Leakage of Nuclear Transcripts Late in Simian Virus 40-Infected CV1 Cells: Quantitation of Spliced and Unspliced Late 19S RNAs

LUIS P. VILLARREAL* AND SUSAN JOVANOVICH

Department of Microbiology and Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262

During the late phase of simian virus 40 infections of CV1 cells, the relative ratios of the spliced to the unspliced RNA molecules of the 19S family were measured. In the cytoplasm, unspliced 19S RNA represented between 1 and 2% of spliced 19S RNA. This ratio could be altered by the use of different methods of RNA extraction such that unspliced RNA was observed at 10 to 20% of spliced RNA. The nuclear ratios of spliced to unspliced 19S RNA were also determined. In contrast to cytoplasmic RNA, nuclear unspliced RNA was several hundred percent that of nuclear spliced 19S RNA. Cytoplasmic unspliced 19S RNA appears to be of nuclear origin, and its presence in the cytoplasmic fraction is due to nuclear leakage during RNA fractionation.

Simian virus 40 (SV40)-infected monkey cells express an early and a late phase of transcription. During the late phase of transcription, two size classes of cytoplasmic RNA molecules are synthesized, 19S and 16S RNAs (1, 17, 28, 29). These RNAs code for the synthesis of the three late viral capsid proteins, VP1, VP2, and VP3 (22-24). Both the 16S and the 19S RNAs are spliced molecules whose structures have been determined. The major 16S RNA consists of a 5'-terminal leader segment between map coordinates 0.721 and 0.760, spliced to a body segment between map coordinates 0.939 and 0.171 (2, 7, 8, 10, 13, 14, 16, 17, 19, 20).

The structure of the 19S RNA has also been reported (10, 18, 27). Initially, it was observed that the cytoplasmic 19S RNA consisted of a family of leaders with 5' coordinates at 0.72, 0.71, 0.695 and 0.69, spliced to a single body segment between 0.765 and 0.17 map units (m.u.) (18). A more detailed analysis, however, by Ghosh et al. reported a significantly more complicated family of cytoplasmic 19S RNAs as well as the presence of an 18.5S RNA (10). At least 15 different cytoplasmic 19S structures were described. Five of these RNAs were unspliced molecules with 5' termini at 0.667, 0.709, 0.714, 0.721, and 0.734 m.u. Only one 19S RNA body was reported containing sequences between map coordinates 0.765 and 0.171. Onto this body are spliced two major and one minor population of leader RNAs. These leader populations are described by the common 3' ends of the leader-body splice junction (see Fig. 1). The leader 3' ends are located at 0.760, 0.730, and 0.714 m.u.

This pattern of cytoplasmic 19S RNA ap-

peared to be overly complicated. Furthermore, the presence of unspliced RNAs in the cytoplasm was in apparent contradiction to other reports (18, 27), some of which implied the necessity of splicing for transporting nuclear RNA into the cytoplasm (12; B. Howard and P. Berg, unpublished data).

To resolve this apparent contradiction, we quantitated the relative levels of spliced and unspliced late 19S RNAs found in the cytoplasm and nucleus of SV40-infected CV1 cells obtained by different methods of fractionating RNA. Two protocols for RNA extraction were used and are henceforth referred to as the isotonic method and hypotonic method.

The protocol for isotonic RNA extraction has been described (11, 15). The cytoplasm was prepared in the presence of 0.15 M NaCl, deoxycholate was omitted, and the Nonidet P-40 concentration was lower (0.2%) than that used in the hypotonic RNA extraction (0.5%). The extracted RNA was then treated with DNase as described below and selected on oligodeoxythymidylic acid-cellulose as described for the polyadenylic acid-terminated sequence (3). Nuclease S1 analysis was as described by Berk and Sharp (4) and modified as follows. DNA fragments (1 μ g, 10^6 cpm) labeled with 32 P_O₄ at the *Eco*RI site (5) were mixed with the appropriate RNA fraction extracted from 10^7 cells (approximately 20 μ g of total unfractionated RNA) and ethanol precipitated. The pellets were dissolved in 40 μ l of deionized formamide, heated to 65°C, and mixed with 10 μ l of 2.0 M NaCl, 0.2 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid); pH 6.4], and 5 mM EDTA. Samples were

FIG. 1. Summary of 19S spliced and unspliced RNA structures seen late in SV40 infection. * Data from Ghosh *et al.* (10).

cooled to 49°C and incubated for 4.0 h. After incubation, samples were cooled on ice, diluted with a 10-fold volume of the appropriate buffer, and digested at 37°C for 45 min with either exonuclease VII or nuclease S1 as described (27). Samples were ethanol precipitated and analyzed on an alkaline agarose gel as described previously (21, 27). Dried gels were exposed on Kodak XR-5 film at -70°C for 14 h with a DuPont Cronex Lightning-Plus intensifying screen for autoradiography. Under these conditions, X-ray film response to ^{32}P is linear (26).

