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# Expression of Early and Late Simian Virus 40 Transcripts in the Absence of Protein Synthesis

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We examined the synthesis of early and late simian virus 40 (SV40) mRNA's in SV40-infected cells treated with two kinds of protein synthesis inhibitors. SV40 stimulated the synthesis of mRNA's for both large and small tumor antigens in cells pretreated with the drug emetine before the addition of virus. Emetine is a stringent inhibitor of protein synthesis and, thus, protein factors necessary for transcription and processing of these mRNA's probably preexist in the cell. Surprisingly, infection of cells pretreated with the protein synthesis inhibitor cycloheximide stimulated the synthesis of about 10-fold-higher levels of early viral mRNA's than did comparable infections of nontreated cells. This amplification of early viral mRNA steady-state levels is probably not due to inhibition of synthesis of the early *A* gene product since the same degree of drug-specific amplification was seen in SV40 *tsA*-infected cells that were cultured at the nonpermissive temperature. However, the most interesting effect of cycloheximide addition on viral mRNA synthesis was its stimulation of the appearance of late mRNA's in the cytoplasm of cells at early periods of infection. The synthesis of late mRNA's does not appear to require the *A* gene product as late RNAs can be found in the cytoplasm of cells infected with SV40 *tsA* mutants which have been maintained at 41°C and continuously cultured in the presence of cycloheximide.

During the early phase of lytic infection by simian virus 40 (SV40), two viral mRNA's are detected in infected cells (2). It is likely that these two mRNA's are generated by removal of internal RNA sequences by RNA splicing during posttranscriptional processing of a common precursor. The smaller mRNA is about 2,200 nucleotides long and is composed of the two noncontiguous segments mapping at 0.67 and 0.59 and 0.54 and 0.17 map units (see Fig. 1). The larger mRNA is about 2,500 nucleotides long and is composed of the two noncontiguous segments mapping at 0.67 and 0.54 and 0.54 and 0.17 map units (2). The 2,500- and 2,200-nucleotide mRNA's code for small and large T-antigens, respectively (5).

We examined the effect of protein synthesis inhibitors on the accumulation of SV40 mRNA's. In particular, we investigated whether protein synthesis is required for expression of early mRNA's and whether an active *A* gene product is a prerequisite for late mRNA synthesis. Inhibiting protein synthesis at the time of addition of SV40 will affect the synthesis of the two known early viral tumor-specific antigens, T and t. Tegtmeyer et al. (15) proposed that the gene *A* product is autoregulated after showing that the synthesis of the 94,000-dalton polypeptide is stimulated when cells infected with temperature-sensitive mutants in gene *A* (*tsA* mutants) are shifted to the nonpermissive temperature. Furthermore, several studies have demonstrated that a shift of SV40 *tsA*-infected cells from the permissive temperature to the nonpermissive temperature results in the overproduction of early SV40 RNA (8, 12). The *A* gene product, T, has been shown to bind to the region of SV40 DNA containing the early promoter (11, 16) and it has been hypothesized that this binding suppresses early transcription. In *tsA*-infected cells, the T polypeptide product is thermolabile at the nonpermissive temperature; thus, the early promoter is left open for initiation of transcription. These observations suggest that the rate of synthesis of early SV40 mRNA might be higher in infected cells treated with an inhibitor of protein synthesis. We used two kinds of inhibitors of protein synthesis in this work. Studies with a more complete inhibitor of protein synthesis, emetine, have indicated that an early viral gene product is not required for the expression of the two early SV40 mRNA's. We also show that both of the early and late SV40 mRNA's are overproduced in infected cells treated with the protein synthesis inhibitor cycloheximide.

## MATERIALS AND METHODS

**Cell culture and virus strains.** The established African green monkey kidney cell line (CV1) was used

in these experiments. SV40 wild-type strain 777, temperature-sensitive mutant *tsA58*, and SV40 wild-type strain 4554, kindly provided by Peter Tegtmeyer, were grown and assayed in CV1 cells.

**Preparation of cytoplasmic RNA.** Subconfluent cultures of CV1 cells were infected with 10 PFU of SV40 per cell and incubated at the indicated temperature. Cytoplasmic RNA was prepared from infected cells early after SV40 infection by the procedures previously described (1).

