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# Effect of a *tsA* Mutation on Simian Virus 40 Late Gene Expression: Variations Between Host Cell Lines

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Infection of AGMK or CV-1 cells by the early simian virus 40 mutant *tsA*58 at the permissive temperature (32°C) followed by a shift to the nonpermissive temperature (41°C) caused a substantial decrease in the levels of late viral RNA in the cytoplasm of AGMK cells but not CV-1 cells. At the translational level, this depression of late viral RNA levels was reflected by a decrease in late viral protein synthesis. Thus, in AGMK cells, an early region gene product (presumably large T-antigen) appeared to be continuously required for efficient expression of the late viral genes. In contrast, late simian virus 40 gene expression, once it is initiated in CV-1 cells, continued efficiently regardless of the *tsA* mutation. The difference in expression of the late simian virus 40 genes in these *tsA* mutant-infected monkey kidney cell lines may reflect a difference in host cell proteins which regulate viral gene expression in conjunction with early viral proteins.

The temporal expression of early and late simian virus 40 (SV40) genes appears to be controlled at the level of transcription (2, 5, 14, 18). A primary factor in determining the transcriptional activity of these genes is one of the early gene products, large T-antigen, which appears to have a repressor-like activity in modulating early transcription (2, 14, 18) and also functions in the initiation of viral DNA synthesis (20, 21). These conclusions are based, in part, on studies with early SV40 temperature-sensitive mutants (*tsA* mutants) which synthesize a thermosensitive large T-antigen (1, 24). At elevated temperatures large T-antigen becomes defective (1, 20, 22-24), causing overproduction of early viral RNA (2, 14, 18) and curtailment of viral DNA synthesis (20, 21). The regulation of late SV40 transcription also seems to be dependent, at least in part, on the activity of large T-antigen or concomitant DNA replication or both (2, 5, 14). If monkey kidney cultures are infected in the presence of inhibitors of DNA replication (7, 14; Alwine, unpublished data) or by *tsA* mutants at the nonpermissive temperature (2, 5, 14), the normal pattern of abundant late transcription cannot be established. If the inhibitors of DNA synthesis are added after the onset of SV40 DNA replication, however, they have little or no effect on established late viral transcription. A more complex effect is seen in *tsA* mutant-infected cells when they are shifted from the permissive to the nonpermissive temperature after DNA synthesis and late transcription have been initiated. Although late transcription continues (2, 5, 14), the level of this synthesis may depend on the specific infected monkey kidney cell line. Although the level of late viral mRNA remains high after shifting *tsA* mutant-infected TC-7 or CV-1 cells (2, 5) to nonpermissive temperatures, a decrease of late RNA was noted in shifted *tsA*58-infected primary African green monkey kidney (AGMK) cells (14). Since these earlier studies employed different techniques and were, in some cases, semiquantitative, we have analyzed late SV40 RNAs from CV-1 and AGMK cells in parallel experiments with both wild-type and *tsA* mutant viruses. Data are presented which indicate: (i) the continuous requirement for an early gene product for normal late viral gene expression, and (ii) a difference in the levels of late viral RNA (and late proteins) between CV-1 and AGMK cells which is under the effect of a temperature-sensitive mutation in the early gene. These observations may indicate that the early gene product interacts with a host cell factor(s) for the control of late gene expression.

The cells used in this study were primary AGMK cells and the established AGMK line, CV-1, which were cultivated in minimal essential medium plus 10% fetal calf serum. Virus strains were the wild-type VA4554 (21) and a temperature-sensitive mutant derived from it, *tsA* 58 (20, 21). Confluent monolayers of cells in 150-cm$^2$ Costar plastic bottles were infected at a multiplicity of 10 PFU/cell in 5 ml of minimal essential medium containing 2% fetal calf serum. After rocking at room temperature for 1.5 h, 20 ml of medium was added, and the cells were incubated at 32°C for 40 h. At this point, cells were harvested (32°C samples) or shifted to 41°C for

