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Transcription of Simian Virus 40. III. Mapping of "Early" and "Late" Species of RNA

(restriction enzymes/RNA-dependent DNA polymerase/strand separation)

JOE SAMBROOK, BILL SUGDEN, WALTER KELLER, AND PHILLIP A. SHARP

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

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ABSTRACT **To determine the orientation of transcription of the E and L strands of DNA from simian virus 40 (SV40), we used linear DNA prepared by cleavage of superhelical viral DNA by endonuclease R·R$_1$ from *Escherichia coli* as a primer·template for DNA polymerase. The resulting molecules, which were labeled only at the 3′ end of each DNA strand, were then cleaved with *Hemophilus parainfluenzae* endonuclease *Hpa* I. The ensuing four DNA fragments, whose locations on the viral genome are known, were separated by electrophoresis, denatured, and hybridized to asymmetric SV40 complementary RNA. From the pattern of hybridization of the fragments containing the labeled 3′ ends, we conclude that transcription of SV40 proceeds in a clockwise direction on the L strand and in a counterclockwise direction on the E strand as drawn on the conventional SV40 map.**

To map the "early" and "late" regions of the viral genome, we extracted RNA from lytically infected cells and hybridized it to the separated strands of the four fragments of ^{32}P-labeled SV40 DNA. Early after infection, RNA complementary to part of the E strand of the contiguous fragments A and C was detected. Late polysomal RNA was complementary to part of the L strand sequences of fragments A and C and to the total L strand sequences of fragments B and D.

During infection of permissive cells by simian virus 40 (SV40), two distinct classes of virus-specific, stable RNA are synthesized. One class is present at both early and late times after infection and is complementary to about 30–35% of the E, or (−), strand of viral DNA. The second class, which becomes detectable only after SV40 DNA synthesis has begun, is complementary to about 65–70% of the sequences of the L, or (+), strand (1–3).

To analyze the pattern of viral transcription in lytically infected cells in more detail, we have used specific fragments of viral DNA. The restricting endonuclease R·R$_1$, isolated from *Escherichia coli* (4), cleaves SV40 DNA at a unique site (5–7) by making single-strand scissions that are four bases apart (8), thereby producing cohesive ends (9). *Hemophilus parainfluenzae* (10) also contains restriction activities (10) that cleave SV40 DNA (11, 12). One of these activities, *Hpa* I, cuts the viral DNA at three sites (13). By digesting SV40 DNA sequentially with endonuclease R·R$_1$ and *Hpa* I, we have isolated four specific fragments of viral DNA, whose location on the SV40 genome is known (11, 13). We have used the fragments to analyze the orientation of RNA synthesis relative to the R·R$_1$ cleavage site on SV40 DNA and to identify the regions of the SV40 genome that generate the "early" and "late" classes of viral RNA.

Abbreviations: Ad2+ND1, an adenovirus type 2 nondefective–simian virus 40 hybrid; SV40, simian virus 40; $C_0t_{1/2}$, DNA phosphate concentration × time required for half reannealing of a denatured DNA solution, expressed as mol of nucleotide × sec/liter; cRNA, RNA complementary to DNA.

MATERIALS AND METHODS

Enzymes. Endonuclease R·R$_1$ was prepared from *E. coli* strain RY-13 as described elsewhere (8). Endonculease *Hpa* I was prepared from *H. parainfluenzae* as described previously (13). DNA polymerase from avian myeloblastosis virus (kindly provided by Drs. Dorothy and Joseph Beard, Duke University), was purified as outlined previously (15).

Preparation of ^{32}P-labeled SV40 DNA. ^{32}P-Labeled SV40 DNA (specific activity 5 × 10^5 cpm/μg) was prepared as described previously (13).

