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Characterization of the Autoregulation of Simian Virus 40 Gene A

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Cells infected by *tsA* mutants of simian virus 40 (SV40) overproduce early RNA. Overproduction results from failure of the temperature-sensitive *A* protein (T antigen) to inhibit early transcription. The amount of early RNA in the cytoplasm, determined quantitatively from the kinetics of hybridization to labeled complementary SV40 DNA, was elevated at both permissive (32°C) and nonpermissive (41°C) temperatures in all the early mutants tested (*tsA*7, -30, -58, and -209), but not in the late mutant *tsB*4. The amount of early RNA in a culture maintained at 32°C for 72 h and then shifted to 41°C was maximum when each cell was infected initially with at least one plaque-forming unit of *tsA*58. Azidocytidine (2'-deoxy-2'-azidocytidine), which inhibits initiation of DNA synthesis, did not cause overproduction of early RNA in cells infected with wild-type SV40, showing that the effect seen with *tsA* mutants is not due to interference with initiation of DNA synthesis per se. In parallel infections at 41°C, the amount of early RNA per copy of viral DNA was as much as 2,000 times greater with *tsA*58 than with wild-type SV40, even though there was no replication of the *tsA*58 DNA. Synthesis of late RNA could not be detected during the first 20 h of an infection by either virus at 32°C, indicating that late and early transcription are under different control. In three cell lines transformed by *tsA* mutants, the amount of early RNA increased moderately after a shift from 32 to 41°C, whereas with homologous cells transformed by wild-type virus, the amount of early RNA decreased, indicating that the *A* protein may be able to repress transcription of integrated SV40 DNA. All the observations are consistent with a simple model in which the binding of *A* protein at the origin of replication blocks either binding of RNA polymerase to the early promoter or its progress through the early gene(s).

In a lytic infection by simian virus 40 (SV40), gene *A* is transcribed before the onset of viral DNA synthesis ("early"), leading to the appearance of early mRNA in the cytoplasm and to its translation into T antigen, the *A* protein (8, 14, 17, 18). Temperature-sensitive *A* mutants fail to initiate viral DNA synthesis at a nonpermissive temperature (21) and produce a more thermolabile *A* protein (1, 26), indicating that this protein is directly involved in initiation. Consistent with this idea is that the *A* protein binds preferentially to the origin of replication in vitro (15).

The *A* protein is synthesized continuously during the course of infection and accumulates in the nucleus (1, 25). Tegtmeyer et al. (22, 25) found that, although the steady-state amount of the *A* protein is very low in cells infected with *tsA* mutants at a nonpermissive temperature, the rate of synthesis of this protein is more rapid than in a corresponding infection with wild-type virus. They proposed that the *A* protein regulates its own synthesis, since faster synthesis might reflect the loss of an inhibitory control. We previously confirmed and extended this initial idea by demonstrating that cells infected with *tsA* mutants overproduce early RNA, that they synthesize it faster, that they do so even at a permissive temperature, and that this effect is amplified greatly after a shift to a nonpermissive temperature late in infection when many DNA templates are present (16). Therefore, autoregulation appears to operate through negative feedback control of the transcription of gene *A*. Since the 5' end of RNA molecules complementary to the early strand of SV40 DNA map near the origin of replication (10; S. I. Reed and J. C. Alwine, Cell, in press), control of transcription may well be achieved by the binding of *A* protein at or near the early promoter, which may in turn be

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near the origin of replication (6). We now present additional information on the autoregulation of gene A and examine more critically its relationship to initiation of viral DNA synthesis.

MATERIALS AND METHODS

Cells. The CV-1 line of African green monkey kidney cells, obtained from the laboratory of Paul Berg, was grown in a CO_2 incubator in Lux plastic plates (100 by 15 mm) in Eagle medium as modified by Dulbecco (GIBCO) with 10% fetal bovine serum (Microbiological Associates).

