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Transcription of Simian Virus 40

V. Regulation of Simian Virus 40 Gene Expression

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RNA "exhaustion type" hybridization was used to measure the complementarity of nuclear and cytoplasmic viral RNA to the early (E) and late (L) simian virus 40 (SV40) DNA strands. This type of hybridization measures the amount of labeled RNA complementary to each of the two DNA strands, rather than the fraction of each SV40 DNA strand that is homologous to SV40 RNA. At 48 h after infection, about 5% of the nuclear newly synthesized viral RNA was complementary to the E-strand (− strand) and 95% was complementary to the L-strand (+ strand). This proportion was independent of the labeling time, indicating similar accumulation of the E- and L-RNA transcripts in the nucleus. The nuclear E- and L-viral RNA transcripts sedimented in a similar manner on sucrose gradients. Of the cytoplasmic viral RNA only about 1% was complementary to the E-strand, these molecules sedimenting at 19*S*, whereas 99% were complementary to the L-strand and sedimented at 19*S* and 16*S*. The abundance of E-RNA transcripts in nuclei of cells infected with serially passaged virus was about four times higher than that in nuclei of cells infected with plaque-purified virus; however, the size and proportion of the corresponding cytoplasmic E- and L-RNA transcripts was independent of the type of virus used to infect the cells. According to these results at least two control mechanisms regulate viral gene expression in productively infected cells, one operates at the transcriptional level and the second at the post-transcriptional level.

Recent work from this and other laboratories has provided the following information concerning the transcription of simian virus 40 (SV40) DNA late in the infection of monkey kidney cells.

(i) SV40 DNA is transcribed symmetrically over most, if not all, of its length (1-4); both early (E)- and late (L)-RNA transcripts are present 48 h after infection (11, 17, 23, 29); a major fraction of the newly synthesized viral RNA is self-complementary (1-4) (but the relative abundance of the complementary strands was not known); and a considerable portion from both of the RNA strands is subsequently degraded (1-4).

(ii) The size of the accumulated viral RNA corresponds to about 50% of the double-stranded viral DNA (i.e., about 1.6 to 1.8 $\times 10^6$ dalton; 17, 29). These stable RNA molecules are transcribed from both SV40 DNA strands (17, 23, 29), however they are not self-complementary (1-4).

(iii) Prior to viral DNA synthesis, the stable RNA encompasses sequences present in 48% of the E-strand; subsequent to viral DNA replication the stable RNA encompasses, in addition, sequences present in 52% of the L-strand (18, 30, 37). The regions of the E- and L-strands transcribed into the stable RNA have been localized on the physical map of SV40 DNA and the direction of transcription has been determined (18, 30, 37), but the physical template for the transcription products has not yet been identified.

(iv) The nuclear transcription products include both discrete RNA species and heterogeneous components (4, 28; Y. Aloni, M. Shani, and Y. Reuveni, Proc. Natl. Acad. Sci. U.S.A., in press). The discrete species are represented by distinct peaks sedimenting in sucrose gradients at about 26*S*, 24*S*, and 19*S* (4; Aloni et al., in press). The heterogeneous RNA consists of components sedimenting from 28*S* to more than 60*S* (4, 28, 34, 38; Aloni et al., in press).

(v) The functional cytoplasmic products include two species having sedimentation coefficients of 19*S* and 16*S* (34, 38). These two components are methylated (5), share common nucleotide-sequences (37), and exhibit a precursor-product relationship (Aloni et al., in press).

A 19S viral RNA species accumulates also early in infection and in transformed cells (34, 38).

Despite this body of information the exact mechanism by which the virus regulates the expression of its genes is still unknown. One possibility is that the shift from E- to L-sequences is mediated at the level of transcription (17, 23, 29). An alternative postulator mechanism involves regulated post-transcriptional processing of viral RNA molecules (1–4).

The present work was undertaken to obtain information concerning the biogenesis of the viral RNAs found late after infection, which are complementary to each of the two SV40 DNA strands. Hybridization tests were performed between nuclear and cytoplasmic RNAs labeled for various lengths of time, and separated SV40 DNA strands. We have used here RNA "exhaustion"-type hybridization, which measures the amount of labeled RNA complementary to each of the two DNA strands, rather than the conventional technique which measures the fraction of each SV40 DNA strand that is homologous to SV40 RNA. The relationship between these findings and our present understanding of the regulation of viral gene expression in productively infected cells is also discussed.

MATERIALS AND METHODS

Virus. Two stocks of SV40, strain 777 (15), were used. The plaque-purified stock was produced by infecting BSC-1 cells at a very low multiplicity of infection (an average of 10 PFU per 4×10^6 cells) with a single plaque isolate which had been previously subjected to two sequential plaque-purification procedures. The serially passaged virus stock was produced by infecting BSC-1 cells at a multiplicity of 0.2 PFU/cell with nonplaque-purified strain 777 virus which has been maintained for several years in this laboratory by serial passage at multiplicities in the range 0.1 to 1 PFU/cell. In cells infected at 100 PFU/cell with the serially passaged virus, a fraction of the closed circular viral DNA progeny molecules contains sequences homologous to cell DNA (12, 21).

Infection and labeling of cells. If not otherwise specified, BSC-1 cultures (4×10^6 cells per culture) were infected with 1 ml of plaque-purified virus in Eagle medium with 2% calf serum. After a 2-h period at 37 C for virus absorption, 7 ml of fresh medium was added to each culture. The infected cells were labeled with [5,6-^{3}H]uridine (200 μCi/2 ml of medium per culture, 40 Ci/mmol; The Radiochemical Centre, Amersham, England) for the intervals postinfection outlined.

RNA extraction. At the end of the labeling period, the cultures were washed three times with phosphate-buffered saline (14). To extract RNA from whole cells, the cells were lysed directly by adding 2 ml of sodium dodecyl sulfate (SDS) buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA, 0.1 M NaCl, and 0.5% [wt/vol] SDS) per plate. Cytoplasmic and nuclear fractions were prepared by lysing the cells in phosphate-buffered saline containing 0.5% of Nonidet P-40 (26). The cytoplasmic lysate was carefully removed by low-speed centrifugation at 4 C. The nuclear fraction was washed further with 2 ml of phosphate-buffered saline containing 1.0% of Nonidet P-40 and 0.5% of sodium deoxycholate, and nuclei were collected by low-speed centrifugation (26).

