Volume 8 Number 1 1980 Nucleic Acids Research

Evidence for the role of double-helical structures in the maturation of Simian Virus-40 messenger RNA

Nancy H.Chiu, Walter B.Bruszewski and Norman P.Salzman

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20205, USA

Received 22 October 1979

ABSTRACT

Simian Virus-40 infected BSC-1 cells were pretreated with glucosamine and briefly pulsed with [^{3}H]-uridine. The labeling can be halted instantaneously by the addition of cold uridine and glucosamine. Under these pulse-chase conditions, the inhibitory effects of the intercalating agent proflavine on the processing of prelabeled nuclear RNA precursors were examined in vivo. Proflavine inhibits the cleavage of viral nuclear RNA precursors. However, turnover of the mature viral mRNAs in the cytoplasm is not inhibited. The effect of proflavine on processing is not a secondary consequence of its inhibition of protein synthesis. The data suggest that base-paired secondary structures in the primary transcripts are important processing signals in the generation of viral mRNA molecules.

INTRODUCTION

Intramolecular base-paired regions in heterogeneous nuclear RNA have been found in various types of cells (1-6). It has been suggested that these double-stranded (DS) regions may be important in the cleavage and processing of the nuclear precursors. Consistent with this idea is the finding that a double-strand specific bacterial enzyme, RNase III, is involved in site specific cleavage of RNA (7,8). Intercalating agents have been shown to increase the metabolic stability of nuclear RNA precurosrs (9-11). Snyder et al. (9) have shown that proflavine inhibits the cleavage which converts nuclear rRNA from 45 to 32S molecules in vivo. Combining [^{3}H]-uridine prelabeled nuclei and surrogate cytoplasm, Yannarell et al. (11) have shown that proflavine inhibits the processing of messenger as well as ribosomal nuclear RNA precursors.

In the present series of experiments with Simian Virus 40 (SV-40) infected cells, we have used proflavine to study the processing of ^{3}H-labeled viral RNA molecules under defined pulse-chase conditions (12-14). Proflavine has specific effects on processing which may implicate DS regions in the regulation of cleavages of transcripts in the production of mature mRNA.

© IRL Press Limited, 1 Falconberg Court, London W1V 5FG, U.K.

MATERIALS AND METHODS

Cells and virus. A plaque-purified stock of wild-type SV 40 (strain 776) was grown in a BSC-1 line of African green monkey kidney cells with an input multiplicity of 0.01 PFU/cell. For all experiments, BSC-1 cells were grown at 37°C in 150-mm plastic petri dishes with Eagle's medium supplemented with 2 mM glutamine, streptomycin (1.35 mg/ml), penicillin (0.62 mg/ml), chlortetracycline (0.2 mg/ml), mycostatin (500 U/ml) and 10% fetal calf serum (FCS). Confluent monolayers of cells were infected with virus at an input multiplicity of 20 PFU/cell. The infected cells were then grown at 37°C in the same medium except that it contained 2% FCS.

Pulse and chase experiments. At 45-50 h postinfection, cells were preincubated for 80 min at 37°C with 20 mM glucosamine in Eagle's medium containing 2% FCS. The cultures were then rinsed with prewarmed Hanks' balanced salt solution and labeled for 5 min at 37°C with 100 µCi of [^{3}H]-uridine per ml (Schwarz/Mann; 33 Ci/mmol) in Hanks' solution. In the chase experiments, the cultures were rinsed with prewarmed Hanks' solution and incubated with prewarmed fresh Eagle's medium containing 2% FCS, 20 mM glucosamine and 1 mM unlabeled uridine at 37°C for varying time intervals, with or without proflavine. The concentration of proflavine used was 80 µM, or as otherwise specified.

Cell fractionation and RNA extraction. Cytoplasmic and nuclear fractions were prepared by hypotonic buffer solution swelling and Dounce homogenization. The preparation of nuclear and cytoplasmic RNA's was described in detail previously (14).

Sucrose gradient sedimentation. To avoid nuclear RNA aggregation, RNA samples were denatured at room temperature for 30 min in 80% dimethyl sulfoxide, 10% dimethyl formamide, and 10% TES buffer (0.01 M EDTA, 0.01 M Tris-hydrochloride, pH 7.4 and 0.2% sodium dodecyl sulfate [15]). After appropriate dilution, the samples were layered on 5-20% sucrose (Schwarz/Mann; RNase free) gradients containing 0.05 M NaCl in TES buffer. Unless otherwise specified, the gradients were centrifuged in a Beckman SW41 rotor at 39,000 rpm for 5.5 h at 20°C. Tubes were punctured at the bottom, and 0.3-ml fractions were collected.

Hybridization. For hybridzation of [^{3}H]-RNA with total SV-40 DNA, viral DNA was isolated from purified virions, and 30 µg was immobilized on 30-mm nitrocellulose filters (Schleicher and Schuell). For hybridization across sucrose gradients, minifilters were used. Square minifilters (3 by 3 mm) were cut from the 30-mm SV-40 DNA (30 µg/filter) immobilized filters. Blank

minifilters without DNA were cut in the same way from 30-mm blank filters. Hybridizations were performed in 4X SSC (1X SSC = 0.15M NaCl, 0.015 M Na_3 citrate) at 60°C for 18 h, and then the filters were washed, RNase treated and washed again. The filters were dried and counted in toluene-based scintillant.