**Preparation of $^{32}$P-labeled restriction fragments of SV40 DNA.** Closed circular SV40 DNA labeled in vivo with $^{32}$P was prepared with a specific activity of $5 \times 10^5$ to $10 \times 10^5$ cpm/$\mu$g as described previously (13). Labeled SV40 DNA was digested completely with the restriction endonucleases indicated in the text and figure legends, purified, and ethanol precipitated. *Eco*RI and *Hin*dIII were purified in our laboratory.

**Analysis of viral RNA.** The technique of Berk and Sharp (2) was used to map the size and positions of virus-specific transcripts. The cytoplasmic RNA mixed with the $^{32}$P-labeled restriction fragments of SV40 DNA in 80% formamide (4) was denatured at 60°C for 10 min and annealed at 49°C for 3 h. The concentrations of RNA and [$^{32}$P]DNA in the reaction mixture were 2.5 to 5 mg/ml and 5 to 10 $\mu$g/ml, respectively. The hybridization mixture was treated with nuclease S1 at 45°C for 30 min and analyzed by neutral or alkaline gel electrophoresis as described previously (2). The gel was thoroughly dried on Whatman 3MM filter paper and exposed to X-ray film (Kodak), using an intensifying screen (Du Pont Cronex) for autoradiography.

## RESULTS

The structures of the two early SV40 mRNA's are shown in Fig. 1. When an excess of *Eco*RI-cut DNA was hybridized to early cytoplasmic RNA and the products were analyzed by electrophoresis in agarose gels after treatment with endonuclease S1, the 2,500-, 2,200-, and 1,900-nucleotide bands (produced by S1 cleavage at the splice site) were reproducibly observed on a neutral agarose gel, and the 1,900-, 630-, and 330-nucleotide bands were observed on an alkaline gel.

**Effects of emetine on early cytoplasmic RNA.** When CV1 cells were treated with emetine 15 min before and throughout the course of infection with SV40, the 2,500- and 2,200-nucleotide mRNA's were detected (Fig. 2, lane a). As shown by densitometry of autoradiographs such as Fig. 2, the relative amounts of 2,500- and 2,200-nucleotide mRNA's were similar to those observed in infected cells not treated with the drug (Fig. 2, lane c). Similar results were obtained in cells treated with the drug 1.5 h after infection (Fig. 2, lane b). Treatment with 50 $\mu$g of emetine per ml, which is a more effective inhibitor of protein synthesis than is cycloheximide (7), decreased the rate of protein synthesis to 3% the rate in untreated CV1 cells (data not shown). Most of this residual protein synthesis is due to mitochondria.

**Effects of cycloheximide on early cytoplasmic RNA.** Emetine inhibition of protein synthesis is nonreversible, whereas cycloheximide inhibition is readily reversible, but not as stringent. Similar experiments following the synthesis of early mRNA's in the presence of cycloheximide were performed. Figure 3 shows that treatment with 25 $\mu$g of cycloheximide per ml 15 min before or 1.5 h after infection resulted in the overproduction of both the 2,500- and 2,200-nucleotide mRNA's. The level of early mRNA in cells treated with the drug 15 min before or 1.5 h after infection was about 7- or 10-fold higher, respectively, compared with the level of early mRNA in untreated cells. (This comparison was made by densitometry of autoradiograms of gels such as that of Fig. 3A.) The amount of early mRNA in cells treated with the drug 5 h after infection was increased only about fourfold compared with that detected in untreated cells (data not shown). In all experiments, the ratio of 2,500-nucleotide RNA to 2,200-nucleotide RNA was similar to that detected in untreated cells.

