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Autoregulation of simian virus 40 gene *A* by T antigen

(autogenous regulation/*A* protein/mRNA/nucleic acid hybridization/temperature-sensitive mutants)

STEVEN I. REED, GEORGE R. STARK, AND JAMES C. ALWINE

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

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ABSTRACT **During lytic infection by simian virus 40, gene *A* is transcribed into early RNA, which is translated into *A* protein (T antigen). Both the rate of synthesis and the intracellular amount of early RNA are higher in cells infected by temperature-sensitive *A* (ts*A*) mutants than in cells infected by wild-type virus. These differences are observed at permissive temperature (32°) and are amplified greatly after a shift to restrictive temperature (41°). For example, at 32° cells infected by ts*A* mutants synthesize early RNA approximately twice as fast as cells infected by wild-type virus. After the shift to 41°, the rate of synthesis in the ts*A* infection increases to 15 times the rate in the wild-type infection. In contrast, cells infected by ts*A* mutants do not overproduce late RNA. We suggest that the *A* protein regulates its own synthesis by negative feedback control of gene *A* transcription.**

In lytic infection by simian virus 40 (SV40), the expression of gene *A* is required to initiate each new round of viral DNA synthesis, as well as to begin late transcription (1). Transcription of gene *A* leads to the formation of an early 19S cytoplasmic messenger RNA which is translated into *A* protein, synonymous with T antigen (2, 3). The *A* protein is synthesized continuously during the course of infection and accumulates in the nucleus (4, 5).

Recent observations suggest that gene *A* is regulated autogenously. Tegtmeyer *et al.* (5) proposed autoregulation of gene *A* on the basis of work showing that the synthesis of *A* protein is stimulated when cells infected by temperature-sensitive mutants in gene *A* (ts*A* mutants) are shifted to a restrictive temperature. We have observed that the intracellular concentration of T antigen is about the same in infections by wild-type or ts*A* virus at 32° but that the lability of the mutant T antigen *in vitro* is greater even at this permissive temperature; therefore, an increased rate of synthesis might compensate for an increased rate of degradation of the mutant *A* protein *in vivo* (4). In the present study we have investigated the autoregulation of gene *A* further by examining its transcription. If the *A* protein regulates its own synthesis by negative feedback control of the transcription of gene *A*, the rate of synthesis of early RNA should be greater and the intracellular concentration of early RNA should be higher in cells infected with a ts*A* mutant because the mutant *A* protein is more labile. We have determined the relative rates of synthesis by selectively adsorbing pulse-labeled SV40 RNA to separated strands of SV40 DNA coupled to cellulose and have determined intracellular amounts by examining the initial rate of hybridization in solution of RNA from infected cells with highly labeled separated strands of SV40 DNA. At permissive temperature, both are higher in cells infected by a ts*A* mutant than in cells infected by wild-type virus and the difference is amplified greatly by a shift to restrictive temperature. These results further support the hypothesis that the *A* protein of SV40 regulates its own synthesis by controlling the transcription of gene *A*.

Abbreviations: SV40, simian virus 40; ts, temperature sensitive; PFU, plaque-forming unit; E-strand, the SV40 DNA strand complementary to early messenger RNA; L-strand, the other SV40 DNA strand, complementary to late messenger RNA; $NaDodSO_4$, sodium dodecyl sulfate; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; WT, wild type.

MATERIALS AND METHODS

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

Virus and DNA. Wild-type SV40 strain VA45-54 (6) and the mutants tsA58 (7) and tsA7 (1) derived from it were used. Nondefective stocks were prepared as described by Estes *et al.* (8). SV40 DNA was prepared from CV-1 cells infected with 0.01 plaque-forming unit (PFU) of wild-type virus per cell. DNA labeled with ^3H was prepared by adding 0.25 mCi of [^3H]thymidine to each plate on the fourth and eighth day after infection. After 11 days, the cells were lysed according to Hirt (9) and SV40(I) DNA (supercoiled circles) was purified (10), dialyzed into 10 mM Tris·HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, and stored at 4°.

SV40 [^3H]DNA with High Specific Radioactivity. SV40(I) DNA was labeled *in vitro* by "nick translation," using *Escherichia coli* DNA polymerase I and bovine pancreatic deoxyribonuclease I according to the procedure of Rigby *et al.** using [^3H]dTTP (45 Ci/mmol). DNA (specific radioactivity 1.6×10^7 cpm/μg) was fragmented at 4° with 6–8 10-sec sonications at setting 7 of a Sonifier Cell Disruptor (Heat Systems–Ultrasonic, Inc.).