Labeling of proteins. At 45-50 h postinfection, cells were rinsed with Hanks' balanced salt solution, and then incubated at 37°C in Eagle's medium free of methionine and supplemented with glutamine, streptomycin, penicillin, chlortetracycline, mycostatin and 2% dialyzed FCS. Proflavine was either absent from this medium or present in concentrations varying from 4 to 80 μM. After 3 h, this pre-treatment medium was replaced with labeling medium which contained 50 μCi of [^{35}S]-methionine per ml (Amersham; 745 Ci/mmol), but otherwise it was identical to the pre-treatment medium. Labeling was carried out either in the absence of proflavine or in the presence of the same concentration used in pre-treatment. Incubation in medium containing [^{35}S]-methionine was for 1.5 h at 37°C.

Preparation of extracts from [^{35}S]-methionine labeled cells. The procedure of Schaffhausen et al. (16) was employed. However, cells were lysed in the absence of glycerol. We found that, after proflavine treatment, adequate cell breakage was achieved only when glycerol was omitted from the lysing solution. Cells in one petri dish were lysed in 1 ml of detergent solution. The lysate was then added to 1 ml of the lysing solution supplemented with 20% glycerol. All operations were carried out at 0°C.

Measurement of incorporation of [^{35}S]-methionine into gross proteins in the extracts. 150-μl aliquots of the extracts were dried onto paper scintillation pads (Whatman 3 MM). The pads were washed in cold 10% trichloroacetic acid and rinsed in cold acetone. After drying, the pads were counted in toluene-based scintillant.

Detection of [^{35}S]-methionine labeled T- and t-antigen in the extracts. The procedure was adapted from that of Schauffhausen et al. (16). The precipitation of immune-reactive material was carried out at 0-4°C. One ml of extract was added to 10 μl of normal sheep serum (Flow Laboratories) and 80 μl of 1:1 (vol:vol) suspension of Sepharose beads with covalently bound staphylococcal protein A (Pharmacia) in a solution of 0.14 M NaCl and 20 mM tris-HCl, pH 9.0. This mixture was agitated in a vortex mixer for 30 min and then centrifuged to remove the beads. The supernatant was then mixed with 80 μl of fresh bead suspension and 10 μl of immune hamster serum. The immune serum was obtained from hamsters bearing SV-40 induced tumors. (The

serum was the kind gift of Dr. Evelynne May.) After 30 min of mixing, the entire suspension was transferred to a plastic micro-dispenser tip plugged with glass wool. Seven ml of washing solution (0.5 M LiCl, 1% β-mercapto-ethanol and 100 mM tris-HCl, pH 9.0) was forced through the beads. Air was then forced through the beads to partially dry them. The end of the tip was sealed and 50 µl of electrophoresis sample buffer (5% sodium dodecyl sulfate, 5% β-mercaptoethanol, 0.05% bromphenol blue, 20% glycerol and 100 mM tris-sulfate, pH 8.0) was added to the beads. They were stirred and then elution of protein from the beads was allowed to occur for 15 min at 20°C. Pressure was applied to express the sample buffer from the end of the tip.

The samples were subjected to electrophoresis on a slab gel which consisted of a stacking gel of 5% acrylamide and 0.13% bisacrylamide and a separating gel of 15% acrylamide and 0.087% bisacrylamide (17). Phosphorylase-a (Sigma; M.W. of 94,000 daltons) and myoglobin (Sigma; M.W. of 17,000 daltons) were used to calibrate the gels. Gels were stained with 0.05% Coumassie Brilliant Blue in 25% methanol and 10% acetic acid. Autoradiograms were made of dried gels.

RESULTS

Effect of proflavine on the kinetics of decay of total cellular RNA. Forty-eight hours after infection cells were pretreated with gulcosamine, pulsed with [^{3}H]-uridine for 5 min and then chased. The cells were harvested and isotope incorporated into RNA was determined at different time intervals during the chase. As shown in Fig. 1A, the TCA precipitable labeled RNA decreased with time of chase. The prompt arrest of incorporation of label shows that the unlabeled uridine chase is effective. Fig. 1B shows results of adding proflavine to a concentration of 80 µM at various intervals during a 90-min chase. While all points represent incorporated label in cells harvested after a total of 90-min chase, the values are plotted on the time axis at the time of addition of proflavine. It is apparent that proflavine has arrested the decay of labeled material upon contact with the cells. Even when the chase is continued for relatively long periods after addition of proflavine, labeled RNA is maintained at the level that existed immediately before addition of the drug.

Effect of proflavine on the kinetics of metabolism of cellular and viral nuclear RNA's. Fig. 2A (open circles) and B (open triangles) represent the labeling and decay of the cellular and viral nuclear RNA's. The incorporation of the isotope into cellular and viral nuclear RNA's after 1.5-, 3- and 5-min pulses was 0.8, 1.7, 5.0×10^6 and 2.3, 4.7, 13.3×10^4 cpm, respectively.