The bands migrating as DNA segments of 1,200 and 890 nucleotides in the gel shown in Fig. 3B were due to hybridization to 19S and 16S late SV40 mRNA's. The synthesis of late mRNA's in cycloheximide-pretreated cells was

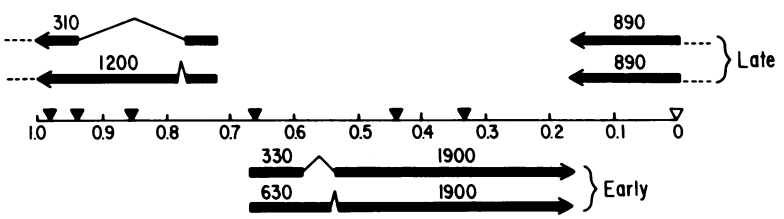


FIG. 1. *Transcription map of SV40. Heavy lines represent the nucleotide sequences included in the early and late mRNA's and indicate the direction of transcription. Numbers above the heavy lines represent the length in nucleotides. Numbers below the narrow lines indicate SV40 genome map units. The cleavage sites of the EcoRI and HindIII endonucleases used in this study are indicated as ▽ and ▼, respectively. Note that the HindIII B fragment is formed by cleavage at 0.655 and 0.43 map unit.*

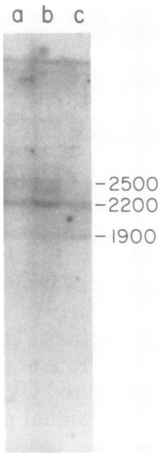


FIG. 2. *S1 gel analysis of early cytoplasmic RNAs isolated from emetine-treated cells. Cytoplasmic RNAs were isolated from cells treated with 50 μg of emetine per ml 15 min before (a) or 1.5 h after (b) infection and from untreated cells (c). The recoveries of total cytoplasmic RNA extracted from emetine-pretreated, emetine-posttreated, and untreated cells were about 0.85, 0.85, and 1 mg/$10^8$ cells, respectively. The RNAs were hybridized with $^{32}$P-labeled EcoRI-cleaved DNA. The hybrids were digested with endonuclease S1, and the digest was analyzed by electrophoresis in a 1.4% neutral agarose gel. To determine the size of the S1-resistant DNA segments, we used HindIII digests of adenovirus type 5 and SV40 DNAs as markers.*

not expected since viral DNA replication and the prerequisite *A* protein synthesis are thought to be required for late transcription (14). To further investigate this phenomenon, we followed the accumulation of early and late mRNA's at different times postinfection. Again, cycloheximide was added to parallel cultures either 15 min before or 1.5 h after infection. Figure 4 (lanes a and b) shows that by 5 h postinfection there was little early mRNA in either control or cycloheximide-treated cells. However, by 10 h postinfection (Fig. 4, lanes c and d), the culture to which cycloheximide had been added yielded a higher abundance of early mRNA, and late mRNA bands appeared. By 15 h postinfection, the untreated culture (Fig. 4, lane f) accumulated large amounts of late mRNA's, whereas the cycloheximide-inhibited culture (Fig. 4, lane g) appeared to continue to accumulate both early and late mRNA's. Thus, the addition of cycloheximide to the culture seemed to prevent the amplification of late mRNA's relative to early mRNA typically seen 10 and 15 h postinfection. However, it is clear that infected CV1 cells treated with 25 μg of cycloheximide per ml, a condition which decreased the rate of protein synthesis by 93% (data not shown), synthesized appreciable levels of late SV40 mRNA's.

**Late mRNA synthesis in SV40 *tsA*-infected cells.** SV40 *tsA* mutants have a thermolabile *A* gene product which is rapidly degraded at the nonpermissive temperature, 41°C (15). SV40 *tsA*-infected cells are defective for synthesis of both viral DNA and late mRNA's when infected and maintained at the nonpermissive temperature (14). However, the addition of 25 μg of cycloheximide per ml to SV40 *tsA*-infected cells induced the synthesis of late mRNA's, even though the cells were cultured at 41°C throughout the experiment (Fig. 5). The