RNA was extracted with phenol-chloroform-isoamyl alcohol (26) at room temperature and collected by ethanol precipitation. The precipitate was resuspended in TKM (0.05 M Tris, pH 6.7, 0.025 M KCl, and 0.0025 M $MgCl_2$) and digested with 25 to 50 μg of DNase per ml (Worthington, RNase-free electrophoretically purified) at 2 C for 60 min. The digest was extracted with SDS-phenol and the extract in either 0.25 M NaCl or SDS buffer was passed through Sephadex G-100 column (1.1 by 55 cm) or fractionated on a sucrose gradient (6), respectively. Similar results were obtained also with RNA extracted by the hot phenol (31) and Pronase-cold phenol methods (1).

Preparation of ^{14}C-labeled SV40 DNA and separation of the strands. To prepare ^{14}C-labeled SV40 DNA, 0.1 μCi of [^{14}C]thymidine ([2-^{14}C]thymidine, 50 mCi/mmol, The Radiochemical Centre, Amersham, England) was added per ml of culture medium 3 h after infection. Seven to 9 days later the virus was purified (11) and the labeled DNA was extracted from the purified virus with phenol-sodium trichloroacetate (12). Component I SV40 DNA was isolated by equilibrium centrifugation in a CsCl density gradient supplemented with ethidium bromide (12), followed by sedimentation through a neutral sucrose gradient (30). The specific activity of the viral DNA was 500 counts/min per μg. SV40 DNA I (120 μg) was converted to single nicked component III by treatment with endonuclease R_1 (a gift of Robert Kamen; 30). Component III DNA (15S) was purified by sedimentation through a 15 to 30% sucrose gradient (6). To prepare unlabeled cRNA, a reaction mixture consisting of 60 μg of SV40 DNA I, 200 U of *Escherichia coli* RNA polymerase (Sigma), 2.4 mg each of ATP, UTP, CTP, and GTP (Sigma), 0.15 M NaCl, 0.04 M Tris (pH 7.9), 0.004 M $MgCl_2$, 0.001 M $MnCl_2$, and 0.0012 M 2-mercaptoethanol was incubated in a final volume of 8 ml at 37 C for 3 h. RNA was extracted by SDS-phenol and chloroform-isoamyl alcohol and collected by ethanol precipitation (26). The RNA was treated with DNase and passed through a Sephadex G-100 column (6). About 1.6 mg of RNA were collected in the void volume.

To separate the DNA strands, 80 μg of DNA III were denatured with alkali (0.2 N, 20 min), neutralized, mixed with 1.6 mg of unlabeled cRNA in 16 ml of 0.05 M NaCl, and incubated at 68 C for 20 min (equivalent to a DNA annealing Cot 0.5×0.2). The sample was then diluted 10-fold with 0.14 M phosphate buffer (pH 6.8) and passed through a hydroxyapatite column (bed volume 5 ml) at 60 C. The column was then washed with 40 ml of 0.14 M

phosphate buffer (pH 6.8) and the eluates were combined. This fraction contained the single-stranded DNA. RNA-DNA hybrids were then eluted from the column by washing with 40 ml of 0.4 M phosphate buffer, pH 6.8 (the radioactivity was equally divided between the two fractions).

The two fractions were concentrated separately by ultrafiltration (Amicon PM-10), followed by dialysis against 0.3 N NaOH and 0.001 M EDTA to remove the cRNA, and then neutralized by dialysis against 0.001 M EDTA, 0.05 M Tris (pH 7.4). The two fractions were self-annealed separately in 1 ml of 1 M NaCl at 68 C for 1 h at a DNA concentration of 30 μg/ml (equivalent to a DNA annealing Cot 0.5 × 1,100).

RNA-DNA hybridization and self-annealing of RNA. Hybridization of ^{3}H-labeled RNA with L- or E-DNA strands was performed in 0.12 ml of 1 M NaCl, 0.01 M Tris (pH 7.4), and 0.001 M EDTA at 68 C for 3 h. At the end of incubation time, the mixture was brought to 1 ml of 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and filtered through membrane filters (Millipore Corp., 25-mm diameter, 0.45-μm pore size). The filters were then washed with 50 ml of 6× SSC at 60 C and incubated in 5 ml of 2× SSC containing 20 μg of pancreatic RNase per ml at 25 C for 1 h. The filters were then washed with 6× SSC as above, dried, and counted. RNA-DNA hybridization with the DNA immobilized on membrane filters (Millipore Corp., 7-mm diameter, 0.45-μm pore size) was carried out as previously reported (1). Self-annealing of the RNA in 60% (vol/vol) formamide, 0.75 M NaCl, 0.5% (wt/vol) SDS, and 0.01 M Tris (pH 7.4) at 37 C, with isolation of the RNase-resistant RNA in 2× SSC, was also reported (1-4).

RESULTS

Hybridization tests with separated SV40 DNA strands. To verify whether E- and L-SV40 DNA strands (see above) contain homogeneous populations of molecules, liquid hybridization tests were performed between excess amounts of the E- and L-DNA strands and ^{3}H-labeled cRNA or ^{32}P-labeled 16S viral RNA. It has been previously shown that the cRNA is synthesized on an E-DNA template (39), whereas the 16S viral RNA is synthesized on an L-DNA template. ^{3}H-labeled cRNA hybridized exclusively with E-DNA strands (Table 1); no detectable hybrids were formed with L-DNA strands. The 16S viral RNA hybridized exclusively with the latter and no detectable hybrids were formed with E-DNA strands. When a mixture of ^{3}H-labeled cRNA and ^{32}P-labeled 16S RNA was used, the two labeled RNAs hybridized with their complementary DNA strands with the same efficiency; about 70% of the ^{3}H-labeled cRNA and 50% of the ^{32}P-labeled 16S RNA were found to be RNase resistant. The relative hybridization efficiency of the two sin-

TABLE 1. *A test for the homogeneity of E- and L-DNA strands*

Origin of labeled RNA	Percentage of input hybridized to:			
	E-strands		L-strands	
	0.05 μg	0.10 μg	0.05 μg	0.10 μg
^{3}H-labeled cRNA[a]	67[b]	71[b]	<0.1[b]	<0.1[b]
^{32}P-labeled 16S viral RNA[c]	<0.1[d]	<0.1[d]	46[d]	46[d]
^{3}H-labeled cRNA + ^{32}P-labeled 16S viral RNA	66[b]	67[b]	44[d]	46[d]
Denatured ^{3}H-labeled cRNA[e]	35	36	37	36

[a] ^{3}H-labeled cRNA prepared as detailed (39) was run through a linear 15 to 30% sucrose gradient and the fractions sedimenting at about 28S were collected. In order to remove L-sequences from the preparation, the ^{3}H-labeled cRNA (10^4 counts/min) was annealed in solution with 0.5 μg of L-DNA strands (see Materials and Methods), and passed twice through membrane filters (Millipore Corp., 0.45 μm), both filtrates being collected.