5 h (shifted samples) before harvesting. Cytoplasmic RNA from infected cells was prepared as previously described (13). Polyadenylated [poly(A)]RNA was selected on columns of oligodeoxythymidylic-cellulose (3). Cytoplasmic RNA pulse-labeled with [$^3$H]uridine was prepared in a similar manner except that it was treated with 40 μg of DNAse I per ml (Worthington, RNase free) for 30 min at 4°C and was not selected for poly(A) sequences (6). For pulse-labeling, cells were infected and incubated as described above. At the end of the incubation period at 32°C or 4 h after the shift to 41°C, each flask of cells was washed with warmed (32 or 41°C) serum-free medium and labeled with 8 ml of warmed serum-free medium containing 1.5 mCi of [5,6-$^3$H]uridine (New England Nuclear, Boston, Mass.). The period of pulse-labeling was 1.25 h at 32°C and 45 min at 41°C. For the preparation of $^{32}$P-labeled SV40 DNA hybridization probes, SV40 DNA was labeled in vivo and purified as previously described (15). Labeled form I SV40 DNA (25 μg) was digested with the restriction endonucleases *Hpa*II and *Bam*HI (New England Biolabs). The strands of the fragments were separated on a 1.4% agarose gel by the method of Hayward (9). Separated strands were electroeluted from gels, self-annealed, and chromatographed on hydroxyapatite. The resulting purified single strands were dialyzed into 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA. Hybridization analysis with single-stranded DNA probes and cytoplasmic poly(A)-containing RNA was performed under conditions of DNA excess. These conditions were established by titration of different samples. Between 0.1 and 5 μg of poly(A) RNA was used in a normal hybridization reaction. For each reaction 10 ng (10,000 cpm) of DNA probe was added. Hybridization samples were dissolved in 30 μl of 50% formamide, 0.35 M NaCl, 0.04 M PIPES [piperazine-*N*-*N*′-bis(2-ethanesulfonic acid)], pH 6.5, heated to 65°C for 3 min and hybridized at 37°C for 24 to 48 h. After hybridization, the samples were treated with nuclease S1 and electrophoresed on 1.4 or 1.8% alkaline agarose gels as described by Berk and Sharp (4). The gels were dried on DEAE paper and autoradiographed at −70°C with Kodak XR-1 X-ray film and a Dupont Cronex lightening-plus intensifying screen. For analysis of pulse-labeled RNA, SV40 DNA was cleaved with the restriction endonucleases *Hpa*II and *Bam*HI, and the strands of the two resultant fragments were separated as described above and transferred onto nitrocellulose by the method of Southern (19). Each filter (10 cm wide) contained 30 μg of SV40 DNA. Strips (0.5 cm) of the separated strand blot were cut and used in each hybridization (approximately 0.35 μg of each strand). Samples containing 4,000 or 12,000 cpm of virus-specific RNA (previously determined) were added to the hybridization mixture containing 5X SSC (0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 300 μg of yeast tRNA in a volume of 1.5 ml. Incubations were at 68°C for 24 to 48 h. After hybridization the strips were treated with RNase A and washed as previously described (19), and then cut into 1-mm pieces and counted in Econofluor (New England Nuclear). For the analysis of SV40 late protein synthesis, confluent monolayers of AGMK or CV-1 cells grown in 50-mm plates (three plates per set) were infected with VA4554 or *tsA*58 at a multiplicity of 10. The infected cells were incubated for 40 h at 32°C. At that point the cells were pulse-labeled for 1 h at 32°C with [$^{35}$S]methionine or shifted to 41°C for 4 h before pulse-labeling for 1 h at 41°C. The pulse-labeling was accomplished by washing the cells three times with appropriately warmed (32 or 41°C) methionine free minimum essential medium containing 2% dialyzed fetal bovine serum and then labeling each plate with 1 ml of prewarmed medium containing 200 μCi of [$^{35}$S]methionine (300 Ci/mmol, Amersham). After labeling, the plates were put on ice, washed three times with cold analysis buffer (Tris-buffered saline, pH 8, containing 1 mM dithiothreitol, 300 μg of phenylmethylsulfonylfluoride per ml, and 150 μg of tosylamid-2-phenylethyl-chloromethyl ketone per ml). The cells were scraped into 1.5 ml of analysis buffer, sedimented at 3,000 rpm for 10 min, resuspended in analysis buffer containing 0.5% Nonidet P-40, and incubated on ice for 30 min, blending vigorously in a Vortex mixer every 5 min. The lysates were sedimented at 15,000 rpm for 60 min, and the supernatant fraction was withdrawn and saved. Portions (0.3 ml) of the supernatants were immunoprecipitated with heat-inactivated *Staphylococcus aureus* and antiserum directed against the SV40 virion proteins (predominantly VP1) as previously described (12). The serum had been titrated previously to determine optimal condition for these samples. Both the serum and *S. aureus* were the gift of Gilbert Jay. Immunoprecipitates (in 15- to 20-μl volumes) were analyzed by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide slab gels (11). Radiolabeled proteins were detected by fluorography (17).