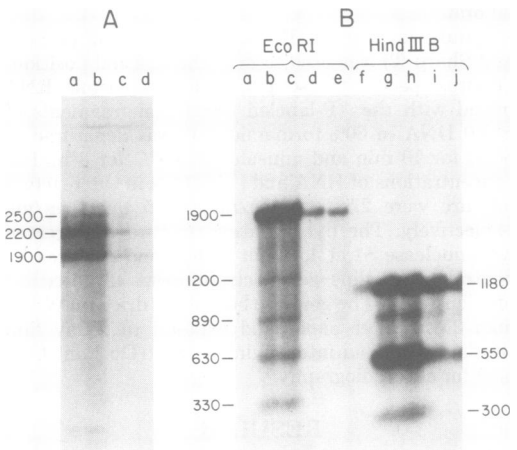


FIG. 3. *S1 gel analysis of early cytoplasmic RNAs isolated from cycloheximide-treated cells. (A) Cytoplasmic RNAs were isolated 10 h after infection from cells treated with 25 μg of cycloheximide per ml 15 min before infection (a), 25 μg of cycloheximide per ml 1.5 h after infection (b), 20 μg of cytosine arabinoside per ml 1.5 h after infection (c), and untreated cells (d). The recoveries of total cytoplasmic RNA extracted from cycloheximide-pretreated, cycloheximide-posttreated, cytosine arabinoside-treated, and untreated cells were about 0.65, 0.80, 1.0, and 1.0 mg/$10^8$ cells, respectively. The RNAs were hybridized to $^{32}$P-labeled EcoRI-cut DNA, and the hybrids were digested with S1 endonuclease, followed by electrophoresis on a 1.4% neutral agarose gel. (B) S1 products of early cytoplasmic RNAs isolated from cells treated with 25 μg of cycloheximide per ml 15 min before (b and g) or 1.5 h after (c and h) infection, from cells treated with 20 μg of cytosine arabinoside per ml 1.5 h after infection (d and i), and from untreated cells (e and j). These RNAs were hybridized to $^{32}$P-labeled HindIII B fragment DNA or EcoRI-cut DNA and were electrophoresed on a 2% alkaline agarose gel. The HindIII B fragment of SV40 spans from 0.655 to 0.43 map unit. [$^{32}$P]DNA was hybridized without RNAs, digested with S1 endonuclease, and electrophoresed (a and f).*

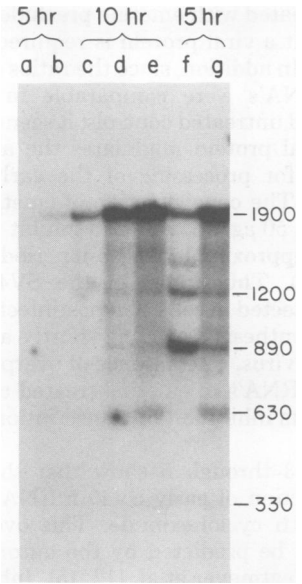


FIG. 4. *S1 gel analysis of the cytoplasmic RNAs isolated from cycloheximide-treated cells at different times postinfection. Cytoplasmic RNAs were isolated 5, 10, or 15 h after infection from cells infected with SV40 strain 777 at 10 (b, d, and g) or 100 (e) PFU/cell and treated with 25 μg of cycloheximide per ml 1.5 h after infection. Control experiments are untreated cells harvested 5 (a), 10 (c), or 15 (f) h after infection with SV40 at 10 PFU/cell. S1 products of the cytoplasmic RNAs hybridized to $^{32}$P-labeled EcoRI-cut DNA were electrophoresed on a 2% alkaline gel.*

$^{32}$P-labeled bands in lanes c and d of Fig. 5 were the result of hybridization of cytoplasmic RNA extracted 10 h postinfection from untreated and treated, respectively, SV40 *tsA*58-infected cells. First, the addition of cycloheximide enhanced the levels of early mRNA in cells infected with *tsA* at the nonpermissive temperature compared with the nontreated control. Second, late SV40 mRNA's appeared in *tsA*-infected cells under conditions where there should be little or no *A* gene activity. Lanes a and b of Fig. 5 show the analysis of RNA from cells 5 h after *tsA* infection. Again, addition of cycloheximide enhanced the accumulation of both early and late mRNA's. Similarly, lanes e and f of Fig. 5 show the RNA synthesized in parallel cultures infected with SV40 wild type and analyzed 10 h later.