[b] Measured as ^{3}H-labeled counts per minute.

[c] ^{32}P-labeled 16S viral RNA was obtained by formamide hybridization and elution of cytoplasmic ^{32}P-labeled RNA (labeled with $^{32}PO_4$ for 6 h at 48 h postinfection). The eluted ^{32}P-labeled RNA was run through a sucrose gradient (6) and the 16S peak was collected. About 2,000 counts/min of ^{3}H-labeled cRNA and 1,000 counts/min of ^{32}P were used in each assay (see Materials and Methods).

[d] Measured as ^{32}P-labeled counts per minute.

[e] ^{3}H-labeled cRNA prepared as detailed (39) was incubated in 60% (vol/vol) formamide, 0.75 M NaCl, 0.5% (wt/vol) SDS, and 0.01 M Tris, pH 7.4, at 37 C for 48 h to allow self-annealing to occur (10). After this incubation, the RNA was treated with pancreatic RNase (80 μg/ml in 2× SSC at 37 C for 30 min), extracted with SDS-phenol, and layered on a Sephadex G-100 column (6). The material excluded in the void volume which contained the dsRNA (1) was pooled. About 2,000 counts/min of denatured ^{3}H-labeled cRNA (100 C 5 min in 0.01× SSC and rapid cooling) were used in the assay. Less than 0.1% of the input counts per minute were hybridized when native dsRNA RNA (2) was used.

gle-strand probes was also determined. For this purpose, double-stranded dsRNA from annealed ^{3}H-labeled cRNA was prepared (1). Denatured dsRNA was subsequently used for hybridization with each of the single-strand probes. The denatured ^{3}H-labeled cRNA hybridized with the same efficiency to each strand (Table 1) resulting in about 70% of the total counts per minute input hybridizing with both of the strands. These results indicate that the E- and L-DNA strands contain homogeneous populations of molecules and that the liquid hybridization technique using separated SV40 DNA strands is a reliable method for the determination of the porportion of labeled RNA complementary to each DNA strand. After these preliminary tests we then applied the method to estimate the abundance of the E- and L-RNA transcripts in the nucleus and cytoplasm of productively infected cells.

Sedimentation pattern of pulse-labeled nuclear SV40 RNA. Forty-eight hours after infection with SV40, BSC-1 cells were exposed to [^{3}H]uridine for 20 min, and the nuclear RNA fraction was subjected to sucrose gradient analysis after treatment with DNase. Figure 1 shows that the bulk of the cellular-labeled RNA in the nucleus occurs in the form of high-molecular-weight polidisperse species sedimenting as a broad band heavier than the 28*S* rRNA marker. The profile of viral RNA was determined from the radioactivity in each gradient fraction capable of hybridizing to filters saturated with SV40 DNA. As was shown previously (4; Aloni et al., in press), more than 20% of the viral RNA sediments together with the polidisperse species, but the great majority of virus-specific RNA is distributed as several characteristic peaks between the 28*S* and 18*S* rRNA markers. Essentially, none of the SV40-specific RNA sediments slower than 18*S* RNA (0.7×10^6 dalton; 24) suggesting that extensive degradation did not occur during extraction and analysis of the RNA. The sharpness and reproducibility of the peaks preclude the possibility that they are the result of random degradation. Molecular weight estimates of the viral RNA suggest that the size of the first recognizable viral RNA peak (26*S*) is compatible with a complete SV40 RNA transcript ($\sim 1.5 \times 10^6$). Two other major peaks have sedimentation coefficients of about 24*S* and 19*S*.

Relative proportion of pulse-labeled RNA hybridizing with L- and E-SV40 DNA strands in different fractions of the sucrose gradient. Gradient fractions were pooled as indicated (Fig. 1) and the material in each pool

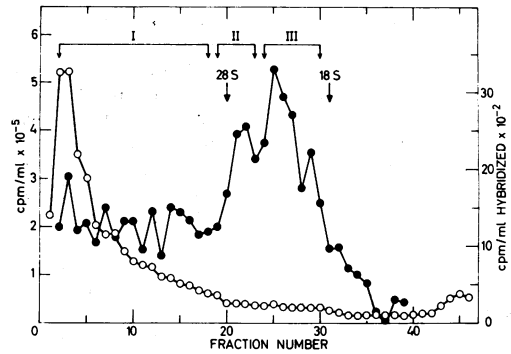

FIG. 1. *Pulse-labeled viral RNA in nuclei of infected cells. At 48 h after infection, 2 × 10^7 BSC-1 cells were labeled for 20 min with [5,6-^{3}H]uridine (0.1 mCi/ml, 40 Ci/mmol). The incorporation was stopped by placing the petri dishes on ice and removing the medium. The cells were washed four times with phosphate-buffered saline (14) and lysed with Nonidet P-40 detergent (26). The nuclei were digested with electrophoretically purified DNase and the RNA was extracted with SDS-phenol. The ^{3}H-labeled RNA was placed on linear gradients of 15 to 30% (wt/wt) sucrose in SDS buffer (6). Centrifugation was for 16 h at 25,000 rpm at 20 C in a Spinco SW27 rotor. Aliquots (50 μl) of each fraction were counted (1). The arrows indicate the positions of ^{32}P-labeled 28S and 18S rRNA markers. Aliquots (50 μl) from each fraction of the gradient in 0.4 ml of 4× SSC were incubated with 7-mm filters containing 0.5 μg of SV40 DNA for 18 h at 68 C. The filters were then treated with RNase (1). The counts per minute hybridized represents the radioactivity remaining bound to the filters; blank filters had less than 5 counts/min. The results are shown as counts per minute per milliliter. With RNA extracted from mock-infected cells, less than 6 counts/min in any one fraction hybridized with SV40 DNA (pattern not shown). Symbols: O, total counts per minute; ●, counts per minute hybridized.*

was collected by ethanol precipitation and centrifugation (6). The plateau levels obtained in hybridization tests between a constant amount of these RNA fractions and increasing amounts of separated strands of SV40 DNA allowed an estimate of the relative proportion of the labeled (i.e., newly synthesized) RNA complementary to the L- and E-SV40 DNA strands. Since the L- and E-RNA transcripts have a similar uridine content (36), the potential RNA transcripts of the two DNA strands would have the same specific activity when labeled with [^{3}H]uridine. Of the labeled viral RNA from each of the three gradient fractions, about 5 to 9% hybridized with the E-strand and 91 to 95% hybridized with the L-strand (Table 2). This indicates that the L- and E-RNA transcripts have similar distributions in the sucrose gradient. A similar conclusion was reached when the

