Transcription Pattern of In Vivo-Labeled Late Simian Virus 40 RNA: Evidence that 16S and 19S mRNA's Are Derived from Distinct Precursor RNA Populations

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The biosynthesis of the two major simian virus 40 mRNA molecules (19S mRNA and 16S mRNA) made at late times in the infective cycle was reinvestigated. By using a modified S1 nuclease technique, we were able to differentiate between pulse-labeled RNA precursor and the spliced mRNA. During a 5-min pulse-labeling with [³H]uridine in vivo, only precursor RNA molecules were detected. Experimental results with polyadenylic acid-selected 5-min pulse-labeled RNA are consistent with the notion that simian virus 40 late RNA can be polyadenylated before final splicing. Finally, 19S mRNA was spliced much more rapidly and appeared more quickly in the cytoplasm than 16S mRNA. Nevertheless, approximately one-half the precursor molecules were destined to become 16S mRNA. Thus, for at least these two viral mRNA's derived from a common transcription unit, the rate of splicing and the rate of nuclear exit are not major determinants of relative mRNA abundance.

Late during simian virus 40 (SV40) lytic infection of monkey kidney cells, two major viral mRNA size classes coding for viral capsid proteins appear in the cytoplasm (1, 15, 19). The two major mRNA's, 16S and 19S mRNA, share the same sequences at their 5' and 3' ends, but differ in the internal sequence as a consequence of differential RNA splicing (9, 10, 16). In the predominant species of 19S mRNA, an untranslated leader of 49 nucleotides from coordinate 0.72 to 0.73 is spliced to a main coding sequence from coordinate 0.78 to 0.17 (Fig. 1) (9). The major species of 16S mRNA contains a leader of 203 nucleotides with the same 5' end as the major 19S mRNA and is spliced to RNA transcribed from coordinate 0.94 to 0.17 (16). The structures of these two major mRNA molecules suggest that they could be produced from a common unspliced precursor RNA by different splicing events. Previous studies by Weinberg et al. (19) and Aloni et al. (1) with a short pulse with [³H]uridine indicated that the 19S mRNA is produced at a faster rate than 16S mRNA. However, it is not clear whether the 19S RNA observed by previous workers during a short pulse with [³H]uridine represents the spliced mature 19S mRNA or a larger polyadenylated precursor. These two RNA molecules differ by only 180 nucleotides out of a total of ~2,550 nucleotides including polyadenylic acid [poly(A)] at the 3' end. Such a small difference in size is certainly beyond the resolution power of sucrose gradient and gel electrophoresis techniques used in the previous studies. In the pres-

ent communication we reinvestigate the kinetics of formation of two spliced late mRNA's by a new technique which can differentiate pulse-labeled unspliced RNA from the spliced products.

S1 nuclease techniques developed by Berk and Sharp (2) have been used successfully to analyze the structure of unlabeled spliced RNA molecules. We adopted and modified this technique for studying pulse-labeled SV40 late RNA molecules. The modified version employs unlabeled DNA probe complementary to the labeled RNA to be studied. Excess probe is hybridized to the labeled RNA, and the resulting hybridization mixture is then digested extensively with both S1 nuclease and pancreatic RNase to remove the unhybridized RNA and DNA. To enhance the detection of small differences in sizes between precursor and spliced mRNA molecules, we used short L (late)-strand DNA probes complementary to the region of RNA near the splice junction.

Specifically, we chose the SV40 DNA fragment 0.67-0.83 obtained by double digestion with *Bgl*I and *Hae*II endonucleases. The L strand of this fragment, complementary to the late RNA transcript, was obtained by strand separation in agarose gel as described by Perlman and Huberman (14).