The cell lines 596HEWT and 596HEA58 (23), hamster embryo cells transformed by SV40 wild-type strain VA45-54 or by the mutant *tsA*58, were the gift of Peter Tegtmeyer. The lines CHLWT15, CHLA209L1, and CHLA239L1 (12), Chinese hamster lung cells transformed by wild-type strain SV-S or by the mutant *tsA*209 or *tsA*239, were the gift of Robert Martin.

Viruses and nucleic acids. The virus strains used included the wild type VA45-54 (24) and temperature-sensitive mutants derived from it: *tsA*7, *tsA*30, *tsA*58, and *tsB*4 (21, 22, 24) and also *tsA*209 (4), derived from the SV-S wild-type strain. Nondefective stocks were prepared as described by Estes et al. (7). SV40 (I) DNA (supercoiled circles) was prepared from CV-1 cells infected for 11 days at 37°C with 0.01 plaque-forming unit (PFU) of VA45-54 per cell as described previously (15). Cytoplasmic RNA was prepared as described previously (16).

Analysis of hybridization kinetics. SV40 DNA was labeled in vitro to high specific radioactivity according to the "nick translation" procedure of P. W. J. Rigby, M. Dieckmann, C. Rhodes, and P. Berg (J. Mol. Biol., in press), using [^{3}H]TTP (45 Ci/mmol, New England Nuclear). The DNA (1.6×10^7 cpm/μg) was fragmented at 4°C by sonically treating it for 6 to 8 periods of 10 s each at setting 7 of a Sonifier cell disrupter. The strands were separated with cRNA according to Sambrook et al. (19).

To determine the fraction of cytoplasmic RNA that was viral, we employed the hybridization kinetics analysis of J. C. Leong, D. E. Garfin, H. S. Smith, P. W. J. Rigby, and H. M. Goodman (manuscript in preparation) as described previously by Reed et al. (16), in which the initial rate of hybridization of viral RNA to highly labeled, separated strands of SV40 DNA is determined. The fraction (f) of viral sequences in the sample is calculated from the slope of a plot of the linear equation $C_0/C = 1 + K_h f(C_r t)$, where C_0 is the initial concentration of complementary single-stranded DNA, C is the concentration of complementary single-stranded DNA at any time (t), C_r is the total concentration of RNA (viral and cellular), and K_h is the second-order rate constant determined with known concentrations of SV40 cRNA. Viral RNA complementary to the E (minus) DNA strand is referred to as E-RNA and viral RNA complementary to the L (plus) strand is L-RNA. Since the coding region for cytoplasmic E-RNA or L-RNA is equal to about half the E-DNA or L-DNA strand (6, 10), C_0 is taken to be half of the input concentration of labeled E-DNA or L-DNA.

The amount of viral DNA in infected cells was determined according to P. W. J. Rigby and P. Berg (manuscript in preparation) by using the hybridization of "nick-translated" SV40 DNA to determine C_0t.

Enzymes and reagents. *Escherichia coli* DNA polymerase I and *E. coli* RNA polymerase holoenzyme were gifts of Leroy Bertsch and Michael Chamberlin, respectively. The S_1 nuclease of *Aspergillus oryzae* was purchased from Sigma Chemical Co., bovine pancreatic DNase I (RNase-free) was from Worthington Biochemicals Corp., and proteinase K was from Merck and Co. 2'-Deoxy-2'-azidocytidine (20) was a gift of Peter Reichard.

RESULTS AND DISCUSSION

Effect of the multiplicity of infection. In previous experiments, CV-1 cells were infected with *tsA*58 virus at high multiplicity (20 to 100 PFU per cell) for 72 h at 32°C and then shifted to 41°C for 5 or 10 h (16). There was a 17-fold increase in cytoplasmic E-RNA after 10 h at 41°C. To examine the possible effects of different multiplicities of infection, CV-1 cells were infected with 0.5 to 50 PFU of *tsA*58 per cell with or without a 10-h shift to nonpermissive temperature, and the fraction of E-RNA in the cytoplasmic RNA pool was determined (Fig. 1). In the experiments with a temperature shift where the overproduction was greatest, the percentage of E-RNA did not increase appreciably when multiplicities greater than about 5 PFU per cell were used. The dashed line in Fig. 1 shows the percentage of host cells initially infected with at least 1 PFU as a function of multiplicity, calculated according to a Poisson distribution. The agreement between this curve and that of shifted samples demonstrates that maximum overproduction of E-RNA in the