TABLE 2. *RNA-DNA hybridization between ^{3}H-labeled RNA from different fractions shown in Fig. 1 and excess amounts of E- and L-DNA strands*[a]

Origin of ^{3}H-labeled RNA	Input (counts/min 10^{-3})	Percentage of viral ^{3}H-labeled RNA hybridized to:				Percentage of input hybridized to the two SV40 DNA strands
		E-strands		L-strands		
		0.05 μg	0.10 μg	0.05 μg	0.10 μg	
I (>30S)	20	5.6	5.6	94.4	94.4	2.9
II (25 to 30S)	2	9.2	9.1	90.8	90.9	36.0
III (18 to 25S)	2	5.5	5.5	94.5	94.5	39.0

[a] Hybridization was performed as described. The background obtained with 0.10 μg of φX174 DNA (a gift of A. Razin) was less than 0.01% of input counts per minute. The background of the scintillation counter was 5 counts/min, and samples were counted for at least 30 min. For each concentration of E- and L-DNA strands the total counts per minute hybridized to both strands was taken as 100%. The experiment was performed three times and yielded similar results.

nuclear RNA was run first under denaturing conditions, in sucrose gradient containing 85% formamide (25), and fractions were pooled as above, or when the RNA in the pooled fractions was subjected to denaturing conditions (100 C, 10 min in 0.01 M Tris, pH 7.4, 0.001 M EDTA, and rapid cooling) prior to hybridization. These two control experiments indicate that no significant amount of labeled dsRNA molecules existed in the preparation. In addition, when ^{3}H-labeled cRNA was included in hybridization mixture containing E-DNA strands and the corresponding concentration of cold RNA extracted from whole nuclei of infected cells, the efficiency of hybridization was found to be the same as with ^{3}H-labeled cRNA alone. In both cases, about 70% of the input counts per minute were found as RNase-resistant hybrids. These results, together with the findings that increasing concentrations of DNA probes gave the same level of hybridization (see tables), show that the hybridization conditions were of the RNA exhaustion type.

If the molecules present in the 25 to 30S region (cut II in Fig. 1) are viral RNA transcripts of the entire SV40 DNA molecule, this would mean that each strand of the SV40 DNA is transcribed over its entire length as has been previously suggested (1–4). Yet the two types of RNA transcripts differ in proportion; L-RNA is about 20-fold more abundant in the nucleus than the E-RNA. This presumably reflects the rate of formation of the two RNA transcripts rather than their relative metabolic stability (see below).

In these experiments using RNA exhaustion hybridization, about 3% of the RNA sedimenting greater than 30S, and 35 to 40% of the RNA sedimenting less than 30S, formed complexes with SV40 DNA, indicating that the cellular and viral RNA molecules have different *S* value distributions. Under the conditions of hybridization used here, a small fraction of complementary sequences of RNA could be expected to form RNase-resistant duplex structures, but these would be eliminated by filtration through the nitrocellulose membrane.

Relationship between labeling time and the proportion of labeled RNA hybridized with the L- and E-DNA strands. At 48 h after infection with SV40, BSC-1 cells were exposed to [^{3}H]uridine for various periods of time ranging from 2 min to 3 h. RNA was extracted from the nuclei, treated with DNase, and subjected to sucrose gradient analysis. Figure 2A shows that up to a labeling time of 20 min, the bulk of the cellular RNA is found as high-molecular-weight polydisperse species, whereas with labeling times of 45 min to 3 h (Fig. 2B), the pre-rRNA (32S) is the predominant species in the nucleus. Similar profiles of nuclear RNA were also obtained with other cells (26), suggesting that no extensive degradation or aggregation occurred during extraction and analysis of the RNA. In Fig. 2A, the profiles were divided into two portions and fractions were pooled as indicated. In Fig. 2B, only those fractions sedimenting faster than 20S were pooled to avoid possible contamination with mature 19S and 16S viral RNAs which comprise the predominant radioactive species at these labeling times (38). About 95% of the ^{3}H-labeled RNA (in counts per minute) hybridized with the L-strands and 5% hybridized with the E-strands with all pooled fractions tested, indicating that an increased labeling time has no effect upon the proportion of labeled E- and L-RNA transcripts (Table 3). It appears, therefore, that there is no preferential accumulation of either the L- or E-RNA transcripts in the nuclei of infected cells. However, in comparison with cellular RNA the proportion of labeled viral RNA decreases with time. Thus, although more than 14% of the total labeled RNA in a 2-min pulse

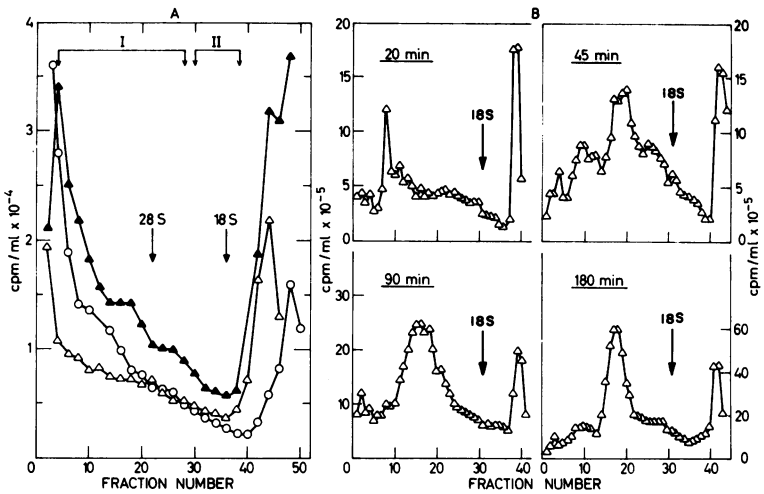

FIG. 2. *Pulse-labeled nuclear RNA of SV40-infected cells. At 48 h after infection, 12 × 10⁷ cells were labeled with [5,6-³H]uridine (0.1 mCi/ml, 48 Ci/mmol) for six different time periods. ³H-labeled RNA was then extracted from the nuclei and run through sucrose gradients as in Fig. 1. Symbols: (A) Δ, 2-min pulse; O, 5-min pulse; ▲, 10-min pulse. (B) Labeling times as indicated in the figure.*