Isolation of Specific Fragments of SV40 DNA. Ten micrograms of ^{32}P-labeled SV40 component I DNA was converted to linear molecules by digestion with endonuclease R·R$_1$ as described earlier (6). The DNA was purified by two extractions with phenol saturated with 0.1 *M* Tris·HCl (pH 7.8) and one extraction with chloroform. The DNA was concentrated by ethanol precipitation and dissolved in 1 ml of 10 m*M* Tris·HCl (pH 7.4), 10 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol. Fifty microliters of the final preparation of *Hpa* I was added and the mixture was incubated for 3 hr at 37°. The reaction was complete, as determined by electrophoresis through 1.4% agarose–ethidium bromide gels (13). The fragments of ^{32}P-labeled SV40 DNA were separated by electrophoresis through 0.7% agarose–2.2% acrylamide gels for 12 hr at 4 V/cm (17). The gels were stained for 30 min a a solution of 0.4 μg/ml of ethidium bromide and examined directly by short-wavelength ultraviolet illumination. The fluoresecent bands of DNA were cut from the gel and the DNA was eluted from the matrix electrophoretically (13).

Preparation of RNA. "Early" RNA was prepared from the cytoplasm of BSC-1 monkey cells 12 hr after infection with SV40 (50 PFU/cell) in the presence of 50 μ*M* 5-fluorodeoxyuridine (3). "Late" RNA was extracted from polysomes (16) prepared from infected cells at 28 hr after infection (16), and total cell RNA was extracted 48 hr after infection, as described earlier (3). SV40 cRNA was synthesized *in vitro* using *E. coli* RNA polymerase (3).

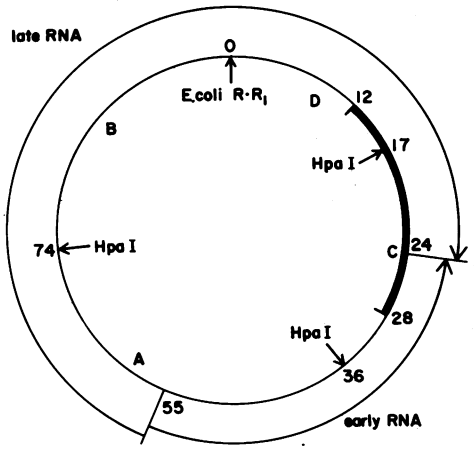

FIG. 1. A map of the SV40 genome. The cleavage site of *E. coli* R·R$_1$ endonuclease is at zero and map distances are given as percentage lengths of SV40 in a clockwise direction. The segment of SV40 contained in the adenovirus-SV40 hybrid Ad2+ND$_1$ (5, 13) is shown as a heavy line. The positions of the *Hpa* I cleavage sites are taken from Danna *et al.* (12) and Sharp *et al.* (13). The assignment of the early and late regions of SV40 DNA, which is described in the text, assumes that early RNA is transcribed from a contiguous portion of the viral genome.

Separation and Purification of SV40 DNA Strands. Reaction mixtures contained from 0.5 to 1.2 μg of heat-denatured DNA of one of the four specific fragments of SV40 DNA, 50 μg of cRNA, 0.02 *M* Tris·HCl (pH 7.4), 0.05 *M* NaCl, and 0.4% sodium dodecyl sulfate, in a total volume of 1 ml. The mixtures were incubated for 1 hr at 68° and the E and L strands of each of the fragments were separated by chromatography on hydroxylapatite and purified as described previously (3).

Ad2+ND$_1$DNA was extracted from purified Ad2+ND$_1$ virions as described earlier (19).

RESULTS

The four specific fragments obtained by digestion of SV40 DNA with endonucleases R·R$_1$ and *Hpa* I correspond to 38, 26, 19, and 17% of the total genome (13). Danna *et al.* (11) have already determined the location of each of the fragments on viral DNA and we have confirmed their order from the following data: (*1*) the length of the largest fragment obtained by cleavage of circular SV40 DNA with *Hpa* I is 43% of the complete viral genome; it is cleaved by endonuclease R·R$_1$ to yield the fragments B (26%) and D (17%) shown in Fig. 2 (13). (*2*) The DNA of the adenovirus-SV40 hybrid Ad^2+ND$_1$ hybridizes with fragments C and D but not with fragments A and B (see Fig. 4), AD2+ND$_1$ DNA contains the segment of SV40 DNA that maps between positions 12 and 28 (5, 13) (see Fig. 1). Thus fragment B must lie counterclockwise, and fragment D, clockwise, from the R·R$_1$ cleavage site. Fragment C must be adjacent to fragment D, and fragment A, therefore, must map between fragments B and C.