Separation of SV40 DNA Strands. cRNA complementary to the E-strand was synthesized *in vitro* using *E. coli* RNA polymerase holoenzyme and SV40(I) DNA, and the strands of SV40 DNA were separated according to Sambrook *et al.* (11).

Preparation of RNA. Infected (100 PFU per cell) or mock-infected cells from 10 plates were harvested after washing by scraping them into Tris–saline buffer (20 mM Tris·HCl, pH 7.6, 1 mM Na_2HPO_4, 8 mM KCl, 0.137 M NaCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$), and were collected by centrifugation at 800 × *g* at 4°. For total cellular RNA, cell pellets were resuspended in 2–3 ml of lysis buffer [20 mM Tris·HCl, pH 7.5, 10 mM EDTA, 0.2 M NaCl, 1% sodium dodecyl sulfate ($NaDodSO_4$)], and lysed by vortex mixing. For nuclear and cytoplasmic RNA, cell pellets were resuspended in 2–3 ml of TKM (20 mM Tris·HCl, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$) containing 0.2 M sucrose and 0.05% of the nonionic detergent NP40 and disrupted with 75 to 80 strokes of a Dounce homogenizer. Nuclei were sedimented three times through 0.32 M sucrose and 0.05% NP40 in TKM. The nuclear pellets were resuspended in lysis

* P. W. J. Rigby, M. Dieckmann, C. Rhodes, and P. Berg, personal communication.

buffer and lysed as described for total cell pellets. The supernatant cytoplasmic fractions were made 0.2 M in NaCl and 1% in $NaDodSO_4$, and vortexed. Proteinase K (100 μg/ml) was added to all RNA preparations and incubated at 25° for 0.5 hr. The digests were made 0.1 M in Tris·HCl, pH 9.0, and were extracted with phenol:chloroform:isoamyl alcohol (1:1:0.02), equilibrated previously with pH 9.0 buffer. The first interphase was back-extracted with 0.1 M Tris·HCl, pH 9.0, 0.2 M NaCl, and the two aqueous phases were combined for two additional extractions. Nucleic acid was precipitated overnight from the final aqueous phase at −20° by adding 3 volumes of cold ethanol and was collected by centrifugation. The pellets were dissolved in 1.5 ml of 20 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM NaCl and 30 μg/ml of bovine pancreatic deoxyribonuclease I, and incubated at 37° for 1 hr. Digestion of the DNA was judged complete when added [^{3}H]DNA became acid soluble. The digest was then made 0.1 M in sodium acetate, pH 5.1, 0.1 M NaCl, 10 mM EDTA, and extracted three times with phenol:chloroform:isoamyl alcohol (1:1:0.02) equilibrated to pH 5.1. Phenol was removed from the aqueous phase by three extractions with water-saturated ether and the RNA was precipitated with ethanol. RNA pellets were dissolved in 99% formamide and the concentrations were determined from the absorbance at 260 nm after dilution into water. In all cases, the ratio A_{260}/A_{280} was 1.95–2.00. Yields of RNA from wild-type and mutant infections were the same within 5%. There was an increase of 60–70% after the shifts to 41°.

Enzymes. *E. coli* DNA polymerase I and *E. coli* RNA polymerase holoenzyme were generous gifts of Leroy Bertsch and Michael Chamberlin, respectively. The S_1 nuclease of *Aspergillus* was purchased from Miles Laboratories, bovine pancreatic deoxyribonuclease I (ribonuclease-free) from Worthington, and proteinase K from Merck.

RESULTS

Viral RNA at permissive temperature

The amounts of RNA complementary to the E- or L-strand of SV40 DNA were compared in cells infected at 32° by either wild-type or tsA58 mutant virus. Late in infection, at 72 hr, cells infected at a multiplicity of 100 PFU per cell were harvested and either total cellular RNA or fractionated cytoplasmic and nuclear RNA were prepared. The percentage of RNA complementary to each SV40 DNA strand was determined by hybridization kinetics according to Leong *et al.*† (Fig. 1). The data are summarized in Fig. 2 as the points at zero time. The percentage of sequences complementary to the E-strand was four times greater in total RNA from cells infected by tsA58 than in total RNA from cells infected by wild-type virus. Similarly, the concentration of sequences complementary to the E-strand was twice as great in cytoplasmic RNA (0.15% compared to 0.08%) and 10 times greater in nuclear RNA from cells infected by the tsA mutant. In contrast, the percentage of sequences complementary to the L-strand in total cellular, nuclear, or cytoplasmic RNA from infections with wild-type virus was about twice as great as in the equivalent fractions from cells infected by the mutant.