**Variation in sequence of early region of SV40 strains.** SV40 *tsA*58 was isolated by Tegtmeyer (14) and has been used extensively in studies of the role of the *A* gene product in viral DNA replication and transcription (3, 6). The parental strain from which *tsA*58 was derived is 4554. Most of the experiments discussed above were done with the SV40 wild-type strain 777. In our initial studies on viral mRNA synthesis after infection with SV40 *tsA*58, the $^{32}$P-labeled strain 777 DNA was used as a probe to detect mRNA's in mutant-infected cells. After digestion of the RNA/DNA hybrids with S1 endonuclease and electrophoresis in an alkaline gel, bands of 1,700, 630, and 330 nucleotides were observed (Fig. 6, lane c). No [$^{32}$P]DNA was found migrating as a 1,900-nucleotide band which was the colinear segment of early mRNA mapping from 54 to 14 map units (Fig. 1). A possible explanation for the generation of the 1,700-nucleotide band is that the two strains of SV40 differed in sequence at a site 200 nucleotides from the 3′ end of the early region and that S1 endonuclease cleaves at this site in RNA/DNA hybrids. That this indeed is the case is shown in Fig. 6, lane i. When SV40 *tsA*58-infected cells were analyzed by hybridization to $^{32}$P-labeled SV40 strain 4554 DNA, the typical set of bands was observed, including the 1,900-nucleotide band. The converse was also seen;

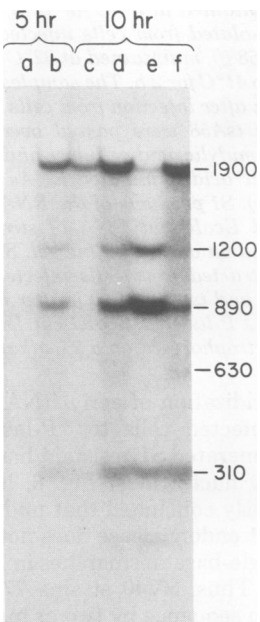


FIG. 5. *S1 gel analysis of cytoplasmic RNAs isolated from tsA-infected cells. CV1 cells were infected with 10 PFU of tsA58 or SV40 wild-type strain 4554 per cell and treated with 25 μg of cycloheximide per ml 1 h after infection. At 5 or 10 h after infection, cytoplasmic RNAs were extracted from tsA58-infected cells treated with cycloheximide (b and d) or 4554-infected cells treated with the drug (f). Control experiments were untreated cells infected with tsA58 (a and c) and 4554 (e). S1 products of the cytoplasmic RNAs hybridized to $^{32}$P-labeled EcoRI-cut DNA of strain 4554 were electrophoresed on a 2% alkaline gel.*

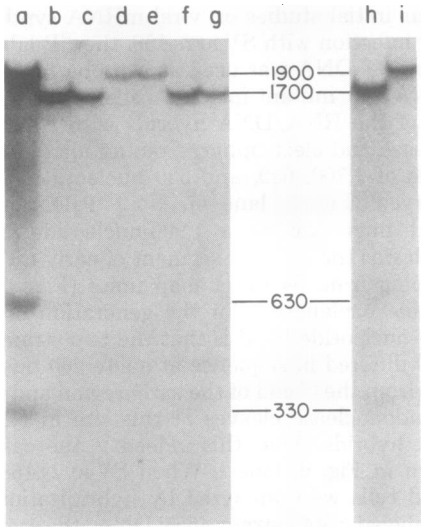


FIG. 6. *Variation in sequence of early region of SV40 strains. Cytoplasmic RNAs were isolated from cells infected with SV40 strain 777 (a), 4554 (b), and tsA58 (c), treated with cycloheximide 1.5 h after infection, and maintained at 37°C for 10 h. Cytoplasmic RNAs were isolated from cells infected with strain 777 (d) or tsA58 (f), maintained at 32°C for 46 h, and then shifted to 41°C for 2 h. The samples of the RNAs extracted 48 h after infection from cells infected with strain 777 or tsA58 were passed over columns of oligodeoxythymidylic acid-cellulose and fractionated to polyadenylic acid-terminated RNAs (lanes e and g, respectively). S1 products of the RNAs hybridized to $^{32}$P-labeled EcoRI-cut DNA of strain 777 were electrophoresed on a 2% alkaline gel. S1 products of the RNAs extracted from cells infected with SV40 strain 777 (h) and tsA58 (a) 10 h after infection and hybridized to $^{32}$P-labeled EcoRI-cut DNA of strain 4554 were electrophoresed on a 2% alkaline gel.*

i.e., the hybridization of early RNA from SV40 strain 777-infected cells to $^{32}$P-labeled strain 4554 DNA generated S1-resistant bands of 1,700, 630, and 330 nucleotides (Fig. 6, lane h). We have previously concluded that under standard conditions S1 endonuclease does not frequently cleave at single-base mismatches in RNA/DNA hybrids (1). Thus, SV40 strains 777 and 4554 must differ in sequence by two or more bases at a site approximately 200 nucleotides from the 3′ end of the early region. The cleavage site was mapped to the 3′ end of the 1,900-nucleotide segment by hybridization with probes cleaved with other restriction endonucleases (data not shown).