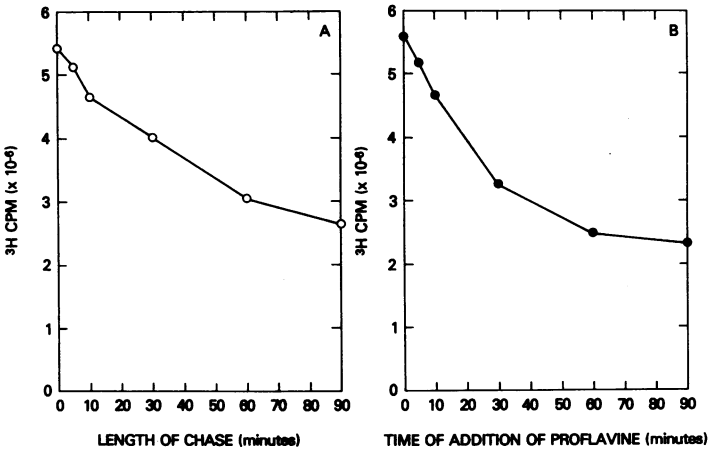

Figure 1. Effect of proflavine on total cellular RNA degradation. (A) Incorporation of radioisotope into infected cells was measured after a 0-, 5-, 10-, 30-, 60- and 90-min chase. The 5-min pulse corresponds to the zero-time chase. (B) Proflavine was added to the pulse labeled infected cells at various times during the chase period. The TCA precipitable label in each sample was measured after a 90-min chase.

Once the chase was initiated, no further uptake of radioisotope occurred and the radioactivity decreased with the time of chase. Curves in Fig. 2A (solid circles) and B (solid triangles) represent the effect of proflavine on the decay of the cellular and viral nuclear RNA's. The chase medium was made 80 μM in proflavine at different times during the chase period and the chase was allowed to continue until 90 min. The TCA precipitable radioactivity of both cellular and viral nuclear RNA's were then determined and both were seen to decrease with time of chase. However, once proflavine was added, the further decay of both classes of nuclear RNA's was immediately inhibited.

Effect of proflavine on the kinetics of labeling of cellular and viral cytoplasmic RNA's. Ribosomal RNA has a half-life of about 20 h. The predominant late 16S and 19S viral mRNA species have half-lives of 2 and 5 h, respectively (14,19). Therefore, the cytoplasmic radioactivity that accumulates from 0-90 min of chase reflects primarily newly synthesized species, and decay of newly synthesized cytoplasmic RNA is not significant. As shown in Fig. 3 (open circles and triangles), the labeling of total and viral cytoplasmic RNA's begins soon after the pulse and reaches a maximum accumulation after a 30-min chase. The closed circle (Fig. 3A) and closed triangle

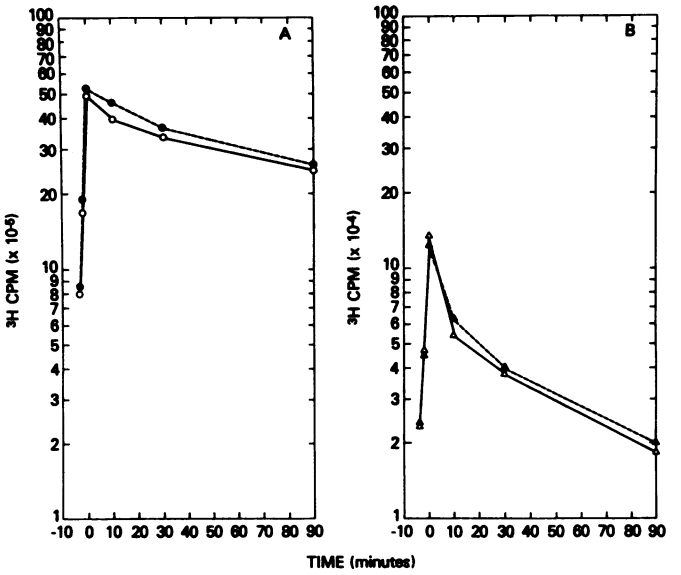

Figure 2. Effect of proflavine on the kinetics of decay of cellular and viral nuclear RNA's. The incorporation of radioisotope per petri dish was measured after 1.5-, 3- and 5-min pulses of [^{3}H]-uridine without proflavine. The 5-min pulse corresponds to the zero-time chase with or without proflavine. After labeling, the decay of the cellular (o——o) and viral (Δ——Δ) nuclear RNAs at various chase time intervals was determined under the chase conditions described in the experimental section. In replicate plates, proflavine was added at different times during the chase period. The proflavine treated samples were all harvested at 90 min after the start of the chase and the amount of label in cellular (●---●) and viral (▲---▲) nuclear RNA was determined. These experimental values are plotted at the time coordinates corresponding to time of addition of proflavine.