In order to examine the rates of synthesis of RNA complementary to E- or L-strand at 32°, cells infected either with wild-type virus or with tsA58 were labeled with [^{32}P]phosphate for 1 hr, 73 hr after infection. Total RNA was hybridized to separated SV40 DNA strands immobilized on cellulose according to the method of Noyes and Stark (12). An excess of DNA was used so that binding of ^{32}P-labeled SV40-specific sequences would be independent of the amount of unlabeled viral RNA present and therefore would be a true measure of the rate of synthesis. As shown in Table 1, the rate of synthesis of RNA complementary to the E-strand is about twice as great in cells infected by the mutant as in cells infected by wild-type virus. In contrast, the rate of synthesis of RNA complementary to the L-strand is about half that of the wild type. Labeled RNA from uninfected cells was used to determine experimental backgrounds.

Viral RNA after a shift from 32° to 41°

The effect of the tsA mutation on the synthesis of viral RNA was determined after a shift from permissive to nonpermissive temperature. Cells infected with 100 PFU per cell of either wild-type or tsA58 virus were incubated at 32° until late in infection (72 hr) and then shifted to 41° for 5 or 10 hr more. The percentages of RNA complementary to the E- or L-strand are shown in Fig. 2 for total cellular, nuclear, and cytoplasmic RNA. The percentage of RNA complementary to the E-strand increases with time at 41° in all fractions from cells infected with tsA virus. The change is greatest in the cytoplasmic RNA, where there is an increase of 17-fold, from 0.15 to 2.5%, during 10 hr at 41°. These increases cannot be attributed to selective degradation of cellular RNA, since the yields of total RNA from cells infected with wild-type virus were not appreciably different (see *Materials and Methods*). In cells infected by wild-type virus, the *apparent* percentage of cytoplasmic RNA complementary to the E-strand decreases from 0.08 to 0.006% during 10 hr at 41°, so that there is an *apparent* 400-fold difference in the percentage of this RNA when mutant and wild-type infections are compared (see *Discussion*). Concurrently, the concentration of sequences complementary to the L-strand increases by a factor of about 2 in both the mutant and wild-type infections.

To determine whether the increase in RNA complementary to the E-strand in cells infected by tsA58 is due to more rapid synthesis, RNA was labeled with [^{32}P]phosphate from hour 72 to hour 73 after a shift from 32° to 41° 71 hr after infection. As shown in Table 1, the rate of synthesis of RNA complementary to the E-strand is about 15 times greater in cells infected with tsA58 than in cells infected with wild-type virus after the temperature shift. The rate of synthesis of sequences complementary to the L-strand is twice as great in the infections by wild-type virus both before and after the temperature shift.

The amount of SV40 early RNA was also determined before and after a shift to nonpermissive temperature for tsA7 (1, 13). In this mutant, viral DNA synthesis and virus production are shut down incompletely at elevated temperatures (1) and the A protein may be less labile *in vitro* than the A protein from tsA58 (4). As expected, the level of early RNA increases in cells infected with tsA7 after a shift to 41°, but the increase (7-fold) is less than in cells infected with tsA58 (17-fold).

Saturation of E-strand by cytoplasmic viral RNA

In infections by wild-type virus, nuclear SV40-specific RNA contains antisense sequences, the cytoplasmic SV40 RNA does not (14, 15). About half the E-strand is complementary to sense RNA and should be resistant to nuclease S_1 after hybridization with saturating amounts of early cytoplasmic RNA (14). It is important to demonstrate that the cytoplasmic RNA overproduced by tsA58 after a shift to 41° is messenger complementary to the E-strand and not antisense RNA. In Table 2 are shown the results of saturation hybridization experiments with E-strand using cytoplasmic RNA from cells infected with tsA58

† J. C. Leong, D. E. Garfin, H. S. Smith, P. W. J. Rigby, and H. M. Goodman, personal communication.