## DISCUSSION

Emetine concentrations of 50 μg/ml essentially completely inhibit protein synthesis in mammalian cells within 5 min of addition. Thus, accumulation of both early SV40 mRNA's in cells pretreated with emetine precludes the possibility that a viral protein is required for their synthesis. In addition, since the ratios of the two early mRNA's were comparable in emetine-treated and untreated controls, it seems unlikely that a viral protein modulates the alternative pathways for processing of the early nuclear precursor. The concentration of emetine added to the cell, 50 μg/ml, will also inhibit transcription after approximately a 1.5-h period (data not shown) (9). Thus, most of the SV40-specific mRNA detected in cells 10 h postinfection is due to RNA synthesis occurring shortly after addition of the virus. The absence of overproduction of early mRNA's in emetine-treated cells probably reflects inhibition of transcription by emetine.

Figures 3 through 5 show that there is an overproduction of early SV40 mRNA's in cells treated with cycloheximide. This overproduction would be predicted by the autoregulation model of Tegtmeyer et al. (12, 15). Inhibition of *A* protein synthesis would leave the early SV40 promoter constitutively on and should result in a maximum level of transcription. However, addition of cycloheximide must also be affecting the accumulation of early mRNA's in other ways since treatment of cultures with this inhibitor also enhances the level of early mRNA's in cells infected with an SV40 *tsA* mutant maintained at the nonpermissive temperature. Inhibition of protein synthesis by addition of cycloheximide might have several effects on cellular physiology and, by this means, on the accumulation of mRNA's. In particular, the levels of mRNA's could also be affected by changes in the half-life of mRNA's due to addition of cycloheximide. Such changes in mRNA half-lives after cycloheximide inhibition have been observed in adenovirus type 2-infected cells (M. C. Wilson and J. E. Darnell, Cold Spring Harbor Symp. Quant. Biol., in press). Note that if cycloheximide was increasing the relative concentration of early viral mRNA's by 10-fold by increasing their half-lives, the half-life of these mRNA's in untreated cells would have to be in the 30-min to 1-h range. This is the case for early adenovirus type 2 mRNA's (Wilson and Darnell, in press).

The observation that late mRNA's were synthesized in *tsA*-infected cells cultured in the presence of cycloheximide at 41°C is most surprising. These conditions should preclude the synthesis of a functional *A* gene product and thus preclude viral DNA replication. The latter is commonly thought to be required for the onset of late transcription. However, these results suggest that late mRNA synthesis can occur in the absence of *A* gene activity. One obvious possibility is that the addition of cycloheximide sta-

bilizes late transcription products, which, in nontreated cells during the early stages of infection, are rapidly degraded. However, this implies that transcription from the late region occurs throughout a normal lytic infection, an idea suggested previously on the basis of other types of experiments (3, 6). Of course, other explanations for the synthesis of late mRNA's in cycloheximide-treated cells are equally possible given the lack of knowledge of events controlling transcription of late SV40 mRNA's.

Fredinand et al. (6), Birkenmeier et al. (3), and Parker and Stark (10) have shown previously that transcription complexes prepared from cells infected at the nonpermissive temperature with SV40 *tsA58* incorporate radioisotopes into RNA complementary to both the early and the late regions. As these in vitro complexes do not initiate transcription, this suggests that early transcription complexes exist in vivo which have an RNA polymerase either actively transcribing the late region or staged in a postinitiation state ready to continue transcription of the late region. More recently, Parker and Stark (10) have investigated the regulation of transcription after transfection with SV40 DNA. These studies proved that a virion component was not responsible for suppressing late mRNA synthesis during the early stage of infection. They further propose that the *A* gene product may directly stimulate late transcription. The results reported here only suggest that the *A* gene product is not required for the synthesis and accumulation of late mRNA's.

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