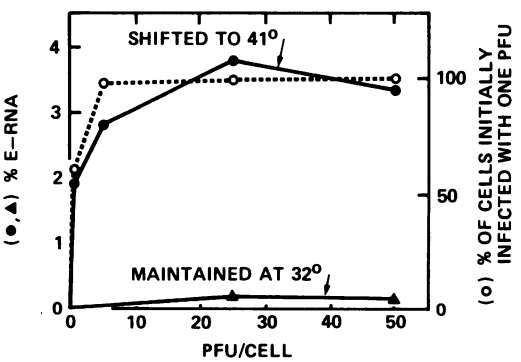

FIG. 1. *Effect of multiplicity of infection on overproduction of E-RNA by tsA58. The amount of E-RNA in the cytoplasmic fraction was determined in CV-1 cells that had been infected for 72 h with* (●) *or without* (▲) *a shift to 41°C for an additional 10 h. The dashed line is the calculated percentage of host cells infected initially with at least 1 PFU.*

culture as a whole is obtained when each cell is infected initially with at least 1 PFU; i.e., infection with more than one virus per cell does not further increase the amount of E-RNA.

Levels of E-RNA in cytoplasmic samples from cells infected with different temperature-sensitive mutants. We previously observed overproduction of E-RNA with both *tsA*58 and *tsA*7 (16). Lai and Nathans (11) showed by the marker rescue technique that *tsA* mutants map in one of three adjacent *Hin*dII+III restriction endonuclease fragments, all derived from the early region of the SV40 genome. For a detailed comparison of mutants, we chose a representative from each group: *tsA*7 (B fragment), *tsA*30 (H fragment), and *tsA*58 (I fragment). These mutants were all derived from the VA45-54 strain, which was used as the wild-type control in the experiments. We also used a mutant in the late region, *tsB*4 (J fragment) and one additional *A* mutant *tsA*209 (I fragment), derived from the SV-S wild-type strain. The percentage of E-RNA was determined in cytoplasmic RNA prepared from CV-1 cells infected at 20 PFU per cell for 72 h at 32°C with or without a 10-h shift to 41°C (Table 1). All the *tsA* mutants overproduced E-RNA, even at a permissive temperature, and the overproduction was amplified upon a shift to restrictive temperature. The late mutant *tsB*4 had the same level of early RNA as did the wild-type control both at 32°C and after a shift to 41°C.

We reported previously that there was a 17-fold increase in E-RNA when cells infected with *tsA*58 were shifted from 32 to 41°C for 10 h late in infection (16), yet in Table 1 there is only a sevenfold increase. The amount of E-RNA was the same in cells that had been shifted to 41°C in the two studies, but there was more in cells maintained at a permissive temperature in the experiments of Table 1 than previously. This discrepancy appeared to be due to a small difference in the permissive temperatures in the two cases: a shift from 31 to 33°C caused more than a twofold increase in the amount of E-RNA. In previous work we found that mutant *A* proteins were more labile than the wild-type *A* protein in vitro at all temperatures (1). If they were also more labile in vivo, an increase in temperature even in the permissive range would increase the rate at which the mutant *A* proteins decay, which in turn would be reflected in increased synthesis of E-RNA.