Hybridization and S1 analysis with polyadenylated and nonpolyadenylated cytoplasmic and nuclear RNA extracted by the isotonic method gave the results shown in Fig. 2. Track 1 is a control experiment performed by hybridizing probe to uninfected RNA. No background due to probe-probe hybridization was observed. Cytoplasmic polyadenylated RNA gave the pattern shown in track 5. Only one major band was seen, with a length of 1.23 kilobases (kb). This corresponds to the major 19S body with a 5' coordinate at 0.765 m.u. The 16S body fragment, which would correspond to a 310-nucleotide fragment, was run off the bottom of the gel in an effort to increase the resolution of the various 19S RNA species. Longer exposure of these autoradiograms showed a faint band with a length of 1.47 kb. This corresponds to a contiguous RNA species with a 5' coordinate of 0.720. Analysis of these hybrids by exonuclease VII, as previously described (27), indicated that greater than 95% of the 19S RNA is spliced (data not shown). This pattern of 19S RNAs was not altered when deoxycholate (0.05%) was present during RNA fractionation. The pattern of RNA in the nonpolyadenylated cytoplasmic fraction was very similar to that described above (Fig. 2, track 4). Again, the major species corresponded to the 19S body fragment (1.23 Kb), with a minor band at 1.47 kb. Nuclear RNA patterns are shown in tracks 2 and 3 of Fig. 2. Nuclear polyadenylated

FIG. 2. Alkaline agarose gel analysis of DNA-RNA hybrids digested with nuclease S1. RNA was extracted 48 h after infection with SV40 at a multiplicity of infection of 10 to 20 by the isotonic method. Fractionated RNA was hybridized to $^{32}\text{PO}_4$ end-labeled SV40 DNA probe cut at the *EcoRI* site and then cut again with *BclI*. After nuclease S1 digestion and alkaline agarose gel analysis, gels were dried and autoradiographed. Track 1, uninfected CV1 RNA; track 2, SV40-infected nuclear nonpolyadenylated RNA; track 3, SV40-infected nuclear polyadenylated RNA; track 4, SV40-infected cytoplasmic nonpolyadenylated RNA; track 5, SV40-infected cytoplasmic polyadenylated RNA; track 6, SV40 DNA markers.

RNA (track 3) showed three major species with lengths of 1.23, 1.47, and 1.75 kb. The 5' coordinates of the RNAs were 0.765, 0.720, and 0.665 m.u., respectively. In general, the 1.23-kb and 1.47-kb species were the most abundant.

The nonpolyadenylated nuclear RNA (track 2) gave a band pattern similar to that of nuclear polyadenylated RNA. In the nonpolyadenylated RNA, the unspliced 1.47-Kb band (0.720 m.u.) was almost always the most abundant species. Other minor species were generally observed in both the polyadenylated and nonpolyadenylated nuclear RNAs. All of these minor bands were larger than the 19S body species (1.23 kb). Some of this RNA was longer than the DNA probe used (even when a linear *EcoRI*-cleaved DNA was used), indicating the presence of nuclear RNA transcripts that were contiguous for more than one unit length of the SV40 DNA. Others have reported large unspliced nuclear polyadenylated transcripts with 5' termini at 0.64 and 0.59 m.u. (18). We have also occasionally observed such molecules, but at very low levels.

Extraction of RNA by the hypotonic method was as follows. Cells were washed twice with a cold solution of Tris-buffered saline, then once

with a cold solution of 10 mM Tris-hydrochloride (pH 7.5) containing 10 mM NaCl and 5 mM $MgCl_2$ (low-salt buffer). The buffer was removed by aspiration and replaced with the same solution plus 0.5% Nonidet P-40 and 0.05% deoxycholate, 1.0 ml per 100-mm culture dish. The cell monolayers were allowed to swell and lyse on ice for 5 min. Cells were then scraped off with a rubber policeman. Cell aggregates were dissociated by repeated rapid pipetting of the cell suspension through a Pasteur pipette. The nuclei were removed by centrifugation in an International centrifuge (3,000 rpm, 3 min). The cytoplasmic supernatant was decanted and subjected to a second centrifugation to remove residual nuclei. This supernatant was decanted and mixed with an equal volume of 0.4 M NaCl, 0.1 M Tris-hydrochloride (pH 8.0), 20 mM EDTA, and 1% sodium dodecyl sulfate (extraction buffer) and extracted twice with an equal volume of water-saturated phenol-chloroform (1:1). The nuclear pellet was suspended in a 15-fold volume of low-salt buffer with Nonidet P-40 and deoxycholate, then subjected to a second centrifugation. This nuclear pellet was again suspended and pelleted. This pellet was suspended in a 10-fold volume of low-salt buffer and mixed with an equal volume of extraction buffer. The DNA was sheared by vigorous shaking and extracted twice with phenol-chloroform. The nuclear and cytoplasmic extracts were precipitated with 2 volumes of ethanol. The precipitates were washed with 70% ethanol, drained, and suspended in a small volume of 20 mM Tris (pH 7.5), 10 mM NaCl, and 10 mM $MgCl_2$. DNase I, freed of RNase by iodoacetate treatment (31), was added (20 μ g of DNase per mg of nucleic acid) and incubated at room temperature for 30 min. Then, 0.5 volume of 80 mM Tris (pH 8.0), containing 50 mM EDTA, 400 mM NaCl, and 0.5% sodium dodecyl sulfate, was added, and the mixture was extracted with an equal volume of phenol-chloroform, followed by precipitation with 2 volumes of ethanol.

