concentration than late transcription, and that a low steady-state level of functional T antigen remains in a *tsA* mutant at high temperature, a level insufficient for DNA synthesis but sufficient for late transcription. A possible structural explanation for this hypothesis is provided by the work of Tjian (43), who has shown that multiple copies of a protein related to large-T antigen bind sequentially at or near the origin of SV40 DNA when the concentration of the protein is increased. Large-T antigen may stimulate late transcription when bound at or near the origin (33) at low concentrations, and additionally may stimulate initiation of viral DNA replication and repress early transcription (34) at higher concentrations. A stable T antigen-DNA complex may also be used repeatedly for late transcription over a long period of time. In

this case, a small portion of T antigen from a *tsA* mutant which functions in late transcription could be stabilized against denaturation and degradation at nonpermissive temperature through its association with DNA. In this context, it is very interesting that Green and Brooks (17) have found that RNA polymerase tends to stay with unreplicated templates for long periods of time (the half-time for loss of polymerase is 6 h).

Future experiments will help to confirm the suggestion we make now that large T-antigen has an active role in late transcription, discrete from its roles in initiating DNA replication and in repressing early transcription. For example, it will be very informative to study the effects of protein synthesis inhibitors on the synthesis of late RNA at early times in infection. If large-T antigen does function in a positive way in late transcription, it need not function at initiation. Laub et al. (25) have observed that late transcriptional complexes, with short nascent RNA chains initiated near the origin of replication, accumulate late in infection and have suggested that late transcription may be blocked at a nearby attenuator site until a stimulatory factor allows transcription to proceed. T antigen could play such a role, either by stimulating transcription through stoichiometric binding to the DNA or by acting catalytically upon an attenuator protein to remove it. The idea that late transcription may be controlled after initiation is supported by the results of Ferdinand et al. (13), who have analyzed the strand specificity of SV40 RNA synthesized from transcriptional complexes isolated with Sarkosyl or Triton-X-100 very early in infection, using either wild-type or *tsA*58 virus. As much as 20% of the RNA synthesized in vitro was late, suggesting that a post-initiation block to late transcription may have been removed during isolation of the complexes in the presence of detergents. Further work with isolated transcriptional complexes will be of great importance in helping to define the mechanism of control of late transcription in more detail and the postulated role of large-T antigen in this process.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant CA 17287 from the National Cancer Institute. B.A.P. was supported by Medical Scientist Training Program grant GM 01922 from the National Institutes of Health.

We are grateful to Luis Villarreal for helpful suggestions and discussions.

LITERATURE CITED

1. **Alwine, J. C., D. J. Kemp, and G. R. Stark.** 1977. Method for detection of specific RNA's in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc. Natl. Acad. Sci. U.S.A. **74**:5350–5354.
2. **Alwine, J. C., S. I. Reed, J. Ferguson, and G. R. Stark.** 1975. Properties of T antigens induced by wild-type SV40 and tsA mutants in lytic infection. Cell **6**: 529–533.
3. **Alwine, J. C., S. I. Reed, and G. R. Stark.** 1977. Characterization of the autoregulation of simian virus 40 gene *A*. J. Virol. **24**:22–27.
4. **Avila, J., R. Saral, R. G. Martin, and G. Khoury.** 1976. The temperature-sensitive defect in SV40 Group D mutants. Virology **73**:89–95.
5. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
6. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNA's by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
7. **Berk, A. J., and P. A. Sharp.** 1978. Spliced early mRNAs of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **75**: 1274–1278.
8. **Birkenmeier, E. H., B. M. F. Radonovich, M. Shani, and N. P. Salzman.** 1977. The SV40 DNA template for transcription and late mRNA in viral nucleoprotein complexes. Cell **11**:495–504.
9. **Chou, J. Y., and R. G. Martin.** 1975. DNA infectivity and the induction of host DNA synthesis with temperature-sensitive mutants of simian virus 40. J. Virol. **15**: 145–150.
10. **Cowan, K., P. Tegtmeyer, and D. D. Anthony.** 1973. Relationship of replication and transcription of simian virus 40 DNA. Proc. Natl. Acad. Sci. U.S.A. **70**:1927–1930.
11. **Crawford, L. V., C. N. Cole, A. E. Smith, E. Paucha, P. Tegtmeyer, K. Rundell, and P. Berg.** 1978. The organization and expression of the early genes of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **74**:117–121.
12. **Estes, M. K., E. S. Huang, and J. S. Pagano.** 1971. Structural polypeptides of simian virus 40. J. Virol. **7**: 635–641.
13. **Ferdinand, F.-J., M. Brown, and G. Khoury.** 1977. Characterization of early simian virus 40 transcriptional complexes: late transcription in the absence of detectable DNA replication. Proc. Natl. Acad. Sci. U.S.A. **74**: 5443–5447.
14. **Fiers, W., R. Contreras, G. Haegeman, R. Rogiers, A. Van de Voorde, H. Van Heuverswyn, J. Van Herreweghe, G. Volckaert, and M. Ysebaert.** 1978. The total nucleotide sequence of SV40 DNA. Nature (London) **273**:113–119.
15. **Frost, E., and J. Williams.** 1978. Mapping temperature-sensitive and host-range mutations of adenovirus type 5 by marker rescue. Virology **91**:39–50.
16. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
17. **Green, M. H., and T. L. Brooks.** 1978. Recently replicated simian virus 40 DNA is a preferential template for transcription and replication. J. Virol. **26**:325–334.
18. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
19. **Khoury, G., J. C. Byrne, and M. A. Martin.** 1972. Patterns of simian virus 40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Natl. Acad. Sci. U.S.A. **69**:1925–1928.
20. **Khoury, G., P. Howley, D. Nathans, and M. Martin.** 1975. Posttranscriptional selection of simian virus 40-specific RNA. J. Virol. **15**:433–437.
21. **Khoury, G., and E. May.** 1977. Regulation of early and late simian virus 40 transcription: overproduction of early viral RNA in the absence of a functional T-antigen. J. Virol. **23**:167–176.
22. **Lai, C.-H., R. Dhar, and G. Khoury.** 1978. Mapping the