(Fig. 3B) curves represent the effect of proflavine on the appearance of cellular and viral cytoplasmic RNA's. The medium was made 80 μM in proflavine at different times during the chase period and each sample was harvested 90 min after the beginning of the chase. Radioactivity contained in cellular and viral cytoplasmic RNA was then determined. Both cytoplasmic RNA's increase with time of chase. However, once proflavine is added, the accumulation of newly synthesized cytoplasmic RNA's is inhibited.

Effect of proflavine on the size distribution of total and viral nuclear RNA's. In the nucleus, the ribosomal precursors are transcribed in the form of 45S RNA and then processed to 32S RNA. With a 5-min pulse of [^{3}H]-uridine and 10 min of chase, the predominant labeled nuclear species is 45S (data not

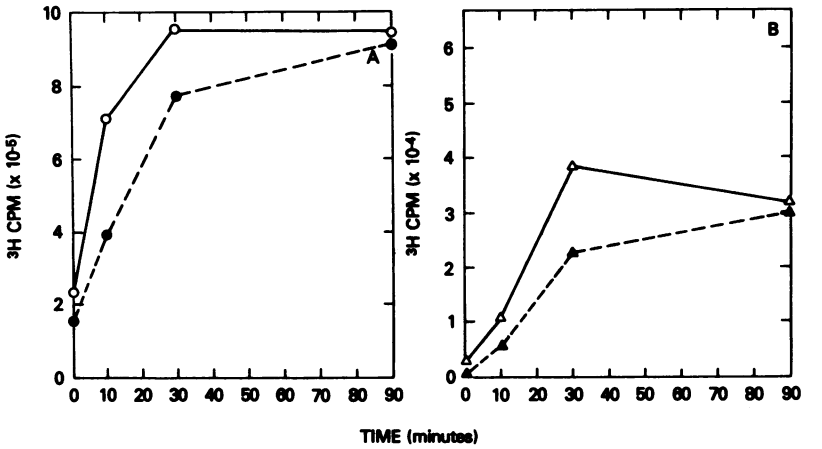

Figure 3. The effect of proflavine on the labeling of cellular and viral cytoplasmic RNA's. The 5-min pulse corresponds to the zero-time chase with or without proflavine. After a 5-min pulse with [^{3}H]-uridine, the labeling of the cellular (o——o) and viral (Δ——Δ) nuclear RNA at various chase time intervals was determined; alternatively, proflavine was added at various chase time intervals and then the labeling of the cellular (•---•) and viral (▲---▲) nuclear RNA of each sample at 90-min of chase was determined. See legend to Fig. 2 for additional details.

shown). After an additional 30-min chase, most of the label sediments as 32S RNA (Fig. 4, open circles). When the chase medium is made 80 μM in proflavine at the end of the initial 10-min chase, 45S RNA accumulates and 32S RNA does not appear during the chase (Fig. 4, closed circles). These results are similar to those reported by Snyder et al. (9).

The size distribution of viral nuclear RNA's after various chase time intervals has been reported in detail previously (14). In brief, with a 5-min pulse and 5-min chase, viral nuclear RNA is accumulated as heterogeneous species between 19 and 26S (data not shown). After a 30-min chase, these larger species are further processed. Besides the predominant 19S RNA there are three additional peaks sedimenting at 17.5, 16 and 2 to 4S (Fig. 5B). After a 90-min chase, 16 to 19S species, which are late-strand mRNA region specific sequences, are transported into the cytoplasm. 2 to 4S RNA's are processed nonspecific viral sequence residues which have a 3-h half-life and are finally degraded in the nucleus (Fig. 5C). When cells were labeled and then chased in the presence of 80 μM proflavine, the large viral precursors accumulated and were not processed (Fig. 5A).

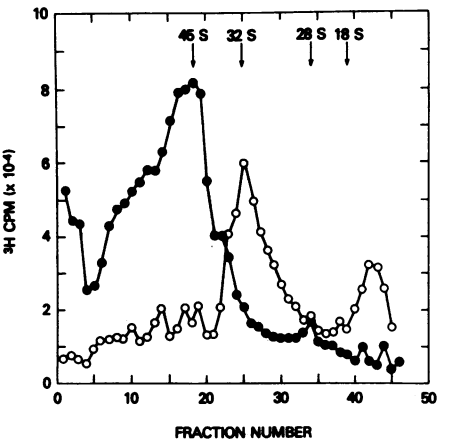

Figure 4. Effect of proflavine on the size distribution of pre-ribosomal nuclear RNA. Cells were pulsed for 5 min with [^{3}H]-uridine and chased for 10 min without proflavine. The cells were then further chased for 30 min in the presence (o——o) or absence (●——●) of proflavine. The nuclear RNA was isolated and analyzed on a 5-20% sucrose gradient in an SW41 rotor for 90 min at 39,000 rpm. The gradients were fractionated and counted.