RNA extracted by the hypotonic method gave alkaline agarose gel patterns as shown in Fig. 3. Track 4 is the pattern obtained from the hybridization of *Eco*RI end-labeled DNA to cytoplasmic polyadenylated RNA. In contrast to the pattern obtained by the isotonic extraction, the levels of unspliced 19S RNA now appeared substantially increased. Although the 1.23-kb band was still the major species, the 1.47-kb band was very much increased. Many other less abundant species were also seen, all of them larger than the 1.23-kb 19S body. Of interest was the presence of very large RNA that protected the full length of the probe. The presence of this very

FIG. 3. *S1* analysis of SV40-infected cell RNA extracted by the hypotonic method. DNA-RNA hybrid analysis was performed as described in Fig. 2 except that the RNA was extracted by the hypotonic method. Track 1, nuclear nonpolyadenylated RNA; track 2, nuclear polyadenylated RNA; track 3, cytoplasmic nonpolyadenylated RNA; track 4, cytoplasmic polyadenylated RNA.

large RNA was variable from one preparation to another. The nonpolyadenylated cytoplasmic fraction gave a pattern very similar to the polyadenylated fraction (track 3). Again the 19S body (1.23 kb) was the major species, but the unspliced RNAs were present in elevated levels, and very large transcripts were present as well. Polyadenylated nuclear RNA is shown in track 2. Here the major species was the unspliced 1.47-kb band. It is of interest that this band seemed equally abundant in the cytoplasmic and nuclear fractions. Very large transcripts were also seen in the nuclear polyadenylated RNA. Nuclear nonpolyadenylated RNA gave a pattern similar to the nuclear polyadenylated fraction (track 1).

Densitometer tracings were obtained from the autoradiograms. To insure that the autoradiographic patterns were true representations of the RNA patterns, hybridization conditions were chosen such that DNA was in excess of RNA (1 μ g of DNA probe was hybridized to the RNA fractions from 10^7 cells). To ensure a linear response of the X-ray film to the dried radioactive gels, several exposure times were examined.

Peak areas from the densitometer tracings were determined by plotting peaks on uniform-weight bond paper, and each peak was cut out and weighed. The area under the major 19S body fragment (1.23 kb) was assigned the value of 100%, and the areas under the other peaks were determined relative to this value. The results of this analysis for cytoplasmic polyadenylated RNA are shown in Table 1. RNA extracted by the isotonic method generally showed

TABLE 1. *Cytoplasmic ratios of unspliced to spliced 19S RNAs^a*

RNA molecule	Ratios (%)			
	Isotonic extraction		Hypotonic extraction	
	Range	Mean	Range	Mean
Spliced 19S (0.765 m.u.)		100		100
Unspliced 19S (0.720 m.u.)	0.7-3.0	1.8	6.0-24	15
Very large transcripts		<0.1	1.0-20	12

^a Peak areas from the densitometer tracings were determined. The area under the spliced 19S RNA peak (5' end at 0.765) is assigned the value of 100%, and other peak areas are determined relative to this value. These values were obtained from 15 separate RNA preparations.

very low levels of unspliced RNA (1.8%), with levels ranging from 0.7 to 3.0%. In no instance were very large transcripts present at levels greater than 0.1%. However, RNA extracted by the hypotonic method often contained substantial quantities (up to 24%) of unspliced RNA. In general, RNA prepared by this method contained almost 10 times as much unspliced RNA, as well as a variable and sometimes large amount of very large transcripts, indicating that leakage of nuclear transcripts into the cytoplasmic fraction was probably occurring during fractionation. Furthermore, the method of RNA extraction was not the sole factor in allowing nuclear leakage of RNA. RNA prepared very late in infection (about 60 h postinfection) or after a very high multiplicity of infection (50 to 100) by the isotonic method also tended to have higher levels of unspliced RNA in the cytoplasm (data not shown). On the other hand, RNA extracted by the hypotonic method earlier in the infectious cycle (24 h postinfection) did not show very high levels of cytoplasmic unspliced RNA.