Cytoplasmic poly(A) SV40 RNA was analyzed by the nuclease S1 procedure of Berk and Sharp (4) followed by alkaline agarose gel electrophoresis and autoradiography. To quantitate the

relative amounts of late mRNA species from the intensity of autoradiographic bands, hybridizations were performed in DNA excess, and incubation times were prolonged to ensure that reactions were essentially complete. The specificity of the reaction was further ensured by using a [$^{32}$P]DNA probe which consisted of the purified late sense (+) strand of the B fragment, generated by cleavage of SV40 DNA with the restriction endonucleases *Hpa*II and *Bam*HI (0.72 to 0.14 on the SV40 map). This strand contains almost all of the known late coding region.

Figure 1 shows the analysis of late RNA from wild-type or *tsA*58-infected cells (CV-1 and AGMK) which had been incubated at 32°C for 40 h after infection (32°C samples) or had been incubated at 32°C for 40 h and then shifted to 41°C for 5 h (shifted samples). In all tracks (Fig. 1C to J), bands representing the "bodies" of the late SV40 19S RNA (0.38 SV40 fractional length) and the late 16S RNA (0.21 fractional length) are present. The small amount of 0.41-fractional-length band seen at the top of some gels is the DNA probe which was either not digested or which annealed with trace quantities of contaminating complimentary DNA strands. Since the same quantities of infected cell RNA were used in matching tracks and since hybridizations were performed in DNA excess, the intensity of the bands reflects the relative amounts of late SV40 RNAs. In wild-type-infected AGMK cells (Fig. 1C and D) and CV-1 cells (Fig. 1G and H), there is a small but definite increase in the quantity of late viral RNAs in cells shifted from 32 to 41°C, compared with the quantity of late RNAs present in cells maintained at 32°C (1.5- to 2-fold increase as measured by microdensitometry tracing). This same increase is also seen in *tsA* 58-infected CV-1 cells (Fig. 1I and J) after a shift from 32 to 41°C. However, *tsA* 58-infected AGMK cells have substantially less late 19S and 16S cytoplasmic RNAs in the shifted sample (Fig. 1F) compared with the 32°C sample (Fig. 1E). This decrease is six- to eightfold as determined by microdensitometry of three separate experiments with different matching sets of RNA.

To further verify the decrease of late SV40 mRNA in *tsA* 58-infected AGMK cells, we annealed pulse-labeled RNA samples from *tsA* 58-infected cells (see above) to the separated strands of the early (E) and late (L) coding regions (*Hpa*II–*Bam*HI-cleaved DNA fragments) which had been bound to nitrocellulose membrane filters (19). The results of this exper-