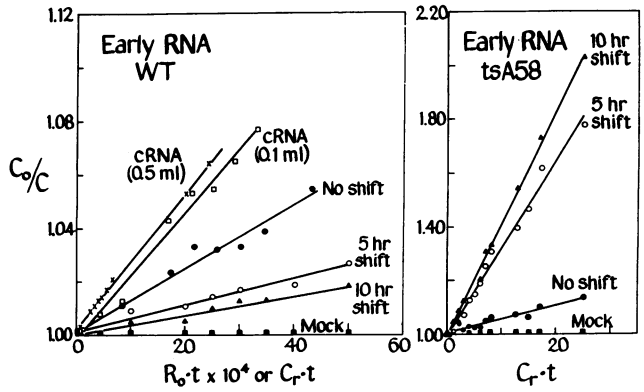

FIG. 1. An example of the quantitation of viral RNA. Left, cRNA and RNA from cells infected with wild-type (WT) virus. Right, RNA from cells infected with ts*A* virus and from mock-infected controls. (●), RNA from cells at 32°; (○), RNA from cells shifted to 41° for 5 hr; (▲), RNA from cells shifted to 41° for 10 hr; (■), RNA from uninfected cells; (×), cRNA in 0.5 ml reaction volume; (□), cRNA in 0.1 ml reaction volume. The concentrations of RNA species complementary to E- and L-strands were estimated by hybridization kinetics according to Leong *et al.*† The initial phase of hybridization between single-stranded DNA and RNA can be described at any concentration by the second-order rate equation:

$$C_0/C = \frac{R_0 e^{k_h t(R_0 - C_0)} - C_0}{R_0 - C_0}$$

where C represents the concentration of single-stranded DNA (as nucleotides) at time t and C_0 is the initial concentration, R_0 is the initial concentration of complementary RNA, and k_h is the second-order rate constant. For the initial stage of hybridization the approximation $e^x = 1 + x$ was used, yielding a simplified rate equation: $C_0/C = 1 + k_h R_0 t$.† A plot of C_0/C versus $R_0 t$ is a straight line with y intercept 1 and slope k_h. The numerical value of k_h can be determined empirically, with known concentrations of RNA and DNA. E-strand labeled *in vitro* to high specific radioactivity and cRNA prepared *in vitro* were used. We have observed that more than 99% of the cRNA hybridizes to the E-strand. These two nucleic acids were mixed and aliquots were placed into silane-treated 13 × 100 mm glass culture tubes so that each tube contained 20 ng of cRNA and 0.25 ng (4000 cpm) of E-strand in 0.5 ml of 50% (vol/vol) formamide buffer [0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) pH 7.5, 0.6 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, and 170 μg/ml of yeast tRNA]. The samples were mixed at 4°, warmed to 37°, and incubated at that temperature for various lengths of time. Samples were hybridized for a maximum of 18 hr because apparent decomposition of the formamide slows the reaction at longer times. After incubation, each sample was diluted with 1.5 ml of buffer (50 mM sodium acetate, pH 4.5, 1.2 mM $ZnCl_2$) and 5000 units of S_1 nuclease were added to digest the single-stranded DNA remaining. After 2 hr at 37°, 60 μg of calf thymus DNA carrier were added and nucleic acid was precipitated (4°) by adding 12 M HCl to a final concentration of 2 M. The samples were diluted to 6 ml with 2 M HCl containing 0.9% sodium pyrophosphate and collected on glass fiber filters. The variable concentration (C) was determined by subtracting the S_1-resistant radioactive material from the input radioactive material. Least square regression analysis was performed to determine the best fit of the line obtained by plotting C_0/C against $R_0 t$. The slope, k_h, was 2.58 ml/A_{260}·hr, where A_{260} is a unit of nucleic acid mass equivalent to 40 μg of RNA. Analogous experiments were then performed to determine the percentage of SV40-specific sequences in RNA from infected cells, except that for experiments with E-strand, C_0 was taken as 50% of the input radioactivity and for L-strand C_0 was taken as 25% of the input for total and cytoplasmic RNA and 50% for nuclear RNA (see the *Discussion*). To determine the percentage of SV40 RNA, C_0/C was plotted against $C_r t$, where C_r is the total RNA concentration of a mixture of SV40-specific and cellular RNA. The concentration of SV40 RNA complementary to either DNA strand is given by $R_0 = fC_r$, where f is the fraction of complementary RNA. Therefore $C_0/C = 1 + k_h f C_r t$ and the slope of the linear plot is $k_h f$. Since k_h is known from the calibration experiments with cRNA, f can be calculated readily.

or wild-type virus. In each case, about half the E-strand is protected from nuclease S_1 digestion by two different amounts of cytoplasmic RNA, showing that the complementary RNA is in excess. When saturating amounts of mutant and wild-type RNA are used in the same hybridization, about half the E-strand still remains undigested, showing that the same DNA sequences were protected in the independent experiments. Since Khoury *et al.* (14) have shown that, in infections by wild-type virus, early cytoplasmic viral RNA is complementary only to the sense half of the E-strand, we conclude that this asymmetry is maintained in our experiments with cytoplasmic RNA from ts*A*58.