TABLE 3. *RNA-DNA hybridization between ³H-labeled RNA from different fractions shown in Fig. 2 and excess amounts of E- and L-DNA strands*[a]

Labeling time (min)	Origin of ³H-labeled RNA (fractions in Fig. 2)	Input (count/min × 10⁻³)	Percentage of viral ³H-labeled RNA hybridized to:				Percentage of input hybridized to the two SV40 DNA strands
			E-strands		L-strands		
			0.05 μg	0.10 μg	0.05 μg	0.10 μg	
2	I (>20S)	25	3.7	3.7	96.3	96.3	14.0
	II (10 to 20S)	5	2.7	2.2	97.3	97.8	35.0
5	I (>20S)	50	3.6	3.6	96.4	96.4	14.0
	II (10 to 20S)	15	4.7	3.8	95.3	96.2	35.0
10	I (>20S)	120	3.4	3.4	96.6	96.6	7.5
	II (10 to 20S)	30	4.9	5.0	95.1	95.0	37.0
20	>20S	50	7.0	5.4	93.0	94.6	2.7
45	>20S	150	8.3	6.7	91.7	93.3	1.4
90	>20S	150	4.2	4.3	95.8	95.7	1.4
180	>20S	150	4.0	3.2	96.0	96.8	1.3

[a] The conditions used for hybridization and analysis of the RNA-DNA hybrids formed were carried out as described in Table 2. The experiment was performed three times and yielded similar results.

hybridized with the viral DNA strands, this proportion decreased to about 1.5% after 45 min of exposure and remained at this level for up to 3 h of labeling. This suggests a greater accumulation of cellular RNA transcripts than viral RNA transcripts. The accumulating cellular RNA species is the 32S pre-rRNA (26; Fig. 2B).

Sedimentation pattern of labeled cytoplasmic SV40 RNA. The sedimentation profile of the virus-specific RNA extracted from the cytoplasm of infected cells labeled between 42 to 48 h after infection with [³H]uridine as determined by hybridization of each fraction with an excess of immobilized SV40 DNA is shown in Fig. 3. As reported previously (4, 38; Aloni et al., in press), two apparently homogeneous RNA components with sedimentation coefficient of 19S and 16S were resolved.

Relative proportion of cytoplasmic viral RNA hybridized with the L- and E-SV40 DNA strands. Analogous experiments to those described with nuclear RNA were performed on peak fractions from the 19S and 16S cytoplasmic viral RNA components. Total cytoplasmic RNA hybridized almost exclusively with the L-strand (Table 4); hybridizable counts per minute with the E-strand amounted to less than

1.0%. Moreover, no detectable hybridization with the E-strand was found with the ^{3}H-labeled 16S RNA component, whereas about 1.4% of the ^{3}H-labeled 19S RNA hybridized with the E-strand. Since no other recognizable RNA components have been found in the cytoplasm (4, 38; Aloni et al., in press), it appears that all the cytoplasmic E-RNA transcripts present late after infection are concentrated in the 19S peak. This viral RNA species is presumably similar to the 19S viral RNA found early in infection and in transformed cells (34, 38).

It can also be seen from Table 4 that, although about 2.5% of total cytoplasmic ^{3}H-labeled RNA after a 6-h labeling period is viral RNA, about 44 and 28%, respectively, of the labeled RNA in the 16S and 19S peaks is virus specific.

The analysis of both cytoplasmic and nuclear viral RNA (see above) suggests that a preferential accumulation of L-RNA transcripts occurs in the cytoplasm. This could be due either to a different rate of transport of the L- and E-RNA transcripts from the nucleus to the cytoplasm or may reflect the higher stability in the cytoplasm of L-RNA transcripts. The present results, together with previous observations indicating that the cytoplasmic viral RNAs are non-self-complementary (1-4, 28) and that they represent the equivalent of 50% of the viral genome (17, 29), suggest that a post-transcriptional control mechanism is operating by ensuring that only the true viral message will accumulate in the cytoplasm. The nature of this control mechanism is as yet unknown.

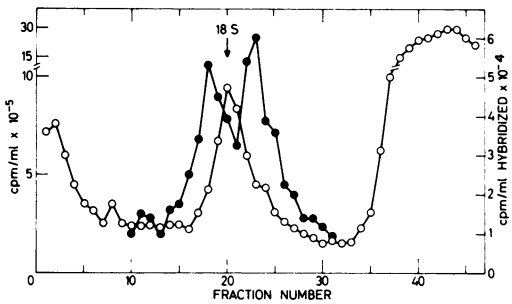

FIG. 3. *Viral RNA in the cytoplasm of infected cells. 10^7 BSC-1 cells were labeled with [5,6-^{3}H]uridine from 42 to 48 h postinfection. Cytoplasmic fractions were prepared with Nonidet P-40 detergent (26) and the ^{3}H-labeled RNA was extracted and placed on a 15 to 30% linear sucrose gradient in SDS buffer (6). Centrifugation was 24 h at 25,000 rpm at 20 C in a Spinco SW27 rotor. Aliquots (5 μl) of each fraction were counted. The counts per minute hybridized in 5 μl of the corresponding fractions were determined as in Fig. 1. The results are shown as counts per minute per milliliter. Symbols: ○, total counts per minute; ●, counts per minute hybridized.*

The nature of the viral dsRNA. We have previously described the ds nature of the RNase-resistant structures formed upon annealing nucleic-acids either from SV40- or polyoma-infected cells (1-4, 10). It was observed in these studies that a relatively high proportion (up to 30%) of the newly synthesized viral RNA can form high-molecular-weight (~10S) dsRNA. This might suggest that the two SV40 DNA strands are transcribed at a similar rate. However, as described above, L-RNA transcripts are about 20 times more abundant in the nucleus than the E-RNA transcripts. The dsRNA formed upon annealing may therefore consist of more unlabeled-labeled hybrid molecules than labeled-labeled hybrid molecules. The following experiments were aimed at clarifying this point.