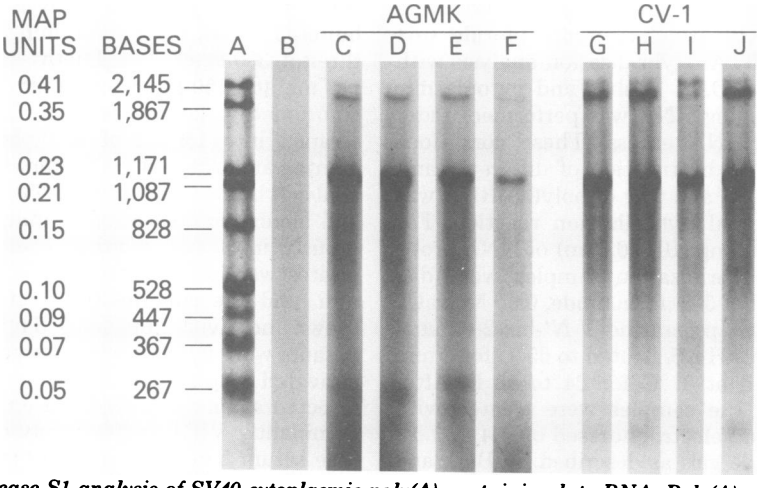


FIG. 1. *Nuclease S1 analysis of SV40 cytoplasmic poly(A)-containing late RNA. Poly(A)-containing cytoplasmic RNA, isolated from AGMK or CV-1 cells infected with WT-SV40 or tsA58 (see text), was hybridized to the late (+) strand of the smaller fragment of $^{32}$P-labeled SV40 DNA generated by digestion with the restriction endonucleases HpaII and BamHI (0.73–0.14 SV40 map units). Samples were hybridized, nuclease S1 treated, electrophoresed, and autoradiographed as described in the text. In these experiments equal amounts of RNA were added to each matching set of samples; thus quantitative comparisons can be made directly within and between matched lanes. (A) SV40 DNA fragment size markers; (B) hybridization with probe DNA alone; (C) 32°C sample from AGMK cells infected with wild-type SV40; (D) shifted sample from AGMK cells infected with wild-type SV40; (E) 32°C sample from AGMK cells infected with tsA58; (F) shifted sample from AGMK cells infected with tsA58; (G) 32°C sample from CV-1 cells infected with wild-type SV40; (H) shifted sample from CV-1 cells infected with wild-type SV40; (I) 32°C sample from CV-1 cells infected with tsA58; (J) shifted sample from CV-1 cells infected with tsA58.*

iment indicate the relative synthetic rates of early and late SV40 RNA. These ratios are summarized in Table 1. In the cytoplasmic fraction of both *tsA*58-infected AGMK cells and CV-1 cells, a shift from 32 to 41°C was accompanied by an increase in pulse-labeled early RNA (see the shift/32°C ratios for early RNA) in agreement with previous results (2, 14, 18). The temperature shift in *tsA*58-infected CV-1 cells led to a small increase in the amount of pulse-labeled cytoplasmic late viral RNA (see the shift/32°C ratio for late RNA). In contrast, pulse-labeled late cytoplasmic viral RNA was significantly decreased in *tsA*58-infected AGMK cells after the temperature shift. Wild-type-infected cells do not show the overproduction of early RNA, and pulse-labeled late RNA does not decrease in AGMK cells after a temperature shift (14, 18; our data not presented).

The pulse-labeled RNA from the nuclear fractions of *tsA*-infected AGMK and CV-1 cells was also assayed for early and late SV40 RNA (data not presented). In agreement with previous results (14, 18), the nuclear early RNA ratios showed an overproduction similar to that seen in the cytoplasm for *tsA*-infected cultures which are shifted to 41°C. However, the very low levels of pulse-labeled nuclear late RNA prevented an accurate determination of the shift/32°C ratios for both cell lines. These low levels of pulse-labeled late nuclear RNA, compared with the late cytoplasmic RNA, are also seen in wild-type-infected cells and agree with previous results by a more sensitive (C,t) hybridization analysis (18). These results suggest rapid transport of newly synthesized SV40 late RNA from the nucleus.

From the results of the nuclease S1 and pulse-labeling analysis of cytoplasmic late RNA, it appears that in *tsA*-infected CV-1 cells, a shift from 32 to 41°C is characterized by an increase (overproduction) in early RNA with little alteration in the levels of late RNA. In AGMK cells, however, the early RNA overproduction, after a shift to 41°C, is accompanied by a substantial decrease in late RNA. The fact that similar data were obtained in experiments designed to examine not only steady-state RNA (Fig. 1) but also pulse-labeled transcripts (Table 1) suggests that the alterations in late RNA levels accompanying temperature shifts with AGMK cells may occur at the level of synthesis; however, other explanations are possible, and a more extensive examination of nuclear late RNA is necessary to establish the mechanism of the decrease.

We next asked whether the specific decrease in cytoplasmic late RNA in shifted samples of *tsA*58-infected AGMK cells affects the synthesis of late viral proteins. To determine this, AGMK and CV-1 cells were infected under the same conditions used previously and were then pulse-labeled with [$^{35}$S]methionine for 1 h at the end of the incubation at 32°C or at the end of the shift period. Samples were immunoprecipitated with serum directed against the SV40 virion proteins and analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels (11, 12). Figure 2 shows the VP1 bands resulting from this analysis. Comparing the 32°C and shifted samples from wild-type-infected AGMK cells (Fig. 2B and C), wild-type-infected CV-1 cells (Fig. 2F, G), or *tsA*58-infected CV-1 cells (Fig. 2H and I), we see a definite increase in the intensity of the band in shifted samples. How-