DISCUSSION

The possible effect of antisense RNA (14, 15) on our results must be considered. We find anti-early RNA (sequences complementary to early mRNA) in all RNA fractions prepared from cells infected by wild-type virus, even at a low level in cytoplasmic RNA. The relative amount of this RNA increases after a shift to 41° (data not shown). Anti-early RNA may account for the *apparent* decline of early RNA in infections by wild-type virus after a shift to 41° through formation of RNA·RNA hybrids, i.e., increasing amounts of anti-early RNA will cause an *apparent* decrease in the concentration of early RNA. However, the small amount of anti-early RNA detected is far too low to account for the large differences between the amounts of wild-type and ts*A*58 early RNAs.

The heterogeneity of SV40-specific RNA requires us to make assumptions in assigning C_0, the initial concentration of complementary DNA in the hybridization kinetics experiments (Fig. 1). Since the initial rate we measure reflects primarily hybridization of the most abundant class of RNA, the effective C_0 will be only the fraction of total SV40 DNA complementary to this class of RNA. We assume that the most abundant species of early RNA is early 19S messenger, complementary to 50% of the E-strand (16). For total cellular or cytoplasmic late RNA, late 16S messenger, complementary to 25% of the L-strand (16), is assumed to predominate. Aloni (15) has reported that nuclear late RNA is 19S or larger, so we assume that the abundant species in the nucleus is late 19S RNA, complementary to 50% of the L-strand. However, since the composition of SV40-specific nuclear RNA is not yet well characterized, the numbers we calculate must be viewed with some caution in this case. Although uncertainty about the value of C_0 may introduce some inaccuracy into the data, the qualitative conclusions of the work remain strong.

Inhibition of viral DNA synthesis in ts*A* mutants at high temperature might affect early RNA synthesis. To evaluate this possibility, we are presently studying the relationship between

(We assume that k_h is the same for hybridizations with cRNA and with RNA made *in vivo*, i.e., any effects of differences in size of these RNAs are neglected.) In the left panel are shown sample data and regression lines for hybridization to the E-strand of total RNA from cells infected with wild-type virus. In this case, the standard procedure was modified by reducing the total volume to 0.1 ml and by performing the experiment in small conical plastic tubes, because the percentage of RNA complementary to the E-strand was low. A separate determination of k_h was made for the hybridizations carried out in 0.1 ml. Digestion with S_1 nuclease was accomplished by washing the contents of the tubes into 2 ml of 30 mM sodium acetate, pH 4.5, 0.5 mM $ZnCl_2$, and 175 mM NaCl and adding 5000 units of enzyme, followed by the treatment already described. For all experiments to determine early RNA, each sample contained 0.5 A_{260} unit of RNA and 0.25 ng (4000 cpm) of E-strand. For late RNAs, there was 0.1 A_{260} unit of RNA and 0.25 ng of L-strand.

Table 1. Hybridization of pulse-labeled total RNA to excess separated strands of SV40 DNA

Virus	Total amount of RNA (μg)	Temperature during infection (°C)	% of RNA bound* (average of two determinations)
		E-strand DNA	
tsA	10	32	0.88 ± 0.07*
tsA	20	32	0.88 ± 0.02
WT	10	32	0.56 ± 0.04
WT	20	32	0.54 ± 0
tsA	5	32 → 41	3.10 ± 0.30
tsA	10	32 → 41	3.15 ± 0.05
WT	20	32 → 41	0.23 ± 0.03
WT	40	32 → 41	0.15 ± 0.04
		L-strand DNA	
tsA	5	32	0.95 ± 0.05
tsA	10	32	0.66 ± 0.05
WT	5	32	1.90 ± 0.10
WT	10	32	2.00 ± 0
tsA	2.5	32 → 41	0.67 ± 0.15
tsA	5	32 → 41	0.63 ± 0.04
WT	2.5	32 → 41	1.70 ± 0.20
WT	5	32 → 41	0.98 ± 0.03