As shown in Fig. 2, when this probe was hybridized in excess to labeled SV40 late RNA and then digested with S1 and pancreatic nucleases, the sizes of the resultant nuclease resistant RNA-DNA hybrids reflected the sequences present at the 5' end of late RNA molecules

FIG. 1. Schematic representation of SV40 genome with the regions coding for late primary transcript and mRNA noted. The genome of 5,227 nucleotides is by convention divided into 1 unit length beginning at the EcoRI cut site. The coarse interrupted line represents the uncertainty about the location of the 3' terminus for the late primary RNA transcript which lies farther than 1,000 nucleotides from the mRNA 3' terminus. The fine interrupted line represents the variability in 19S leader structure (see reference 5 for more detail) which has been observed for a minority of 16S RNA as well (12).

studied. The nuclease treatment did not cleave the RNA opposite the DNA gap left after digestion of the spliced out region (data not presented). Examination of SV40 DNA and mRNA sequences (6, 9, 10, 11, 17) shows that the major late mRNA species, 19S and 16S mRNA, will form RNA-DNA hybrids of 280 and 200 nucleotides with this probe, respectively, whereas an unspliced late RNA with the same 5' end as the major late mRNA will form a hybrid 500 nucleotides long. The size difference between the RNA-DNA hybrids of the major 19S mRNA and unspliced precursor was now 500 versus 280 nucleotides instead of 2,550 versus 2,370 for the total RNA lengths. The different sizes of RNA-DNA hybrids can be easily separated by electrophoresis in an agarose gel, and the presence of labeled spliced 19S mRNA or its unspliced precursor can be easily distinguished. When SV40 bulk poly(A)-containing late mRNA's labeled with [3 H]uridine were analyzed by this method, two RNA-DNA species of expected lengths were indeed observed (Fig. 3B). Control experiments with labeled mRNA from uninfected cells showed no nuclease-resistant label in the gel. If this RNA is hybridized with a different probe, 1.0/0.0 $\rightarrow$ 0.67, one measures the distance between the 3' end of late mRNA's and coordinate 1.0/0.0 (EcoRI cleavage site). As predicted from the work of other investigators (20), a hybrid length of 875 nucleotides was observed (Fig. 3A).

To examine the biosynthesis of SV40 late RNAs, nuclear and cytoplasmic RNAs were pulse-labeled for 5 min *in vivo* with [3 H]uridine at 48 h postinfection. By using the 0.67–0.83 probe, one detected a single hybrid of 500 nucleotides in length (Fig. 4A). As mentioned above, this was the expected value for an unspliced late RNA with the same, 5' end as the major late mRNA. To exclude the possibility that the hybrid detected was the result of the hybridization with a small completed RNA transcribed from the region 0.67–0.83, we used another probe, 0.67 $\rightarrow$ 1.0/0.0. With this probe a hybrid of 1,450 nucleotides was observed, whereas no significant radioactivity migrating at the position of 500 nucleotides in agarose gel could be seen (Fig. 5). This result indicated that the SV40-specific late RNA labeled for 5 min

FIG. 2. Schematic representation of the technique used in this work. SV40 DNA restriction fragments complementary to the 5' end of late RNA were hybridized in excess with labeled RNA extracted from SV40 infected CV1 cells. The unhybridized portions of DNA and RNA were digested with S1 nuclease as well as pancreatic RNase. The resulting RNA-DNA hybrids were then separated by electrophoresis in agarose gel.