Effect of proflavine on the occurrence of cytoplasmic viral RNA species. There are at least two main categories of viral late mRNA's, 19S and 16S. The 19S species appears first in the cytoplasm and 16S molecules are detected 20 min later (14,18,19). After a 20-min [^{3}H]-uridine pulse, the predominant labeled species in the cytoplasm is 19S (Fig. 6A). When the 20-min pulse is followed by a 4-h chase, label is present almost entirely in the form of 16S molecules (Fig. 6B). However, when this 4-h chase incubation takes place in 80 μM proflavine, a predominant peak of 16S mRNA does not appear (Fig. 6C). The drug has inhibited the maturation and/or transport from the nucleus of the 16S mRNA. The proflavine has not, however, blocked the degradation of a significant portion of the 19S material, as judged by a decrease in the amount of labeled RNA in the sedimentation peak. Proflavine may thus block specific cleavages of RNA which contribute to maturation of mRNA, but not the turnover of the mature mRNA's in the cytoplasm.

Effect of inhibition of protein synthesis on processing. Fig. 7 shows the effect of 80 μM proflavine on translation in vivo. Infected cells were incubated with various concentrations of proflavine for a total of 4.5 h. During the last 1.5 h of this period, [^{35}S]-methionine was present for incorporation into protein. Whereas 4 and 8 μM proflavine concentrations have no

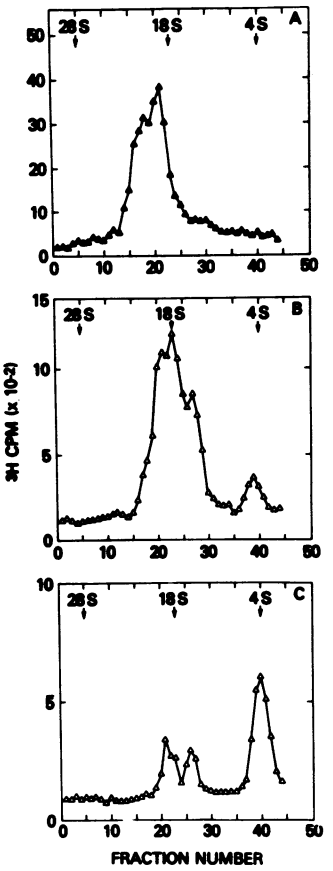

Figure 5. Effect of proflavine on the size distribution of viral nuclear RNA. At 45 h postinfection, cells were pulsed for 5 min with [^{3}H]-uridine and chased for 5 min with glucosamine and unlabeled uridine. (A) Chase was continued in the presence of 80 μM proflavine for 80 min. (B) A 20-min chase was carried out in the absence of proflavine. (C) An 80-min chase was carried out in the absence of proflavine. Nuclear RNA was isolated from each sample; and the samples were analyzed by sucrose gradient centrifugation. Hybridization was carried out on each gradient fraction.

effect on translation, 80 μM proflavine causes substantial inhibition of protein synthesis. We thought it possible that the effects on RNA processing shown in the presence of 80 μM proflavine might be due to inhibition of synthesis of the enzymes that metabolize the RNA species. If this were the case, it should be possible to produce the effects that we attribute to proflavine by using cyclohexamide, an inhibitor of protein synthesis. Cells were labeled with [^{3}H]-uridine and then chased for 4.5 h either in the presence or absence of cycloheximide (100 μg/ml). The drug inhibited amino acid incorporation by 90% within 1 min of addition. The profiles of sedmentation of cellular and viral cytoplasmic RNA were determined. The data presented in Table 1 were obtained by measuring the area under the appropriate peaks of sedimentation profiles. Whereas appearance of mature rRNA species in

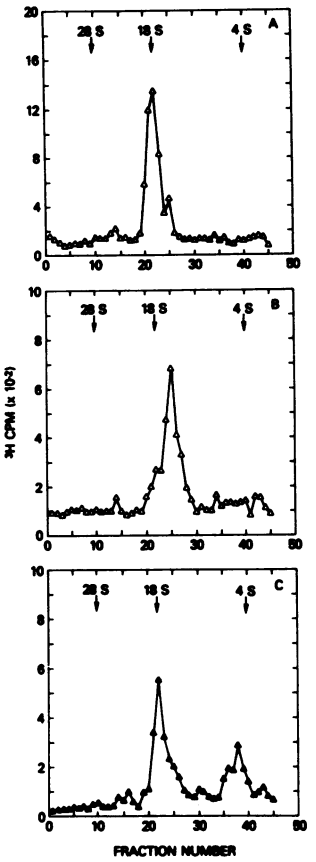

Figure 6. Effect of proflavine on the generation of cytoplasmic viral RNA's. At 45 h postinfection, cells were pulsed for 20 min with [^{3}H]-uridine. RNA was then prepared and analyzed on a sucrose gradient, or the cells were chased for 4 h in the presence or absence of proflavine. Size distribution of viral RNA sequences was determined by hybridization across the sucrose gradients with SV-40 DNA. (A) 20-min pulse; (B) 20-min pulse and a 4-h chase in the absence of proflavine; (C) 20-min pulse and a 4-h chase in the presence of proflavine.

the cytoplasm is inhibited 55% by blocking translation, appearance of 16S SV-40 mRNA is not blocked. During the 4.5 h drug treatment, synthesis of proteins required for viral nuclear RNA processing is apparently not significantly affected.