At 48 h after infection, BSC-1 cells were exposed to [^{3}H]uridine for 20 min. RNA was extracted from either whole cells or nuclei (26). dsRNA was obtained by self-annealing the two labeled RNA preparations at 37 C in 60% formamide with subsequent RNase digestion (1-4, 10). As a control, a portion of the whole cell ^{3}H-labeled RNA was also treated with RNase but with no prior self-annealing. The molecular

TABLE 4. *RNA-DNA hybridization between cytoplasmic ^{3}H-labeled RNA and excess amounts of E- and L-DNA strands*[a]

Origin of ^{3}H-labeled RNA	Input (counts/min × 10^{-3})	Percentage of viral ^{3}H-labeled RNA hybridized to:				Percentage of input hybridized to the two SV40 DNA strands
		E-strands		L-strands		
		0.05 μg	0.10 μg	0.05 μg	0.10 μg	
Total cytoplasmic	25	<1.0	<1.0	>99.0	>99.0	2.4
16S	4	<0.1	<0.1	100.0	100.0	44
19S	3	1.4	1.4	98.6	98.6	28

[a] Total cytoplasmic ^{3}H-labeled RNA, and ^{3}H-labeled RNA of the 19S and 16S peak fractions of Fig. 3 were hybridized with the separated DNA strands and analyzed as described in Table 2. The experiment was performed four times and yielded similar results.

weights of the resultant viral RNAs were then estimated by sucrose gradient centrifugation (Fig. 4). The main peak of RNase-resistant structures for both whole cell and nuclear RNAs sediment at about 4*S*; however, in both preparations there were heavier RNA molecules up to about 18*S*. The ds nature of the fast sedimenting RNA was verified by a test of its susceptibility to RNase as a function of temperature. Hybrids of ^{3}H-labeled cRNA and E-DNA strands were used as a control for RNA-DNA duplexes. Sharp melting profiles, with T_m values of 79 and 70 C in 0.1× SSC, were obtained for the RNase-resistant structures and RNA-DNA hybrids, respectively (Fig. 5). The T_m value for the RNase-resistant structures is in a good agreement with the melting curve of duplex SV40 RNA previously observed (1–4).

The fast sedimenting whole cell and nuclear dsRNA's which represent between 15 to 30% of the rapidly labeled viral RNA (1, 4) were divided into fractions with various *S* values (Fig. 4), and the relation of native and denatured samples towards the L- and E-DNA strands was tested by RNA exhaustion type hybridization (Table 5). No hybridization was registered with either the L- or E-DNA strand, when native RNA was used emphasizing the duplex nature of the RNA. However, when the samples were first denatured (100 C, 5 min in 0.01× SSC and rapid cooling) about 15% of the input ^{3}H-labeled RNA formed RNase-resistant hybrids with viral DNA strands (Table 5). Of the viral RNA sequences, about 20% were complementary to E-strands

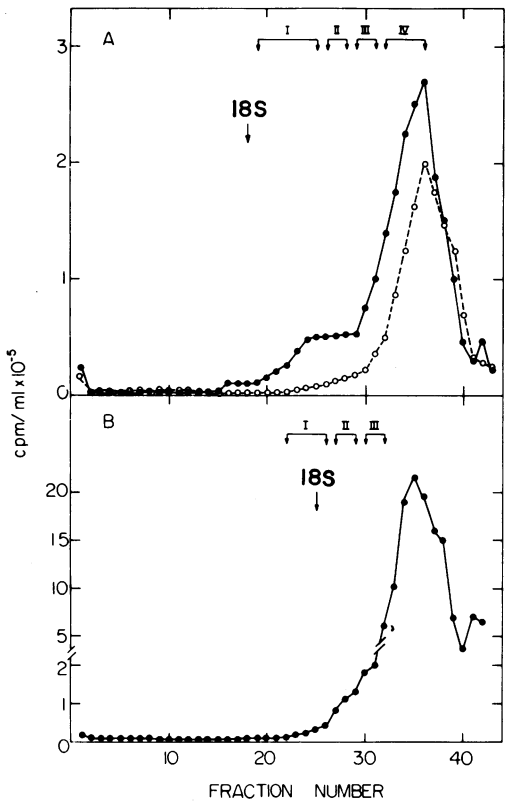

FIG. 4. *Sedimentation of RNase-resistant RNA through sucrose gradients. At 48 h after infection, 4 × 10⁷ BSC-1 cells were labeled for 20 min with [5,6-³H]uridine (0.1 mCi/ml 40 Ci/mmol). ³H-labeled RNA was extracted from whole cells (A) or from nuclei (B) (prepared as in Fig. 1), self-annealed for 36 h in 0.2 ml of 60% (vol/vol) formamide, 0.75 M NaCl, 0.5% SDS (wt/vol), and 0.01 M Tris (pH 7.9), digested with 5 μg of pancreatic RNase per ml in 2× SSC for 10 min at 25 C, extracted with SDS-phenol, and collected by ethanol precipitation. A portion of whole cell RNA was treated with RNase as above with no prior self-annealing. (A) RNA was sedimented through a linear 15 to 30% (wt/vol) sucrose gradient in SDS buffer in a Spinco SW27 rotor for 24 h at 25,000 rpm at 20 C with 18S ³²P-labeled rRNA serving as a sedimentation marker. At the end of the run, 20-μl fractions were collected, precipitated in 10% trichloroacetic acid, and counted. Symbols: ●, self-annealed and treated with RNase; ○, treated with RNase with no prior self-annealing. (B) Nuclear ³H-labeled RNA was self-annealed, treated with RNase, and analyzed as in (A). The sucrose gradient was centrifuged in a Spinco SW27 rotor for 16 h at 25,000 rpm at 20 C.*