Analysis of densitometer data from nuclear RNA extracted by the isotonic method is shown in Table 2. Nuclear polyadenylated RNA contained large amounts of unspliced 1.47-kb RNA, on an average of 110% of the spliced RNA, as well as variable but substantial amounts of larger species, the most abundant being an RNA with a 5' coordinate at 0.665 m.u. In the nuclear nonpolyadenylated fraction, large unspliced RNAs were almost always considerably more abundant than spliced RNA, up to 700% for the 1.43-kb species. The 1.75-kb species and the very large transcripts were also generally more abundant than the spliced 19S RNA. RNA extracted from nuclei washed with deoxycholate (0.05%) retained similar levels of spliced 19S RNA. If matched sets of nuclear and cytoplasmic RNA hybridization were quantitated as described above, unspliced nuclear RNA was found to be

TABLE 2. *Ratios of major 19S nuclear RNAs^a*

RNA 5' coordinates	Ratio (%)			
	Polyadenylated		Nonpolyadenylated	
	Range	Mean	Range	Mean
0.765 (spliced)		100		100
0.720 (unspliced)	87-385	110	63-660	350
0.665 (unspliced)	15-65	40	27-145	120
Very large transcripts	5-50	35	15-240	150

^a Nuclear RNA was prepared by the isotonic method.

about 20 times more abundant than unspliced cytoplasmic RNA on an absolute basis (data not shown). Leakage of much of this RNA into the cytoplasm would be expected to raise the level of unspliced cytoplasmic RNA about 10-fold.

The report of Ghosh et al. indicated that late cytoplasmic 19S RNA was very heterogeneous at the 5' terminus and also that various unspliced 19S RNAs were present in the cytoplasm (10). Canaani et al. also observed 5' heterogeneity (6). Others, however, saw much less heterogeneity and very little unspliced 19S RNA in the cytoplasm (18, 27). The apparent differences between these reports are more quantitative than qualitative. We have observed many of the 19S RNA structures described by Ghosh et al. and are in general agreement with those structures. Our data, however, indicate that unspliced 19S RNAs are of nuclear origin, and that their presence in the cytoplasm is most easily explained by nuclear leakage during RNA fractionation.

The levels of unspliced 19S RNAs in the cytoplasm are very low. On an absolute basis, about 5% of the total unspliced 19S RNAs are cytoplasmic and 95% are nuclear. The relative ratios of spliced to unspliced 19S RNA, however, are dramatically different in the nucleus and the cytoplasm. The nucleus has a ratio of unspliced to spliced RNA several hundred times higher than the cytoplasm. The observation that this ratio can be altered by the method of RNA extraction, the multiplicity of infection, and the time after infection at which RNA is extracted indicates that loss of nuclear integrity probably allows leakage of nuclear transcripts. In support of this is the observation that even under the most leaky extraction conditions, the levels of cytoplasmic unspliced 19S RNA were no higher than nuclear levels. Conversely, levels of spliced cytoplasmic RNA were always much higher than levels of spliced nuclear RNA. The possibility of obtaining some cytoplasmic RNA preparations free of unspliced 19S RNA is strong evidence that unspliced 19S RNA has no required cytoplasmic function. The different methods and conditions of RNA extraction em-

ployed by Lai et al. (18) and Ghosh et al. (10) could well account for the apparent differences in the complexity of the cytoplasmic 19S RNA seen. The significance of the residual unspliced cytoplasmic 19S RNA that is often seen is unclear. Possibly, this RNA represents the level of mitotic cells that have lost their nuclear integrity.

The common difference between spliced and unspliced 19S RNAs is that all of the spliced RNAs have lost the 32-nucleotide sequence between 0.760 and 0.765 (5' ends and 3' ends for many of the spliced and unspliced 19S RNAs are identical; see Fig. 1). An implication is that this 32-nucleotide sequence may be responsible for "holding" the unspliced transcripts within the nucleus and that its removal by splicing allows the RNA to be transported into the cytoplasm. A prediction would be that a deletion of this holding sequence might then allow expression of unspliced cytoplasmic 19S RNA. Such a deletion has recently been constructed and does appear to have this phenotype (R. T. White and P. Berg, unpublished data).

There is, however, an apparent contradiction to this theory. Deletion mutants which have lost the 16S intron (0.760 to 0.939 m.u.) appear unable to transport nuclear RNA into the cytoplasm (13; Howard and Berg, unpublished data). These mutants have also lost the putative 19S holding sequence and might be expected to transport nuclear RNA. It may be that the additional information lost in these 16S intron deletions (0.765 to 0.939 m.u.) has further regulatory functions that affect RNA transport.

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