spliced and unspliced late lytic SV40 RNAs. Cell 14: 971-982.
23. **Lai, C. J., and D. Nathans.** 1975. A map of temperature sensitive mutants of simian virus 40. Virology **66**:70-81.
24. **Laub, O., and Y. Aloni.** 1975. Transcription of simian virus 40. V. Regulation of simian virus 40 gene expression. J. Virol. **16**:1171-1183.
25. **Laub, O., S. Bratosin, M. Horowitz, and Y. Aloni.** 1979. The initiation of transcription of SV40 DNA at late time after infection. Virology **92**:310-323.
26. **Lindstrom, D. M., and R. Dulbecco.** 1972. Strand orientation of SV40 transcription in productively infected cells. Proc. Natl. Acad. Sci. U.S.A. **69**:1517-1520.
27. **Manteuil, S., and M. Girard.** 1974. Inhibitors of DNA synthesis: their influence on replication and transcription of simian virus 40 DNA. Virology **60**:438-454.
28. **Manteuil, S., H. Kopecka, J. Caraux, A. Prunell, and M. Girard.** 1974. *In vivo* incorporation of cytosine arabinoside into simian virus 40 DNA. J. Mol. Biol. **90**: 751-756.
29. **McDonell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119-146.
30. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835-4838.
31. **Mertz, J. E., and P. Berg.** 1974. Defective simian virus 40 genomes: isolation and growth of individual clones. Virology **62**:112-124.
32. **Paucha, E., R. Harvey, and A. E. Smith.** 1978. Cell-free synthesis of simian virus 40 T-antigens. J. Virol. **28**: 154-170.
33. **Reed, S. I., J. Ferguson, R. W. Davis, and G. R. Stark.** 1975. T-antigen binds to SV40 DNA at the origin of DNA replication. Proc. Natl. Acad. Sci. U.S.A. **72**:1605-1609.
34. **Reed, S. I., G. R. Stark, and J. C. Alwine.** 1976. Autoregulation of simian virus 40 gene A by T antigen. Proc. Natl. Acad. Sci. U.S.A. **73**:3083-3087.
35. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237-251.
36. **Robb, J. A., and R. G. Martin.** 1972. Genetic analysis of simian virus 40. III. Characterization of a temperature-sensitive mutant blocked at an early stage of productive infection in monkey cells. J. Virol. **9**:956-968.
37. **Sambrook, J., P. A. Sharp, and W. Keller.** 1972. Transcription of simian virus 40. J. Mol. Biol. **70**:57-71.
38. **Tabak, H. F., and R. A. Flavell.** 1978. A method for recovery of DNA from agarose gels. Nucleic Acids Res. **5**:2321-2332.
39. **Takemoto, K. K., R. L. Kirschstein, and K. Habel.** 1966. Mutants of simian virus 40 differing in plaque size, oncogenicity, and heat sensitivity. J. Bacteriol. **92**:990-994.
40. **Tegtmeyer, P.** 1972. Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. **10**:591-598.
41. **Tegtmeyer, P.** 1974. Altered patterns of protein synthesis in infection by SV40 mutants. Cold Spring Harbor Symp. Quant. Biol. **39**:69-84.
42. **Tegtmeyer, P., M. Schwartz, J. K. Collins, and K. Rundell.** 1975. Regulation of tumor antigen synthesis by simian virus 40 gene *A*. J. Virol. **16**:168-178.
43. **Tjian, R.** 1978. The binding site on SV40 DNA for a T antigen-related protein. Cell **13**:165-179.
44. **Villarreal, L. P., R. T. White, and P. Berg.** 1979. Mutational alterations within the SV40 leader segment generate altered 16S and 19S mRNA's. J. Virol. **29**:209-219.
45. **Zimmerman, S. B., and G. Sandeen.** 1966. The ribonuclease activity of crystallized pancreatic deoxyribonuclease. Anal. Biochem. **14**:269-277.