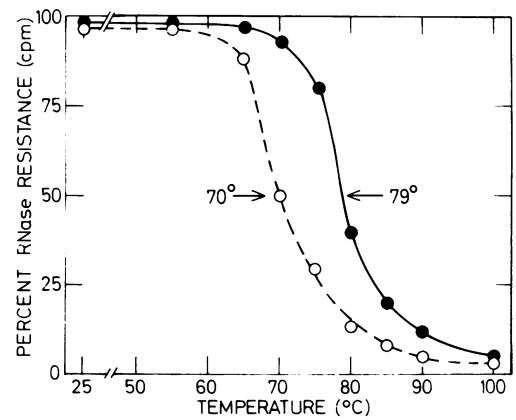

FIG. 5. *RNase susceptibility of self-complementary RNA as a function of temperature. Samples (0.5 ml, 2,000 counts/min) of ³H-labeled RNA from fraction II in Fig. 4A and ³H-labeled cRNA: E-strand hybrids were brought to 0.1× SSC, heated for 5 min at the indicated temperatures, and rapidly cooled. The samples were then brought to 2× SSC, treated with 50 μg of pancreatic RNase per ml for 30 min at 25 C, precipitated in 10% trichloroacetic acid, and counted on membrane filters. The amount of acid-precipitable material before treatment with RNase was assigned a value of 100%. Symbols: ●, ³H-labeled RNA of fraction II in Fig. 4; ○, ³H-labeled cRNA:E-strands hybrids.*

TABLE 5. *Hybridization of self-annealed whole cell and nuclear RNA with E- and L-DNA strands*[a]

Expt	Origin of ^{3}H-labeled RNA	Input (counts/min $\times 10^{-3}$)	Percentage of viral ^{3}H-labeled RNA hybridized to:				Percentage of input hybridized to the two SV40 DNA strands
			E-strands		L-strands		
			0.05 μg	0.10 μg	0.05 μg	0.10 μg	
I	Whole cell RNA	13	3.1	3.3	96.9	96.7	4.1
II	Whole cell RNA after self-annealing RNase treatment and purification on Sephadex G-100	2	0	0	0	0	0
III	As in expt II but after denaturation	2	17.6	18.7	82.4	81.3	14.6
IV	As in expt II but RNase-resistant structures purified on sucrose gradient (Fig. 4A) and then denatured						
	4-7*S*	24	17.2	17.5	82.8	82.5	1.5
	8-9*S*	3	19.2	17.9	80.8	82.1	13.0
	10-11*S*	2	21.3	20.9	78.7	79.1	19.0
	12-16*S*	2	24.0	24.0	76.0	76.0	23.3
V	Nuclear RNA after self-annealing, RNase treatment, purification on sucrose gradient (Fig. 4B), and denaturation						
	13-14*S*	5	28.1	31.9	71.9	68.1	8.0
	15-16*S*	3	35.3	34.6	64.7	65.4	16.2
	17-18*S*	3	38.5	37.7	61.5	62.3	25.8

[a] A portion of whole cell ^{3}H-labeled RNA was used for hybridization before self-annealing. The remainder was self-annealed and the RNase-resistant material was purified by Sephadex G-100 column chromatography (1) or by sedimentation through sucrose gradients (Fig. 4). Denaturation of the dsRNA was in 0.01× SSC at 100 C for 5 min after which it was rapidly cooled and used for hybridization as in Table 2. The experiment was performed twice and yielded similar results.

and 80% were complementary to L-strands (experiment III in Table 5). Moreover, the percentage hybridized with the E-strands increased in proportion to size of the dsRNA (experiment IV in Table 5). The highest proportion (30 to 38%) of hybridization with E-strands was obtained with nuclear denatured dsRNA, the hybridization increasing linearly with the size of the dsRNA (experiment V in Table 5). Whole cell RNA preparations contain the cytoplasmic 19*S* and 16*S* RNA components. The E- and L-19*S* RNA and L-16*S* RNA have molecular weights of approximately 900,000 and 600,000, respectively (37, 38). These viral RNA components are non-self-complementary (1-4, 28), they accumulate in the cytoplasm (4; Aloni et al., in press), and they are very poorly labeled during short labeling periods (4; Aloni et al., in press). It appears, therefore, that the dsRNA formed with whole cell RNA contains many more unlabeled cytoplasmic RNA-labeled newly synthesized RNA than labeled newly synthesized-newly synthesized hybrids. The observation that the largest RNase-resistant nuclear dsRNA's approximate the size of the entire genome and contain labeled E- and L-RNA transcripts in approximately equal amounts indicate that the two types of primary viral RNA products are larger than the mature cytoplasmic RNA components. These data are therefore consistent with the idea that the two SV40 DNA strands are completely or almost completely transcribed (1-4), and that a post-transcriptional control mechanism is then operating to ensure that only the true viral message will accumulate in the cytoplasm.

The proportion of L- and E-RNA transcripts in cells infected with serially pas-

saged virus. It has been shown that infecting cells with serially passaged SV40 stocks leads to the production of substituted and deleted viral DNA molecules (12, 21, 33). Based on an analysis of high-molecular-weight viral RNA in infected cells, it has been suggested that although the serially passaged SV40 stocks integrate at a wide variety of sites in the cellular genome, integration sites for plaque-purified viral DNA molecules may be much more restricted (28). It appears, therefore, that qualitative differences may exist between the populations of the viral DNA templates in cells infected with the two types of viruses. The next series of experiments were performed to determine whether these template differences have any effect on the proportion of E- and L-RNA transcripts present in the nucleus and cytoplasm of infected cells. The ^{3}H-labeled RNA preparations (Table 6) were hybridized with excess amounts of the separated strands of SV40 DNA, and the formation of RNase-resistant hybrids was determined. It can be seen that although the E-RNA transcripts are three to four times more abundant in the nuclei of cells infected with serially passaged virus than in nuclei of cells infected with plaque-purified virus, the size and proportion of the cytoplasmic E-RNA transcript does not differ. The relatively high occurrence of E-RNA transcripts in the nuclei of cells infected with serially passaged virus may result from transcription of substituted DNA molecules that have gained new promoter sites on the E-DNA strands and/or from deleted molecules which have lost promoter sites on the L-DNA strand. Alternatively, the high occurrence of E-RNA transcripts in the nuclei of cells infected with serially passaged virus could result from co-transcription of cell/viral templates, where transcription initiates at cellular promoters and proceeds to transcribe the E-DNA strands. Whatever the reason may be, these results are consistent with the existence of a control mechanism of SV40 gene expression that operates both on the transcriptional and post-transcriptional levels.

DISCUSSION

We have recently shown that late in infection the newly synthesized viral RNA in either SV40- or polyoma-infected cells comprises extensive, if not complete, transcripts of both viral DNA strands (1–4, 10). Symmetrical transcription has been confirmed for polyoma (16) and has also been demonstrated in adenovirus- (27) and herpesvirus-infected cells (13, 19).