Direction of Synthesis of cRNA on SV40 DNA. To determine the direction of RNA synthesis relative to the R·R$_1$ cleavage site in SV40 DNA we carried out the experiment shown in Fig. 2. ^{32}P-Labeled SV40 component I DNA was cleaved with endonuclease R·R$_1$ to yield linear molecules, each end of which contained a 3′-hydroxyl group and a protruding 5′-phosphoryl single-strand tail (8). Because these molecules serve as primer·templates for DNA synthesis (8) we were able to incorporate [^{3}H]dTMP at the ends of the molecules using RNA-dependent DNA polymerase isolated from avian myeloblastosis virus. The products of the reaction were isolated and digested with *Hpa* I, and the four resulting DNA fragments were separated by electrophoresis through 0.7% agarose–2.2% acrylamide gels. Only fragments B and D contained ^{3}H-radioactivity (Fig. 3). Incorporation of dTMP was therefore confined to the two fragments of SV40 DNA that flank the R·R$_1$ cleavage site. Because of the nature of the staggered ends produced by endonuclease R·R$_1$ (8, 9), the ^{3}H must be present in one of the two strands of the DNA of fragment B and in the opposite strand of fragment D. Thus, the ^{3}H from only one of the two fragments should hybridize to asymmetric SV40 cRNA. The fragments were eluted from the gel, denatured by boiling, and reannealed, either alone, or in the presence of 100-fold excess asymmetric cRNA, for a time approximately equivalent to $C_0t_{1/2} \times 0.1$. The hybridization mixtures were then assayed on hydroxylapatite columns. After annealing in the absence of cRNA, less than 6% of the ^{32}P and ^{3}H chromatographed as double-stranded DNA (see Table 1). As expected, approximately 50% of the ^{32}P of all four fragments behaved as hybrid after annealing in the presence of cRNA. At the same time, less than 6% of the ^{3}H in fragment D and greater than 90% of the ^{3}H in fragment B behaved as hybrid. Therefore, the strand of DNA of fragment B that contains ^{3}H is complementary to cRNA. Given that: (*1*) fragment B is located to the left and fragment D to the right of the endonuclease R·R$_1$ cleavage site (Fig. 1); (*2*) cleavage of DNA by endonuclease R·R$_1$ produces molecules with 3′ OH groups and protruding 5′ tails (8, 9); and (*3*) incorporation of 5′-deoxytriphosphates occurs in a 5′–3′ polarity (20*a* and *b*), then DNA polymerase from avian myeloblastosis virus must synthesize DNA in a clockwise direction on one DNA strand of fragment B, and in a counterclockwise direction on the other DNA strand of fragment D. Because the ^{3}H-labeled 3′ end of fragment B is complementary to SV40 cRNA, the synthesis of SV40 cRNA must occur in a counterclockwise

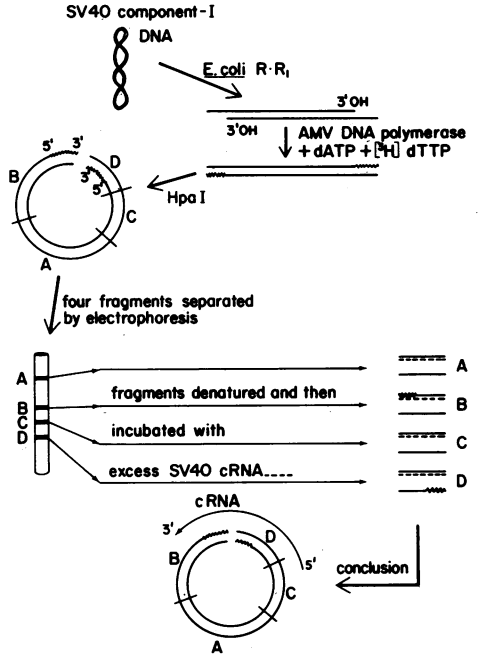

FIG. 2. Determination of the direction of synthesis of SV40 cRNA. See *text* for experimental details.