FIG. 3. Gel fractionation of nuclease-resistant hybrid of poly(A)-terminated SV40-infected cytoplasmic RNA and SV40 late template strand of (A) 0/1.0–0.67 and (B) 0.67–0.83. Cells were labeled with [³H]uridine (100 Ci/ml) for 5 h. The cells were fractionated into nuclei and cytoplasm by blending the cells in a Vortex mixer in 0.03 M Tris (pH 8.5)–0.1 M NaCl–0.01 M CaCl₂–25 μ g of Spermidine per ml which was made 0.6% in Nonidet P-40 (8). The cytoplasmic fractions were phenol extracted and RNA ethanol precipitated as described previously (4). The RNA was passed over polyuridylic acid-Sepharose as described by Wilson et al. (18). The single-strand DNA probe was prepared as described by Perlman and Huberman (11). The RNA-DNA hybridization reaction was performed in 200 μ l of 0.2 M NaCl–0.2% sodium dodecyl sulfate–0.01 M Tris (pH 7.4)–0.01 M EDTA at 65°C. To remove the sodium dodecyl sulfate, the solutions were made 0.3 M KCl and centrifuged at 15,000 rpm for 1 min in an Eppendorf centrifuge. The supernatant was then diluted 10 times with 0.3 M NaCl–0.03 M sodium citrate and digested with 2.5 g of pancreatic RNase per ml at 30°C for 30 min. The mixture was phenol extracted and ethanol precipitated with 100 g of yeast RNA as carrier. After centrifugation at 15,000 rpm for 15 min the hybrid was resuspended in 250 μ l of 0.01 M Tris (pH 7.4)–0.01 M NaCl. The sample was diluted 10 times with 0.05 M sodium acetate (pH 5.0)–0.3 M NaCl–0.001 M ZnSO₄ and was digested with 500 U of S1 per ml at 37°C for 30 min. The sample was diluted with an equal volume of 0.01 M Tris (pH 7.4)–0.2% sodium dodecyl sulfate–0.01 M EDTA and phenol extracted. After ethanol precipitation, the samples were analyzed by electrophoresis through 2% agarose gels in 80 mM Tris–90 mM boric acid (pH 8.3)–2.5 mM EDTA. The gels were sliced into 2 mM disks and counted after treatment with Protosol (New England Nuclear).

FIG. 4. Pulse and chase of SV40 late RNA. (A) Fractionation of nuclease-resistant hybrid of nuclear RNA labeled for 5 min with 1 mCi of [³H]uridine in vivo and SV40 late template strand of 0.67–0.83. Nuclei were prepared as in Fig. 3. The nuclei were digested in 0.01 M Tris (pH 7.4)–0.5 M NaCl–0.05 M MgCl₂–0.033 M CaCl₂ with 100 μ g of DNase I per ml for 1 min at 37°C. The mixture was then adjusted to 0.05 M sodium acetate (pH 5.1)–0.01 M EDTA–0.2% sodium dodecyl sulfate and phenol extracted and ethanol-precipitated as described elsewhere (4). Remaining DNA was removed by resuspending the ethanol precipitated pellet in water and diluting with equal volume of 4 M LiCl. After 8 h at 4°C, the RNA was pelleted by centrifugation for 1 h at 10,000 rpm. RNA prepared from 5×10^7 cells was hybridized with single strand of SV40 restriction fragment 0.67–0.83 corresponding to 30 μ g of form I SV40 DNA. (B) Count profile for total cytoplasmic RNA from 10^8 cells labeled for 5 min in vivo with [³H]uridine and analyzed as in A. (C) Parallel sample to that shown in A and B but one which was chased after labeling period. After a 5-min exposure to 1 mCi of [³H]uridine per ml, the 20 mM glucosamine, 5 mM uridine, and 5 mM cytidine, and the cells were incubated an additional 30 min before harvesting (9). Total cytoplasmic RNA from 5×10^7 cells was hybridized as for A and B. For all these samples the material was digested with nucleases as in Fig. 3 and then fractionated on a 1.4% agarose gel.

FIG. 5. Gel fractionation of nuclease-resistant pulse-labeled RNA hybridized to the SV40 late template strand 0.67-1.0/0. The total nuclear RNA from 2×10^7 cells was labeled *in vivo* for 5 min with 1 mCi of [^3H]uridine per ml. The RNA, after LiCl precipitation, was then hybridized with DNA corresponding to 10 μg of SV40 form I DNA. After digestion of the sample with nuclease, the resistant material was fractionated on a 2.0% agarose gel. The dashed line represents an equivalent RNA sample which is not hybridized to DNA but is subjected to digestion with nuclease before fractionation on a 2.0% agarose gel.