TABLE 1. *Production of E-RNA by several SV40 temperature-sensitive mutants*[a]

Virus	Percentage of cytoplasmic RNA that is E-RNA		Ratio of percentages
	32°C	32°C → 41°C	(32°C → 41°C)/32°C
VA45-54 (WT)[b]	0.039	0.023	0.59
*tsB*4	0.050	0.018	0.36
*tsA*58	0.400	2.80	7.00
*tsA*30	0.130	0.710	5.46
*tsA*7	0.150	0.320	2.13
*tsA*209	0.240	1.20	5.00

[a] The percentage of E-RNA was determined in the cytoplasmic fractions of CV-1 cells infected at 20 PFU per cell for 72 h at 32°C, with or without a 10-h shift to 41°C.

[b] WT, Wild type.

Phenotypically, *tsA* mutants are unable to initiate viral DNA replication and do not produce virus at restrictive temperatures. Different *tsA* mutants vary in the extent to which they are defective: *tsA*7 is the most leaky mutant, *tsA*58 is the least leaky, and *tsA*30 seems to be intermediate (21, 22). This relative order is maintained when the amounts of E-RNA overproduced by the *tsA* mutants are compared, both at permissive and at restrictive temperatures (Table 1). The similarity of the relative effects on initiation of viral DNA synthesis and on control of the transcription of gene *A* within this set of three *tsA* mutants indicates that the *A* protein is involved in both processes.

Effects of inhibitors of DNA replication on synthesis of E-RNA in infections by wild-type SV40. Although the *A* protein appears to have coupled functions in initiating viral DNA synthesis and in controlling gene *A* transcription, overproduction of E-RNA might occur indirectly as a consequence of the cessation of viral DNA synthesis. In this case, inhibitors of DNA synthesis should cause overproduction of E-RNA in an infection by the wild-type virus. We reported previously that cytosine arabinoside causes no overproduction (16). Another inhibitor of DNA synthesis, 2-azidocytidine (20), appears to inhibit synthesis of polyoma DNA at initiation (G. L. Bjursell, L. Skoog, L. Thelander, and G. Söderman, Proc. Natl. Acad. Sci. U.S.A., in press), and, therefore, its action is more similar to the defect in a nonpermissive infection by a *tsA* mutant at 41°C than is the action of cytosine arabinoside, which inhibits primarily elongation. CV-1 cells were infected with wild-type virus at 20 PFU per cell for 72 h at 32°C, and some of the cells were shifted to 41°C. At the time of the shift, samples at each temperature were treated with inhibitory concentrations of 2'-azidocytidine, and the cells were harvested 6 h later. As shown in Table 2, blocking viral DNA synthesis with this inhibitor made no difference in the amount of early or

TABLE 2. *Effect of 2'-azidocytidine on SV40 RNA in infections by wild-type virus*[a]

Temp (°C)	E-RNA (%)		L-RNA (%)	
	−AzidoC	+AzidoC	−AzidoC	+AzidoC
32	0.030	0.030	1.05	1.04
32 → 41	0.021	0.021	2.70	2.97

[a] The percentages of E-RNA and L-RNA were determined in the cytoplasmic fractions of CV-1 cells infected with VA45-54 at 20 PFU per cell for 72 h at 32°C with or without a shift to 41°C. At the time of the shift, the medium above some of the cells was made 2 mM in 2'-deoxy-2'-azidocytidine (AzidoC), and incubation at 32 or 41°C was continued for an additional 6 h. The inhibitory effect of 2'-azidocytidine had been previously tested in CV-1 cells infected for 24 h at 37°C with wild-type virus at 50 PFU per cell. Various amounts of inhibitor were added for 4 h, followed by a 15-min pulse of [^{3}H]thymidine. The amount of label in viral DNA was taken to be the same as the label in supernatants prepared according to Hirt (9). At 0.3 mM, 2'-azidocytidine inhibits SV40 DNA synthesis by at least 95%, an effect similar to the one reported with polyoma (G. Bjursell et al. Proc. Natl. Acad. Sci. U.S.A., in press).

late viral RNA in the cytoplasm at either temperature. Therefore, blocking viral DNA synthesis at initiation does not result in overproduction of E-RNA in the presence of normal *A* protein. This result supports a model in which failure to initiate DNA synthesis and overproduction of E-RNA are both related to rapid loss of a more thermosensitive *A* protein.