Specific inhibition of synthesis of T- and t-antigen. A common transcript of the early region of the SV-40 genome (0.67 to 0.14 map units) is spliced in two different ways to generate two cytoplasmic mRNA's which code for the 94,000 dalton (94K) T-antigen and the 17,000-20,000 dalton (17-20K) t-antigen (20). Messenger for T-antigen is formed by excision of a segment of transcript corresponding to 0.54 to 0.59 map unit. Messenger for t-antigen is formed by a small splice between 0.54 and 0.545 map unit. Reddy et al. (21) have found translation termination codons in all three reading frames in the region between 0.54 and 0.59 map unit. Thus, the generation

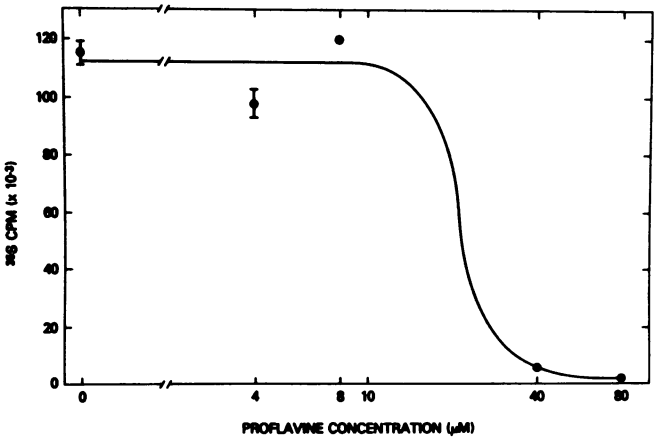

Figure 7. Effect of various concentrations of proflavine on translation in vivo. Rate of translation is reflected by rate of incorporation of [^{35}S]-methionine into acid-insoluble material. Values plotted are the means of three to six determinations. Where the standard error of the mean was relatively large, this value is represented by a vertical bar.

of mRNA for T-antigen must involve deletion of the termination codon. Unspliced messenger could only direct translation of a 17-20K peptide. In vitro translation of SV-40 complementary RNA (cRNA) produces material similar to authentic t-antigen but T-antigen-like material (22) is not formed. Some 60-70K peptides are produced, presumably by translation which initiates beyond the termination codons in the 0.54 to 0.59 region.

It was of interest to determine whether proflavine would specifically inhibit the synthesis of T-antigen, a protein translated from an mRNA which must be spliced. We compared the effect of proflavine on synthesis of T-antigen, gross cellular protein and t-antigen. The mRNA for t-antigen may

TABLE 1. Effect of inhibition of translation on maturation of cellular and viral RNA species

RNA species	Cycloheximide[a]	Control
28S+18S rRNA[b]	45	100
16S SV-40 mRNA[c]	120	100

[a] Relative amount of [^{3}H]-RNA; control is taken as 100%.

[b] Sum of [^{3}H]-RNA in the two sedimentation peaks.

[c] Amounts of SV-40 mRNA are not comparable to amounts of rRNA.

be translated properly without an obligatory requirement for splicing. For these experiments, we used 8 μM proflavine. Fig. 7 shows that in vivo synthesis of total cellular proteins is unaffected by this concentration. When higher concentrations were used, labeling of proteins was so depressed that it was not possible to make meaningful measurements. Fig. 8 shows the extent of labeling of T- and t-antigen during a 1.5-h pulse of [^{35}S]-methionine in the presence and absence of proflavine. It is apparent that the rate of synthesis of T-antigen is greatly reduced while synthesis of t-antigen is unaffected. These data suggest that proflavine specifically inhibits the splicing necessary for maturation of mRNA for T-antigen.

DISCUSSION

Glucosamine can rapidly empty the intracellular UTP pool. Therefore, a true pulse-chase condition can be established when glucosamine is added prior to and after labeling the cells with [^{3}H]-uridine. Glucosamine does not affect normal cellular RNA metabolism (12,13). We have reported the metabolism of pulse labeled late nuclear SV-40 RNA at 45 h postinfection under these

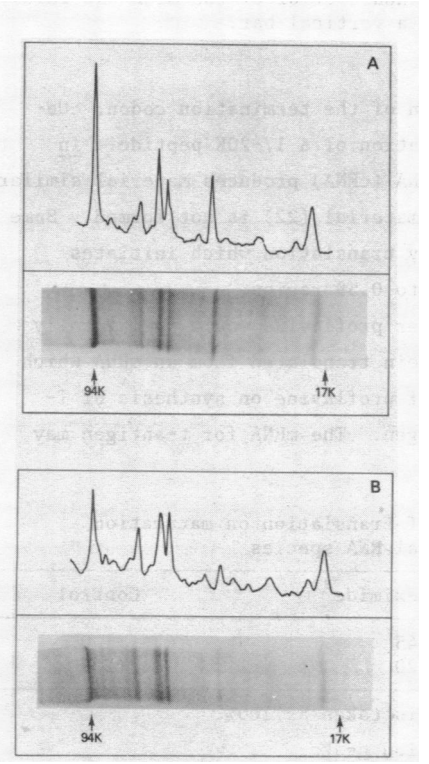

Figure 8. Effect of 8 μM proflavine on rate of synthesis of T-antigen and t-antigen. At 45 h postinfection, cells were treated for 3 h with proflavine and then labeled with [^{35}S]-methionine for an additional 1.5 h in the presence of proflavine. Immune precipitates were then prepared from extracts of the cells and analyzed by polyacrylamide gel electrophoresis. An autoradiogram was made of the dried gel. Densitometric tracings are aligned with the strips of X-ray film from which they were made. Arrows indicate the position of stained molecular weight markers in the gel. (A) control; (B) proflavine.