Cells were washed three times with phosphate-free medium 71 hr after infection with wild-type or tsA58 virus at 32°. Cells to be labeled at 41° were incubated with 2 ml of phosphate-free medium for 1 hr at 41° and cells to be labeled at 32° were incubated with 2 ml of phosphate-free medium for 2 hr at 32°. The medium from each plate was then replaced with 1 ml of preheated phosphate-free medium containing 4.5 mCi of [^{32}P]phosphate. After 1 hr at either 32° or 41°, the total RNA was extracted. The specific radioactivities were 100,000–125,000 cpm/μg for RNA samples labeled at 41° and 50,000–75,000 cpm/μg for RNA samples labeled at 32°. RNA was hybridized in DNA excess according to Noyes and Stark (12). Ten micrograms of either strand were coupled to 18 mg (dry weight) of diazotized cellulose in the presence of 130 μg of purified yeast tRNA carrier (phenol-extracted). The mixed nucleic acids were precipitated with ethanol and dissolved in 0.5 ml of dimethylsulfoxide plus 0.1 ml of 0.2 M borate buffer, pH 8. More than 90% of the DNA was coupled covalently in each case. Addition of carrier was required for efficient coupling of this small amount of DNA. To help achieve DNA excess, the DNA coupled to cellulose was fragmented to about 500 nucleotides, a much smaller size than that of the RNA. Since each small fragment of DNA can bind a much larger molecule of RNA, only a number excess of DNA fragments to RNA molecules is required, allowing one to do hybridizations in DNA excess with a relatively small amount of DNA. A disadvantage of this approach is that ribonuclease digestion cannot be used to lower the backgrounds. To establish experimentally that the condition of DNA excess had been met, two different amounts of each RNA sample were hybridized. As expected, the percentage of input counts bound was the same for each, within experimental errors. Hybridizations were done in duplicate in small conical plastic tubes containing 0.15 μg of E- or L-strand bound to cellulose. The buffer contained 0.1 M Tes, pH 7.5, 0.6 M NaCl, 10 mM EDTA, 0.1% $NaDodSO_4$, 50% formamide, 100 μg of yeast tRNA, and 40 μg of poly(rA) in a total volume of 200 μl. After 36 hr at 37°, the cellulose was washed by centrifugation four times with 40% formamide in the same buffer, five times with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate), and one additional time with 40% formamide buffer. RNA was eluted by incubating the cellulose twice in 50% formamide at 80° for 20 min. The pooled supernatant solutions plus 30 μg of carrier tRNA were precipitated with 2 M HCl, and the precipitates were collected on glass fiber filters and measured for radioactivity.

* Corrected for backgrounds determined in each experiment with two different amounts (ranging from 2.5 to 20 μg) of labeled RNA from uninfected CV-1 cells. The specific radioactivities of these RNAs and of RNAs from infected cells did not differ by more than a factor of 2. The percentage of RNA bound in the blanks ranged between 0.04 and 0.22% and the average value was 0.11% for eight determinations.

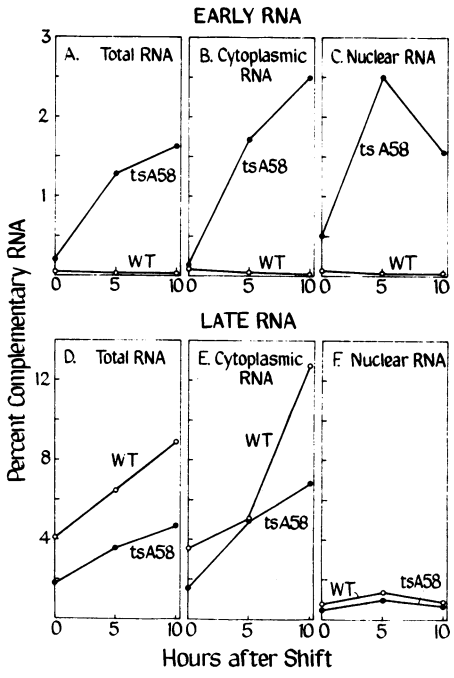

FIG. 2. Viral RNA after a temperature shift from 32° to 41°. Cells were infected with wild-type or tsA58 virus at a multiplicity of 100 PFU per cell and incubated at 32°. The cells were collected 72 hr after infection or shifted to 41° for 5 or 10 hr more. Total cellular, cytoplasmic, and nuclear RNAs were prepared and the fraction of RNA complementary to E- or L-strand was determined as described in Fig. 1. (○), wild type; (●), tsA58. (A) Total cellular RNA complementary to E-strand; (B) cytoplasmic RNA complementary to E-strand; (C) nuclear RNA complementary to E-strand; (D) total cellular RNA complementary to L-strand; (E) cytoplasmic RNA complementary to L-strand; (F) nuclear RNA complementary to L-strand.