has its 5' end located at coordinate 0.72 as the major late RNA. This RNA was synthesized as a species that was at least 1,450 nucleotides long. The data in Fig. 4 suggest that during a 5-min pulse with [^3H]uridine *in vivo*, the newly synthesized SV40 late RNA has not yet undergone final splicing. This RNA would correspond to the unspliced RNA detected by using Berk-Sharp alkaline gel analysis in examining bulk SV40 RNA (12). However, the present technique can not resolve truly unspliced RNAs from RNAs with less than 10% of the nucleotides removed by splicing.

When the poly(A)-containing fraction of the 5-min pulse-labeled RNA was selected by polyuridylic acid-Sepharose chromatography and hybridized to the 0.67-0.83 DNA probe, a single 500 nucleotide hybrid again was detected (Fig. 6). Since the polyuridylic acid column was extensively washed with 10% formamide, a procedure which has been shown to quantitatively remove poly(A)⁻ RNA (16), it is unlikely that

the hybrid observed is due to contamination from poly(A)⁻ fraction. In addition the poly(A)⁻ fraction from a 5-min *in vivo* labeling showed no peak of nuclease-resistant hybridization corresponding to that of either mature mRNA. These results therefore suggest that poly(A) addition can precede RNA splicing. This conclusion is consistent with that arrived at by Lai et al. (12) analyzing bulk unlabeled RNA with the classical Berk-Sharp method.

We then analyzed the fate of newly synthesized SV40 RNA. When RNA was pulse-labeled for 5 min *in vivo* with [^3H]uridine and then chased for 30 min in the presence of 20 mM glucosamine, 5 mM cytidine, and 5 mM uridine, spliced RNA corresponding to the mature mRNA was detected (Fig. 4C). Since during the chase period the specific activity of SV40 nuclear RNA decreased while that in the cytoplasm increased (Table 1), these results strongly suggest that the spliced late mRNA is derived from the unspliced nuclear precursor observed during

FIG. 6. Gel fractionation of nuclease-resistant pulse-labeled poly(A)⁺ and poly(A)⁻ nuclear RNA. RNA was prepared as in Fig. 4. Polyuridylic acid-Sepharose selection was performed after Wilson et al. (16). The column was washed with 10 column volumes of 10% formamide-0.01 M Tris (pH 7.4)-0.01 M EDTA-0.2% sodium dodecyl sulfate before elution. RNA was hybridized to SV40 late template strand 0.67-0.83. (A) Poly(A)⁻ nuclear RNA from 5×10^6 cells labeled for 5 min *in vivo* with [^3H]uridine and hybridized with DNA corresponding to 10 μg of SV40 form I DNA. (B) Poly(A)⁺ nuclear RNA from 5×10^7 cells hybridized with DNA corresponding to 10 μg of SV40 form I DNA. Both samples were subjected to nuclease digestion and then fractionation on 1.4% agarose gels.

the 5-min pulse (Fig. 4A).

Because of the previous reports (1, 4) which suggested that 19S mRNA is produced more rapidly than 16S mRNA but which involved techniques that probably could not distinguish between precursor 19S RNA and mature 19S mRNA, we were encouraged to examine the kinetics of 19S and 16S mRNA appearance in the cytoplasm. We chose not to use glucosamine in these additional studies since glucosamine may alter the kinetics of mRNA synthesis or turnover. The initial finding from the work summarized in Table 2 is that the precursor-sized SV40 RNA species in the cytoplasm is not poly(A) terminated. As work by Sawicki et al. has shown *de novo* polyadenylation in HeLa cells occurs in the nucleus (17). For the cytoplasmic precursor-sized molecule to give rise to mature 19S mRNA and 16S mRNA, it would have to undergo cytoplasmic polyadenylation. Such a biosynthetic step seems quite unlikely although not impossible. More likely is the possibility that the cytoplasmic precursor size represents nuclear leakage. SV40-infected cells may have quite leaky nuclear membranes. By douncing SV40-infected nuclei, it has been possible to recover intact SV40 virions in the cytoplasmic compartment (5, 8). Smaller structures such as

ribonucleoprotein particles might emerge in the cytoplasm even more easily. Although we believe nuclear leakage to occur, we were unable to eliminate the cytoplasmic precursor RNA molecule despite a variety of nucleus-cytoplasm fractionation procedures (data not presented). Thus, we cannot exclude the possibility of a biologically significant role in the cytoplasm for the precursor RNA.