conditions (14). Using the same technique we now report the effect of the intercalating dye proflavine on the processing of nuclear viral RNA precursors. Since this drug binds to double-helical regions of RNA, it may provide information on the role of the secondary structure in processing of viral nuclear RNA.

There are two main catagories of SV-40 late mRNA species, 16S and 19S. The 19S mRNA's have a number of different 5' ends located between 0.67 and 0.72 map unit. The predominant 19S molecule has a splice between 0.73 and 0.765 map unit (24,26). The 16S mRNA's have 5' ends between 0.67 and 0.75 map unit. The predominant 16S molecule has a splice between 0.76 and 0.95 map unit (23-25). Both 16S and 19S mRNA's have a common 3' end at 0.17 map unit.

During a short period of labeling late in the infectious cycle, viral nuclear RNA's sediment between 19 and 26S and contain sequences specific to both the 16S and 19S messengers (0.735 to 0.16 map unit, clockwise) and to the nonmessenger region (0.16 to 0.735 map unit) of the late strand (14). After various chase periods, these large RNA species are reduced in size and two new catagories of viral RNA's appear in the nucleus, 16 to 19S and 2 to 4S. The 2 to 4S molecules arise by processing of larger species and do not leave the nucleus. The 16 to 19S nuclear RNA's are specific to the late messenger region, and are a mixture of the immediate precursors of both 16S and 19S mRNA.

The intercalating dye, proflavine, preserves high molecular weight RNA's in the nucleus and consequently blocks the appearance of cytoplasmic viral mRNA's (Figs. 1-3). These results may be the result of either an inhibition of RNA processing or an inhibition of the transport of RNA from the nucleus. Since most nuclear RNA's are transported immediately after processing, it seems most likely that proflavine acts by blocking processing. While the nuclear viral RNA's that accumulate in proflavine treated cells are heterogeneous, almost all of the molecules sediment at 19S or greater. Nuclear intermediates such as 17.5, 16 or 2 to 4S molecules which appear in the normal chase (Fig. 5) are not formed in the presence of proflavine. The inhibition of processing by proflavine is not due to a secondary inhibitory effect on protein synthesis. When cycloheximide is added to infected cells it does not have a significant effect on the metabolism of viral RNA's (Table 1). Therefore, it is possible that the effects of proflavine are the consequence of intercalation in secondary structures present in the viral nuclear precursor RNA's which may be important signals for processing. This hypothesis is

consistent with sequencing data of Ghosh et al. (26), which show base pairing between the 5' donor and 3' acceptor sequences that are present in unspliced precursors of both major cytoplasmic viral 16S and 19S mRNA's. However, there may be other mechanisms which function during in vivo experiments and produce an inhibition of RNA processing in the presence of proflavine. The physical chemical studies in which binding properties of pure solution of RNA and proflavine have been examined are clearly different than the protein rich environment when drug binding occurs in the nucleus. These uncertainties can be better resolved when an RNA in vitro assay is available which contains purified substrates and processing enzymes.

When labeled cytoplasmic viral mRNA's are examined after a short labeling period, 19S mRNA is seen first and 16S mRNA appears later. When proflavine is added to labeled cells after 19S mRNA is present in the cytoplasm, but prior to the accumulation of 16S mRNA, the drug blocks the appearance of 16S RNA. The drug has presumably inhibited the maturation or transport of 16S mRNA from the nucleus. The sequencing data of Ghosh et al. (26) indicate that the major cytoplasmic viral 19S RNA has a splice between 0.73 and 0.765 map unit and cannot be the precursor of the major cytoplasmic 16S RNA's which are spliced between 0.76 and 0.93 map unit. We detect 16S RNA in the nucleus after a pulse and a 30-min chase (14), and, by S1 treatment (27) or reverse transcriptase mapping (25), other investigators detect viral 16S RNA in the nucleus. The present finding shows that proflavine blocks appearance of viral 16S cytoplasmic mRNA. While it is well documented that viral 16S RNA is cleaved from some larger RNA species, the site where maturation of viral 16S mRNA occurs is unclear.