Viral DNA synthesis and control of transcription in infections with no temperature shift. To examine further the linked functions of the *A* protein in controlling viral DNA synthesis and transcription of E-RNA, CV-1 cells were infected with wild-type or *tsA*58 virus at a multiplicity of 20 PFU per cell for times ranging from 0 to 98 h at 32°C or from 0 to 48 h at 41°C. The amounts of E-RNA and L-RNA in the cytoplasmic fractions are shown in Fig. 2 and 3. After 0 and 72 h at 32°C, and after 0 and 48 h at 41°C, the number of SV40 genomes per cell was determined by C_0t analysis (Table 3). As expected, less viral DNA was made in the *tsA*58 infection than in the wild-type infection at 32°C, and there seemed to be some loss of input *tsA*58 DNA after 48 h at 41°C. The impaired ability of *tsA*58 to grow even at 32°C can be attributed to instability of the mutant *A* protein. Overproduction of E-RNA by *tsA*58 occurred even at 41°C, where the C_0t data show that there was no net synthesis of DNA (Fig. 2). It can be estimated that *tsA*58 produced approximately 180 times more cytoplasmic E-RNA per genome than did wild-type virus at 32°C, whereas at 41°C *tsA*58 produced approximately 2,000 times more.

Initiation of L-RNA synthesis has been reported to depend on the initiation, but not the continuation, of viral DNA synthesis (5). As shown in Fig. 3, accumulation of L-RNA did not start until 24 h after infection at 32°C, after

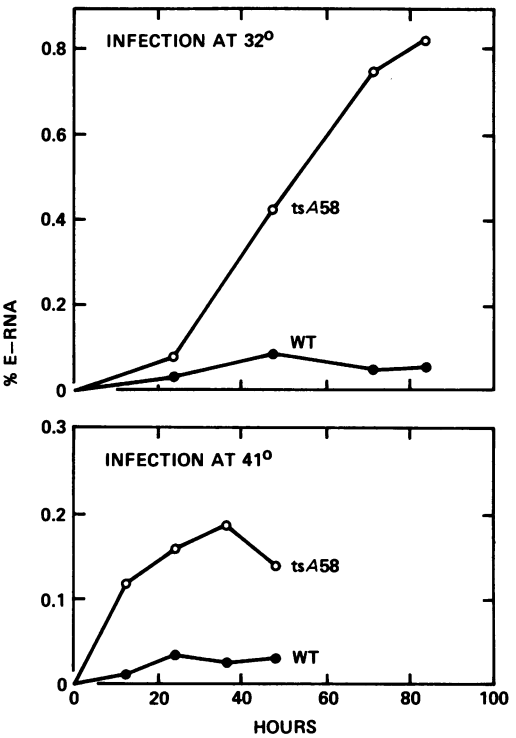

FIG. 2. *Amount of cytoplasmic E-RNA in cells infected with tsA58 or wild-type virus as a function of time at 32 or 41°C. CV-1 cells were infected with tsA58 (○) or VA45-54 (●) virus at a multiplicity of 20 PFU per cell at either 32 or 41°C.*

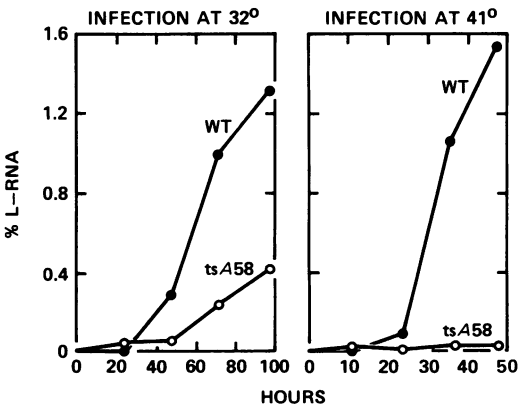

FIG. 3. *Amount of cytoplasmic L-RNA in cells infected by tsA58 or wild-type virus as a function of time. The experiment is the same as described in the legend to Fig. 2.*

the start of DNA synthesis, and the accumulation was diminished in the case of *tsA*58, probably because there were fewer gene copies. At 41°C, accumulation of L-RNA in the infection by wild-type virus started after about 12 h, and very little L-RNA was formed in the *tsA*58 infection, as expected, since *tsA*58 DNA is not synthesized at this temperature.