overproduction of early message and SV40 DNA synthesis. In a preliminary experiment with wild-type virus in which DNA synthesis was inhibited with cytosine arabinonucleoside, there was no overproduction of early viral RNA.

The present results, with two different tsA mutants, help to account for several prior observations on the regulation of A protein synthesis in lytically infected cells. Tegtmeyer *et al.* (5) showed that the rate of synthesis of A protein after a shift from 32° to 41° is appreciably greater in cells infected by tsA mutants than in cells infected by wild-type virus. We have shown previously that the concentration of A protein is comparable in cells infected with tsA or wild-type virus at permissive temperature, even though the mutant A protein is more labile *in vitro* (4). These results are consistent with our present findings that both the rate of synthesis and the intracellular concentration of early RNA are higher in cells infected by A mutants than in cells infected by wild-type virus at both permissive and restrictive temperatures. All the data are consistent with a model in which greater lability of the mutant A protein *in vivo* at permissive and at restrictive temperatures results in derepression of early transcription. The ratios of the rates of early transcription in infections by tsA58 and wild-type virus are 2:1 at 32° and 15:1 at 41°. According to the model, in a mixed infection with wild-type and tsA mutant viruses the more stable wild-type A protein should block derepression of the transcription of mutant early RNA, and therefore wild-type A protein should pre-

Table 2. Hybridization of SV40 E-strand with excess cytoplasmic RNA

RNA	RNA/reaction (A_{260})	Hr at 41°	% Saturation
WT	2.24	0	43
WT	5.60	0	50
tsA58	0.34	0	47
tsA58	1.70	0	50
WT	5.00	10	46
WT	12.00	10	49
tsA58	0.22	10	50
tsA58	1.08	10	51
WT, tsA58	3.36, 0.19	0, 0	46
WT, tsA58	6.00, 0.14	10, 10	48
tsA58, tsA58	0.51, 0.51	0, 10	50

After 72 hr at 32°, cells infected with wild-type or tsA58 virus (100 PFU/cell) were harvested or shifted to 41° for 10 hr more before harvesting. Cytoplasmic RNA was prepared and hybridization reactions (0.1 ml) were performed using the SV40 E-strand probe (500 cpm/reaction). After 36 hr at 37° the samples were digested with nuclease S_1.

dominate even at permissive temperature, as we have already observed (4).

Autogenous regulation has been observed previously in the control of prokaryotic biosynthetic pathways (17), and in the regulation of viral genes. For example, in infections with ts *43* mutants of phage T4, there is overproduction of the gene *43* protein (T4 DNA polymerase) (18) and there is a similar situation with gene 2 of phage M13 (19). In each of these two cases as well as with SV40, the gene controlled must be expressed for viral DNA replication to be initiated. Autogenous regulation of at least some of the genes controlling initiation of viral DNA replication may be general. This type of regulation would allow a virus to have a very efficient early promoter, so that it could initiate infection effectively, and yet allow it to retain the capacity to repress this promoter later in infection, to avoid competing with the expression of late functions.

Since the early promoter(s) of SV40 has not yet been located, it is somewhat speculative to propose a detailed model to explain autogenous regulation of gene *A*. However, the 5′ end of the stable early 19S messenger RNA is complementary to a region at or very near the origin of replication (14, 20). Therefore, early promotion might occur at a position also convenient for controlling DNA replication, possibly at a single site on the viral chromosome. The *A* protein of SV40 binds preferentially to SV40 DNA *in vitro* at or near the origin of replication (10). If the *A* protein binds to the SV40 chromosome at the origin of replication *in vivo*, viral DNA molecules could be diverted from early transcription into DNA replication by means of this single event.

SV40 provides a simple system for studying eukaryotic gene expression and its control, since there are only a small number of early and late messenger RNAs and they can be distinguished easily.

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