The two major early 19S mRNA's share common 5' ends at 0.67 and 3' ends at 0.14 map unit (20). The 19S mRNA which codes for t-antigen has a small gap at 0.54 map unit, while the 19S early mRNA which codes for T-antigen has a gap from 0.54 to 0.59 map unit. A common early viral transcript generates these two mRNA molecules. Cells were treated with 8 μM proflavine, a concentration which has no effect on translation. We found that the drug caused an inhibition of the synthesis of T-antigen but not t-antigen. The sequencing data on the 0.54 to 0.59 region in SV-40 do not suggest an obvious base pairing between the 5' donor and 3' acceptor sequences of 19S T-antigen mRNA precursor (21,29-31). However, our results suggest that some secondary or tertiary structure in the precursor may be important as a splicing signal. We propose that, once proflavine is added, production of T-antigen messenger is blocked and no new mRNA enters the cytoplasm. Synthesis of T-antigen that is

observed results from the mRNA which was present in the cytoplasm at the time of proflavine addition, and which has only partially decayed. Since the synthesis of t-antigen is not affected by proflavine, the production of a small splice in 19S early mRNA of t-antigen may not be influenced by the drug. Alternatively, this small splice may not be a rigid precondition for this RNA to be transported and translated. In an in vitro protein synthesis study with cRNA, Paucha et al. (22) have shown that splicing is not required for the translation of t-antigen. Further characterization of the early nuclear and cytoplasmic viral RNA's in cells treated with proflavine will shed some light on the role of splicing in transport and translation of mRNA's.

REFERENCES

1 Montaignier, L. (1968) C.R. Acad. Sci. D267, 1417-1421
2 Stern, R. and Friedman, R.M. (1970) Nature 226, 612-616
3 Harel, L. and Montaignier, L. (1971) Nature New Biol. 229, 106-108
4 Kimble, P.C. and Duesberg, P.H. (1971) J. Virol. 7, 697-704
5 Jelinek, W. and Darnell, J.E. (1972) Proc. Natl. Acad. Sci. USA 69, 2537-2541
6 Robertson, H.D., Dickson, E. and Jelinek, W. (1977) J. Mol. Biol. 115, 571-589
7 Nikolaev, N., Birge, C.H., Gotoh, S., Glazier, K. and Schlessinger, D. (1975) Brookhaven Symp. Biol. 26, 175-193
8 Robertson, H.D. and Dickson, E. (1975) Brookhaven Symp. Biol. 26, 240-266
9 Snyder, A.L., Kann, H.E. and Kohn, K.W. (1971) J. Mol. Biol. 58, 555-565
10 Brinker, J.M., Madore, H.P. and Bello, L.J. (1973) Biochem. Biophys. Res. Commun. 52, 928-934
11 Yannarell, A., Niemann, M., Schumm, D.E. and Webb, T.E. (1977) Nucl. Acids Res. 4, 603-611
12 Scholtissek, C. (1971) Eur. J. Biochem. 24, 358-365
13 Wertz, G.W. (1975) J. Virol. 16, 1340-1344
14 Chiu, N.H., Radonivich, M.F., Thoren, M.M. and Salzman, N.P. (1978) J. Virol. 28, 590-599
15 Derman, E. and Darnell, J.E. (1974) Cell 3, 255-264
16 Schaffhansen, B.S., Silver, J.E.S. and Benjamin, T.L. (1978) Proc. Natl. Acad. Sci. USA 75, 79-83
17 Laemmli, U.K. (1970) Nature 227, 680-685
18 Weinberg, R.A., Warnaar, S.O. and Winocour, E. (1972) J. Virol. 10, 193-201
19 Aloni, Y., Shani, M. and Reuveni, Y. (1975) Proc. Natl. Acad. Sci. USA 72, 2587-2591
20 Berk, A.J. and Sharp, P.A. (1978) Proc. Natl. Acad. Sci. USA 75, 1274-1278
21 Reddy, V.B., Thimmappaya, B., Dhar, R., Subramanain, K.N., Zain, B.S., Pan, J., Ghosh, P.K., Celma, M.L. and Weissman, S.M. (1978) Science 200, 494-502
22 Paucha, E., Harvey, R. and Smith, A. (1978) J. Virol. 28, 154-170
23 Subramanian, K.N., Dhar, R. and Weissman, S.M. (1977) J. Biol. Chem. 252, 355-367

24 Reddy, V.B., Ghosh, P.K., Lebowitz, P. and Weissman, S.M. (1978) Nucl. Acids Res. 5, 4195-4214
25 Bina-Stein, M., Thoren, M., Salzman, N. and Thompson, J.A. (1979) Proc. Natl. Acad. Sci. USA 76, 731-735
26 Ghosh, P.K., Reddy, V.B., Swinscoe, J., Lebowitz, P. and Weissman, S.M. (1979) J. Mol. Biol., in press
27 Lai, C.J., Dhar, R. and Khoury, G.T. (1978) Cell 14, 971-982
28 Bina-Stein, M., personal communication
29 Fiers, W., Contreras, R., Haegeman, G., Rogiers, R., Van de Voorde, A., Van Heaverswyn, H., Van Herreweghe, J., Volkaert, G. and Ysebaert, M. (1978) Nature 273, 113-120
30 Thompson, J.A., Radonowich, M.F. and Salzman, N.P. (1979), submitted for publication
31 Reddy, V.B., Ghosh, P.K., Lebowitz, P., Piatak, M. and Weissman, S.M. (1979) J. Virol., in press