Control of E-RNA transcription in transformed cells. Cells transformed by *tsA* mutants lose both phenotypic indicators of transformation and detectable T antigen (*A* protein) upon incubation at nonpermissive temperatures (3, 12, 13, 23). Furthermore, the *A* protein from cells transformed with a *tsA* mutant is more thermolabile than the *A* protein from cells transformed by wild-type virus (2, 26). Therefore, the *A* protein is strongly implicated in maintaining the SV40 transformed state. We tested several cell lines transformed by wild-type virus and *tsA* mutants to see whether the *A* protein controls transcription of E-RNA in these cells. Transformed cells were grown to confluency at 32°C, and some were shifted to 39.5 or 41°C for 20 h. As shown in Table 4, both CHL cell lines transformed by *tsA* mutants overproduced E-RNA moderately after the shift to a nonpermissive temperature. The hamster embryo line transformed by *tsA*58 did not survive well at nonpermissive temperatures, perhaps accounting for its failure to produce more E-RNA after a temperature shift. However, the amount of E-RNA did not decrease, as it did with both lines transformed by wild-type virus. These results may indicate that the *A* protein can function to control E-RNA synthesis in transformed cells as well as in infected cells. In transformed cells such regulation could function at the viral promoter, at a cellular promoter, or in conjunction with another factor(s) involved in transformation.

TABLE 3. *Copies of SV40 DNA per cell after infection at 32 or 41°C*[a]

Conditions	Wild type (copies/cell)	*tsA*58 (copies/cell)
Input DNA, zero time	250	230
72 h at 32°C	19,400	2,500
48 h at 41°C	43,100	110

[a] The number of SV40 genomes per cell was determined by C_0t analysis of total DNA from CV-1 cells infected with VA45-54 or *tsA*58 at 20 PFU per cell at 32 or 41°C. The amount of input DNA was determined after 2 h of adsorption at 32°C.

TABLE 4. *E-RNA in SV40-transformed cells*[a]

Cell line	% E-RNA[a]			Ratio (32°C → 41°C)/32°C
	32°C	32°C → 41°C	32°C → 39.5°C	
CHLWT15	0.035	0.024		0.70
CHLA209L1	0.040	0.120		3.00
CHLA239L1[b]	0.036	0.084		2.33
596 HE(WT)[c]	0.051		0.028	0.55
596 HE(*A*58)	0.062		0.063	1.00

[a] The perecentage of E-RNA was determined in cytoplasmic fractions of cells grown to confluency at 32°C with or without a shift to 39.5 or 41°C for 20 h before harvest.

[b] In the experiment with CHLA239L1, total RNA was used instead of the cytoplasmic fraction.

[c] WT, Wild type.

In summary, a *tsA* mutant has diminished ability to synthesize viral DNA at both permissive and restrictive temperatures, and this property can be correlated quantitatively with the amount of E-RNA overproduced, indicating the dual function of the *A* protein in initiation of viral DNA synthesis and in negative feedback regulation of E-RNA synthesis. The coupled effects are consistent with a simple model in which the *A* protein binds at or near the origin of replication and blocks early transcription from a promoter at or near the origin. The large amount of E-RNA made per genome in infections by *tsA*58 at 32°C, and especially at 41°C, indicates that the early promoter is very efficient, giving the virus the advantage of being able to initiate early transcription, and the processes that follow and depend on it, with high probability. With an efficient early promoter, efficient control becomes necessary later, to divert genomes from early transcription to replication, late transcription, and packaging of complete virions.

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