TABLE 1. *Hybridization to cRNA of fragments of SV40 DNA specifically labeled at the R·R$_1$ cleavage site*

DNA fragment	−cRNA				+cRNA			
	^{32}P		^{3}H		^{32}P		^{3}H	
	cpm*	% Double-strand	cpm*	% Double-strand	cpm*	% Double-strand	cpm*	% Double-strand
A	272/3972	6.8	—	—	1694/3810	44.4	—	—
B	184/2805	6.5	17/261	6.5	1467/2957	49.6	260/285	91.2
C	139/2014	6.9	—	—	931/1835	50.7	—	—
D	143/2183	6.5	12/215	5.6	1083/2175	50.8	7/221	3.2

Linear SV40 [^{32}P]DNA produced by R·R$_1$ treatment was labeled with [^{3}H]dTMP at the R·R$_1$ cleavage site and the four specific fragments of the DNA were isolated as described in the legend to Fig. 3. Approximately 0.02 μg of the DNA of each fragment was denatured by boiling and allowed to reanneal in the absence or in the presence of 2 μg of cRNA. The reaction was carried out in 1 ml of 0.14 M sodium phosphate (pH 6.8), 0.4% sodium dodecyl sulfate, for 15 min at 68°, and the hybrids were assayed by chromatography on hydroxylapatite.

* (cpm behaving as duplex)/(total cpm).

direction as drawn on the conventional SV40 map. Since cRNA and early RNA are complementary to the same strand of SV40 DNA, early RNA must also be synthesized in a counterclockwise direction.

Mapping of Early and Late Regions of the SV40 Genome. ^{32}P-Labeled SV40 component I DNA was digested sequentially with endonuclease R·R$_1$ and *Hpa* I. After separation through 0.7% agarose–2.2% polyacrylamide gels, the four fragments were denatured by boiling and incubated at 68° in the presence of a 50- to 100-fold excess of SV40 cRNA for a time equivalent to a DNA annealing of $C_0t_{1/2} \times 0.1$. The mixtures were then fractionated by chromatography on hydroxylapatite. The resulting E and L strands of each of the fragments were self-annealed and purified as described previously for unit-length SV40 DNA (3).

Control experiments showed that the level of contamination of one strand of DNA by the other, and of one fragment of DNA by another, was less than 2%.

To determine which regions of the SV40 genome correspond to early and late functions, ^{32}P-labeled preparations of E and L strand DNA from each of the four fragments were hybridized with RNA extracted from the cytoplasm of BSC-1 cells at different times after infection. Before hybridization, the DNAs were boiled in 0.3 N NaOH—a treatment which reduced their length to about 200 bases, as judged by sedimentation through alkaline sucrose gradients (not shown). The results of the hybridization experiments are shown in Fig. 4 (*left-hand column*). "Early" RNA hybridized only to a portion of the E strand of fragments A and C, and not to DNA derived from any other region of the viral genome. At saturating levels about 50% of the E strand sequences of fragment A and 60% of the E strand sequences of fragment C entered hybrid. The sum of these percentages is equivalent to 31% of the sequences of the entire E strand of SV40 DNA—a figure which agrees well with the previously published estimates of the length of early region of the viral genome (1–3). Assuming that early RNA is transcribed from a contiguous region of the viral genome, and knowing the direction of RNA synthesis, we calculate that the 5′ end of the stable species of early SV40 RNA maps at position 55, and the 3′ end at position 24, on the SV40 map (see Fig. 1).

To check the accuracy of these estimates, we annealed sheared ^{32}P-labeled E and L strand DNA from each of the four fragments to increasing amounts of unlabeled sheared Ad2^{+}ND$_1$ DNA for a time calculated to be equivalent to $C_0t_{1/2} \times 10$ for the [^{32}P]DNA alone. As expected, fragments A and B did not anneal to Ad^{2+}ND$_1$ DNA. However, at saturation 62% of the sequences of the E and L strands of fragment C and 32% of the sequences of the E and L strands of fragment