TABLE 1. *Ratios of viral early and late RNA in tsA58-infected cells shifted to 41°C compared with cultured maintained at 32°C (shift/32° ratios)*

| Cell line | Shift/32° ratio | |
|---|---|---|
| | Early RNA | Late RNA |
| AGMK | 4.5[a] | 0.1 |
| CV-1 | 12.8 | 1.1 |

[a] The numbers represent the ratio of the amounts of early or late RNA in *tsA*58-infected cultures pulse-labeled after a 5-h shift to 41°C compared with pulse-labeled cultures which had been maintained at 32°C (see text). The percentage of total viral RNA in the various samples was between 5.0 and 6.0% in AGMK cells and between 1.0 and 2.0% in CV-1 cells.

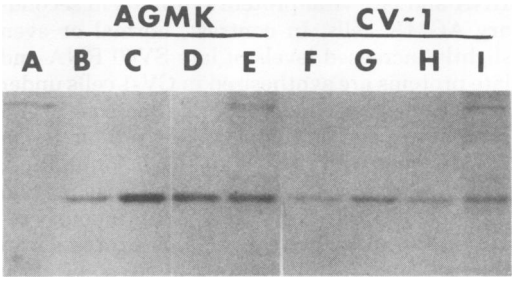


FIG. 2. *Analysis of [$^{35}$S]methionine pulse labeled VP1. AGMK and CV-1 cells were infected with wild type or tsA58 and pulse-labeled with [$^{35}$S]methionine as described in the text. Immunoprecipitated samples were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gels, and bands were visualized by fluorography. (A) Mock-infected sample; (B) 32°C sample from AGMK cells infected with wild-type SV40; (C) shifted sample from AGMK cells infected with wild-type SV40; (D) 32°C sample from AGMK cells infected with tsA58; (E) shifted sample from AGMK cells infected with tsA58; (F) 32°C sample from CV-1 cells infected with wild-type SV40; (G) shifted sample from CV-1 cells infected with wild-type SV40; (H) 32°C sample from CV-1 cells infected with tsA58; (I) shifted sample from CV-1 cells infected with tsA58.*

ever, this increase in intensity is not seen in the shifted sample from *tsA* 58-infected AGMK cells (Fig. 2D and E). Microdensitometer tracing, in fact, shows a decrease. By this analysis the decrease in VP1 synthesis in AGMK cells does not appear to be as great as the decrease in late mRNA levels. The reasons for this difference are unclear. Since translation is affected by many factors besides mRNA concentration, a direct correlation of protein synthesis and RNA concentration may not always be expected. The synthesis of VP1 is reduced only in *tsA* 58-infected AGMK cells after a shift from the permissive to the nonpermissive temperature. Because this correlates with a specific decrease in late mRNA, we conclude the reduced VP1 synthesis is a reflection of the depression in the late mRNA levels. The other late viral proteins VP2 and VP3 are present in much lower amounts than VP-1 and measurements of their band intensities are less accurate. However, with very long exposure, VP2 appears to have a similar decrease in its synthetic rate in *tsA* 58-infected AGMK cells shifted to the nonpermissive temperature (data not shown). This suggests that the decrease in late RNAs affects all late proteins in *tsA* 58-infected AGMK cells at 41°C.

In conclusion, we have shown that the amount of late SV40 RNA in the cytoplasm of *tsA* 58-infected cultures, shifted to 41°C, depends largely on the host monkey kidney cell line. Comparing 32°C samples to shifted samples, we have found a decrease in the level of late SV40 RNA and late viral protein synthesis in secondary AGMK cells. In contrast, normal or even slightly increased levels of late SV40 RNA and late proteins are synthesized in CV-1 cells under similar infection conditions. The decrease in late RNA levels and late viral protein production in AGMK cells, in response to the *tsA* mutation, indicates that a functional early viral protein (presumably large T-antigen) is continuously required for normal late viral gene expression in this cell line. Variations in the effect of early SV40 gene products in different permissive host cell lines have been demonstrated in the recent report that the mitogenic effect of the SV40 *A* gene product differs between AGMK and TC-7 cells (10). In addition, there have been several recent reports suggesting possible interactions between large T-antigen and host proteins (8, 16, 25). These observations suggest that the contrasting results between AGMK and CV-1 cells may be due to differences in host cell factors which interact with early viral proteins for the regulation of late gene expression.

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