Based on these and other results, two major models for SV40 and polyoma transcription during productive infection have been postulated (16): the first suggests that SV40 gene expression is regulated exclusively at a post-transcriptional level. In this case, both strands

TABLE 6. *Hybridization of ^{3}H-labeled RNA of cells infected with plaque-purified or serially passaged virus with E- and L-DNA strands*[a]

Virus used to infect cells	Origin of ^{3}H-labeled RNA	Input (counts/min $\times 10^{-3}$)	Percentage of viral ^{3}H-labeled RNA hybridized to:				Percentage of input hybridized to the two SV40 DNA strands
			E-strands		L-strands		
			0.05 μg	0.10 μg	0.05 μg	0.10 μg	
Plaque purified	Nuclear RNA from cells labeled for 10 min	40	4.3	4.8	95.7	95.2	2.4
	Nuclear RNA from cells labeled for 6 h	75	4.5	4.4	95.5	95.6	2.0
	Cytoplasmic 19S component	5	2.1	2.2	97.9	97.8	45
	Cytoplasmic 16S component	7	0	0	100	100	72
Serially passaged	Nuclear RNA from cells labeled for 10 min	42	12.4	11.3	87.6	88.7	9.5
	Nuclear RNA from cells labeled for 6 h	100	16.8	19.3	83.2	80.7	2.5
	Cytoplasmic 19S component	6	1.4	1.5	98.6	98.5	29.0
	Cytoplasmic 16S component	5	0	0	100	100	60.0

[a] The conditions used for hybridization and the analysis of the RNA-DNA hybrids formed were carried out as described in Table 2. The 19S and 16S components were taken from the peak fractions of gradients as in Fig. 3. The experiment was performed three times and yielded similar results.

of the viral DNA are fully transcribed at similar rates during the whole cycle of infection. Before the onset of viral DNA replication, the entire L-RNA transcript and about 50% of each of the E-RNA transcripts are destroyed in the nucleus and only the remaining halves of the E-RNA transcript are exported to the cytoplasm. Replication of viral DNA leads to an increase in the overall production of E- and L-viral RNA transcripts. However, late in infection, a major portion of the E-RNA transcripts never leaves the nucleus such that the level of E-RNA transcripts in the cytoplasm is unaltered during the entire cycle of infection. The L-RNA transcripts, on the other hand, are only partially degraded and the remaining portion, which comprises about 50% of each molecule, is efficiently exported to the cytoplasm.

The second model suggests that in addition to post-transcriptional controls an additional mechanism determines the frequencies of the transcription of the E- and L-strands during the lytic cycle. Thus, both DNA strands are completely or almost completely transcribed during the entire cycle of infection, but early in the infection the E-DNA strands are transcribed at higher rates whereas late after infection the L-strands are more efficiently transcribed. At both times, processing enzymes degrade the appropriate sequences, and the remaining portions which contain poly(A) at their 3′ ends are exported to the cytoplasm. This model implies that the shift from early to late sequences, which occurs at the onset of viral DNA replication, is regulated at the level of transcription, whereas a post-transcription control mechanism determines the stability of the primary gene product and the fragments of the total transcripts that will reach the cytoplasm.

The experiments described in the present paper were undertaken to distinguish between these two models. For this purpose, RNA exhaustion-type hybridization experiments were performed between the separated strands of SV40 DNA and nuclear and cytoplasmic RNA labeled for various length of times at 48 h postinfection. This type of hybridization measures the amount of RNA complementary to each of the two DNA strands, rather than the fraction of each SV40 DNA strand that is homologous to SV40 RNA. The results obtained indicate that, late after infection, SV40 DNA is symmetrically transcribed over most, if not all, of its length, because nuclear viral RNA molecules with a molecular weight comparable to a complete transcript of the SV40 genome (~26*S*) hybridized to both the E- and L-DNA strands. Moreover, these RNA transcripts were shown to be self-complementary and to form dsRNA molecules which were composed of labeled-labeled molecules. Yet of the two types of viral RNA transcript the L-RNA type is formed about 20 times more efficiently than the E-RNA type, as shown by the fact that 5 and 95% of the newly synthesized viral RNA hybridized with excess amounts of the E- and L-DNA strands, respectively. Furthermore, increasing the labeling time from 2 min to several hours did not change the proportions between the amounts of labeled E- and L-RNA transcripts, suggesting that there is no preferential accumulation of either RNA transcripts in the nucleus of infected cells. The results of the present study therefore favor the second model which emphasizes control of strand selection at the transcriptional level and invokes post-transcriptional processing only in the choice of specific fragments of the total transcripts ultimately transported to the cytoplasm. The first model would be excluded by the present study if the labeled RNA used here would represent the primary RNA transcripts and not the products of RNA processing. This seems to be the case since during the shortest labeling time used (2 min) about 10,000 nucleotides could be synthesized (22), and this number of nucleotides is the equivalent of only two RNA transcripts of the entire SV40 DNA molecule. A different approach would be needed, however, to identify RNA molecules of an extremely short half-life. The identification of such a class of newly synthesized viral RNA is complicated by the delay in equilibration of added labeled precursor with the internal pools of ribonucleotide triphosphates. This delay could be longer than the half-time of synthesis of a single chain in vivo. Experiments done in vitro suggest that such molecules do not exist (Laub and Aloni, unpublished data). It should be emphasized that we have not demonstrated a precursor-product relationship between the various nuclear RNA components and the relation of the symmetric nuclear RNA to the viral mRNA's. The possibility exists, for example, that the symmetric nuclear transcripts are the products of co-transcription of integrated genomes which could be self-complementary or complementary to the true precursors of viral mRNA's. The transcription of integrated genomes may have little to do with mRNA synthesis or they might even be the products of an error process. A different approach would be needed to identify the physical templates of the symmetric viral RNA.

The experiments presented in the present paper show that the symmetrical transcription

mechanism of SV40 DNA in productively infected cells operates differently to the symmetrical transcription mechanism of mitochondrial DNA in HeLa cells (7-9). In the latter system the two DNA strands are transcribed at similar rates (7, 8) and presumably concurrently on the same DNA molecules (8). These differences in the mechanisms of transcription could be related to the marked differences of the molecular weights between the mitochondrial and nuclear RNA polymerases (20, 32).

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