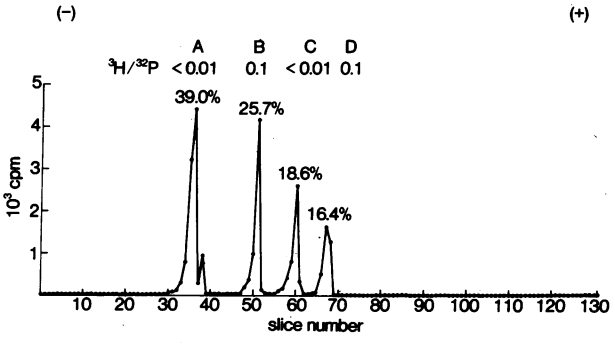

FIG. 3. Radioactivity profile of an 0.7% agarose–1.4% polyacrylamide gel after separation of the four specific fragments of SV40 DNA. ^{32}P-Labeled component I SV40 DNA was digested with endonuclease R·R$_1$ and used as template primer for the incorporation of [^{3}H]dTMP by RNA-dependent DNA polymerase. The reaction mixture contained in a total volume of 0.1 ml: 0.05 M Tris·HCl (pH 7.9), 0.05 M KCl, 0.01 M $MgCl_2$, 0.001 M dithiothreitol, 10% glycerol, 0.1 mM dATP, 5 μCi of [^{3}H]TTP (specific activity 45 Ci/mmol), 2 μg of linear SV40 [^{32}P]DNA produced by R·R$_1$ digestion (specific activity 10^5 cpm/μg), 7 units (15) of avian myeloblastosis virus DNA polymerase. The mixture was incubated for 30 min at 37°. The double-labeled DNA (specific activity of ^{32}P, 10^5 cpm/μg); ^{3}H, 10^4 cpm/μg) was extracted with phenol, concentrated by ethanol precipitation, and cleaved with *Hpa* I. After separation of the resulting four fragments by electrophoresis the gel was sliced and the distribution of ^{32}P was determined by Cerenkov counting. The slices containing the four fragments were pooled and the DNA was eluted electrophoretically. The ratio of ^{32}P to ^{3}H counts in an aliquot of each of the fragments was determined after trichloroacetic acid precipitation. Numbers above the peaks are the percent of total ^{32}P applied to the gel.

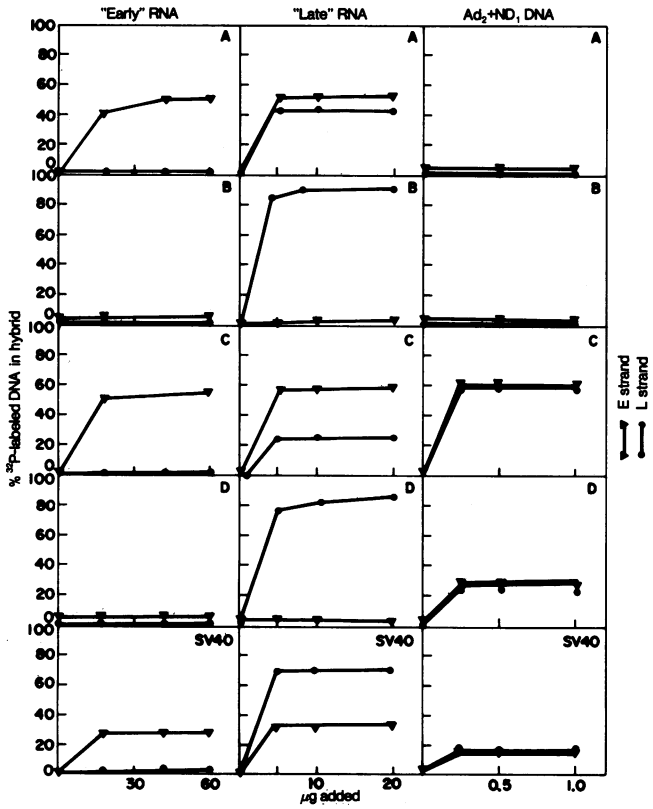

FIG. 4. Hybridization of "early" and "late" RNA to the separated strands of the R·R$_1$ *Hpa* I fragments of SV40 DNA. The separated E and L strands of ^{32}P-labeled SV40 fragments were boiled for 30 min in 0.3 N NaOH, neutralized, and hybridized for 36 hr at 68° to increasing amounts of "early" RNA (*left column*), "late" RNA (*center column*), or Ad2+ND$_1$ DNA (*right column*). Before hybridization the Ad2+ND$_1$ was boiled for 35 min in 0.3 N NaOH. Each hybridization mixture contained about 250 cpm of ^{32}P-labeled DNA in a total volume of 0.1 ml of 0.14 M sodium phosphate (pH 6.8), 0.4% sodium dodecyl sulfate, 1 M NaCl. The proportion of denatured and hybrid ^{32}P-labeled DNA was determined by hydroxylapatite chromatography (18). Control experiments showed that treatment of the RNA preparations for 12 hr at 37° with 0.3 N NaOH completely abolished the hybridization and that RNA extracted from uninfected cells did not hybridize to either strand of any of the SV40 fragments.