The final interpretation of the kinetics of 16S and 19S mRNA cytoplasmic accumulation is dependent on three facts.

(i) Table 2 shows that after 30 min in the nucleus there is no preferential accumulation of labeled 16S mRNA over labeled 19S mRNA. If one compares the molar ratio after 30 min of labeling for 19S RNA and 16S mRNA in the nucleus to that for both species in the cytoplasm, one can see that there is relatively more 19S than 16S mRNA in the nucleus as compared with the ratio for both RNA species in the cytoplasm. The lack of 16S mRNA in the nucleus during short periods of incorporation with radiochemical label has been observed before (11; J. Aloni, personal communication).

(ii) Workers using two very different approaches (1, 4) have published data which has been used to determine the half-life of 19S mRNA to be 2 to 3 h and that of 16S mRNA to be 5 to 6 h.

(iii) 19S mRNA detected in hybrid to 0.67–0.83 DNA after short labeling times (280 nucleotides) corresponds to the majority of 19S mRNA in bulk mRNA and both must have a leader ~30 to 50 nucleotides in length to accommodate the main coding sequence from 0.78–0.83 (250 nucleotides). Therefore, there can be no conversion of majority 19S mRNA to 16S mRNA, which requires a leader of 200 nucleotides encoded in the genome at 0.72–0.76.

TABLE 1. *Pulse and chase of SV40-specific RNA^a*

Method	Nuclear RNA (cpm)	Cytoplasmic RNA (cpm)
5-min pulse	8,500	200
5-min pulse and 30-min chase with 20 mM glucosamine	5,900	1,500

^a [³H]uridine-labeled RNA corresponding to 3×10^6 cells was hybridized with 1 μ g of SV40 DNA filters at 60°C for 48 h as in (4).

TABLE 2. *Kinetics of 19S and 16S mRNA appearance in the cytoplasm*

RNA	Time of labeling (min)	Glucosamine pretreatment	Duration of glucosamine, uridine, cytidine chase (min)	Poly(A) selected	cpm in precursor	cpm in 280-nucleotide 19S RNA leader	cpm in 200-nucleotide 16S RNA leader	Molar ratio, 19S/16S
Nuclear	5	+		—	10,100	<50	<50	
	30	—		—	4,300	1,280	400	2.4
Cytoplasmic	5	+		—	1,630	<10	<10	
	7	—		+	<10	75	<10	>5.4
	10	—		—	300	230	50	3.3
	30	—		—	1,400	1,600	550	2.0
	45	—		+	<10	780	430	1.3
	5	+	30	—	1,100	870	500	1.2

^a RNA was isolated and hybridized as in Fig. 4. Counts per minute represent total radioactivity, after subtracting background detected in appropriate gel fraction. All samples represent pulse-labeled RNA from 5×10^7 cells except for 45-min Poly(A)⁺, which represents RNA from 2×10^7 cells.

With these three facts as background, the kinetic data for the cytoplasm from Table 2 show that the molar ratio of 19S/16S mRNA changes from >5.4 to 1.2 during 30 min of chase or a time equal to from $\frac{1}{4}$ th to $\frac{1}{2}$ th of the half-life of the mRNA with the shorter half-life (19S mRNA).

The past work (1, 4) which suggested that 19S mRNA is produced at a faster rate than 16S mRNA is both confirmed and extended. There was no preferential retention of 16S mRNA in the nucleus, and only $\frac{1}{4}$ th to $\frac{1}{2}$ th of the less stable mRNA was degraded during 30 min of chase. Thus, there might be a mechanism which prevents the precursor molecules from being converted in the same ratio to 19S mRNA and 16S mRNA at 7 min of pulse-labeling and also after 30 min of chase. Such a mechanism which "protects" 16S mRNA precursor molecules from being made into 19S mRNA is evidence for the existence of distinct precursor pools for 19S mRNA and for 16S mRNA.

The data presented in Fig. 3 and 4 of Chin et al. (4) are consistent with the model of separate precursor pools for 19S and 16S mRNA. They show that in the interval between 15 and 60 min of glucosamine chase (13) after a 5-min pulse with [3 H]uridine, the cytoplasmic 16S SV40 RNA peak increases from 500 to 1,200 cpm. If the nuclear precursor pool is randomly producing both 19S and 16S mRNA, there should be a much larger decrease in radioactivity to account for the 19S mRNA which would also be made between 15 and 60 min of glucosamine chase. However, the nuclear 19S RNA only declines from 1,300 to 600 cpm during this time interval, and the cytoplasmic 19S mRNA increases only from 900 to 1,100 cpm between 15 and 60 min of chase. Consistent with other evidence that 19S mRNA and 16S mRNA are not rapidly turning over (2, 14), there is no substantial loss of radioactivity between the nucleus and cytoplasm at 15 min of chase and the nucleus and cytoplasm after 60 min of chase. This result indicates that there is little short-lived 19S or 16S mRNA. These facts are again in agreement with the notion that 19S mRNA is derived more rapidly from a pool of precursor molecules than 16S mRNA, which is produced more slowly from a separate set of precursor molecules.

If different precursor RNAs did not exist for 19S mRNA and 16S mRNA, one would expect the relative abundance of labeled 19S mRNA and 16S mRNA in the nucleus and cytoplasm initially to reflect the relative rate of splicing and then change according to the relative difference in mRNA half-lives between the two mRNA species. 19S mRNA, which is more than 5.4 times more rapidly spliced and 40 to 50% as

stable as 16S mRNA, would then become the major late SV40 mRNA. Since this does not happen, one can speculate that distinct precursor mRNA pools serve to eliminate the rate of splicing, at least for SV40 late RNA, as a determining step in mRNA abundance. Such a mechanism allows for the observed preferential accumulation of 16S mRNA over 19S RNA in steady-state RNA (1, 20).

In conclusion, how does any apparently single size class of precursor molecules (unspliced 19S) include at least two different precursor pools? One more reasonable possibility is suggested by the work of Canaani et al. which showed that 19S and 16S mRNA are methylated differently (3). If the methylation pattern is set on the precursor molecules, then methylation would be a mechanism for establishment of compartments for precursor molecules which lead independently to 16S and 19S mRNA. Another possibility is a critical difference in the protein make-up of the precursor heterogeneous nuclear riboprotein particle which would direct the RNA molecule to become either 19S mRNA or 16S mRNA. Finally, the present work can not distinguish precursor molecules which have begun the process of splicing. Therefore, the pool of 16S mRNA precursor molecules may accumulate because the splicing process is slower for 16S mRNA than for 19S mRNA.

In any case, this is the first report to suggest that mRNA's generated by the same transcription unit are derived from distinct populations of precursor molecules. Thus, for 16S and 19S mRNA's, the rate of splicing is not an important determinant of relative mRNA abundance.

We appreciate the help and encouragement of J. E. Darnell, Jr. throughout this project. We thank A. English and L. Cousseau for aid in preparing the manuscript.

This work was supported by Public Health Service grants CA 16006-06 and CA 19073-03 from the National Cancer Institute and the American Cancer Society grant VC295H. J.F. held a Public Health Service fellowship (GM05836-02) from the National Institute of General Medical Sciences.

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