D formed duplexes with Ad2+ND$_1$ DNA (see Fig. 4, *right-hand column*). Ad2+ND$_1$ DNA contains the SV40 sequences located between map positions 12 and 28. The border between fragments C and D is located at map position 17. Thus, 58% of the sequences of fragment C and 29% of the sequences of fragment D are present in Ad2+ND$_1$ DNA. The values obtained from the hybridization experiments are very close to those predicted. For this reason, we believe that our estimates for the percentages of fragments A and C that are transcribed into stable species of early RNA are not seriously in error.

When ^{32}P-labeled E and L strand DNA of each fragment was hybridized with polysomal RNA extracted from cells at late times after infection, the results shown in Fig. 4 (*center column*) were obtained. At saturating levels of RNA, 45% of the L strand and 52% of the E strand of fragment A, 90% of the L strand and 0% of the E strand of fragment B, 26% of the L strand and 60% of the E strand of fragment C, and 92% of the L strand and 0% of the E strand of fragment D behaved as hybrid.

Thus polysomal RNA extracted late in lytic infection contains sequences complementary to a total of 64% of the sequences of the L strand and 31% of the sequences of the E strand of SV40 DNA. These figures are in reasonable agreement with results obtained with unfractionated SV40 DNA (refs. 2, 3, and Fig. 4), when 70% of the L strand and 34% of the E strand sequences formed hybrids with polysomal RNA extracted at late times in lytic infection. A summary of the results of these hybridization experiments is shown in Table 2. It is clear: (*1*) that all of the early functions of SV40 DNA are located within fragments A and C, (*2*) that all of the sequences of fragments B and D and part of the sequences of A and C code for late functions, and (*3*) that the strand switches between early and late RNAs occur at positions 55 and 24, assuming that early RNA is transcribed from a contiguous region of the SV40 genome (see Fig. 1).

Hybridization of ^{32}P-Labeled SV40 R·R$_1$-Hpa I Fragments to Late Total Cell RNA. When ^{32}P-labeled E and L strand DNA of each of the four specific SV40 fragments was hybridized with total cellular RNA extracted from cells 48 hr after infection, results different from those shown in Fig. 4 were obtained. For each of the fragments, the sum of the transcripts of the E and L strands amounts to more than the equivalent of one DNA strand. At saturating levels of RNA, 68% of the L strand and 50% of the E strand of fragment A, 92% of the L strand and 24% of the E strand of fragment B, 75% of the L strand and 60% of the E strand of fragment C, and 90% of the L strand and 25% of the E strand of fragment D behaved as hybrid (see Table 2). Thus, total cell RNA extracted 48 hr after infection contains sequences complementary to 77% of the L strand and 44% of the E strand of entire SV40 DNA. These estimates are slightly greater than those previously published (3), with unfractionated SV40 DNA strands when 72% of the L strand and 40% of the E strand sequences entered hybrid with saturating amounts of late RNA. All these results suggest that symmetric transcripts corresponding to at least 20% of the sequences of fragment A, 14% of fragment B, 15% of fragment C, and 17% of fragment D are present in cells at late times after infection.

TABLE 2. *Hybridization of the separated strands of SV40 DNA fragments to late polysomal or late total cell RNA*

Fragment	Strand	% of ^{32}P-labeled DNA entering hybrid at saturating levels of: Late polysomal RNA	Total cell RNA
A	E	52	50
A	L	45	68
B	E	0	24
B	L	90	92
C	E	60	60
C	L	26	75
D	E	0	25
D	L	92	90
SV40	E	34	44
SV40	L	70	77

DISCUSSION

Using the staggered breaks introduced by endonuclease R·R_1, we have shown that *E. coli* RNA polymerase transcribes SV40 DNA in a counterclockwise direction, as drawn on the conventional map. Because "early" RNA is made from the same strand of SV40 DNA as cRNA (1–3), it, too, must be synthesized in a counterclockwise direction. Khoury *et al.* (14) have reached a similar conclusion on the basis of experiments using exonuclease III.

Early RNA maps between positions 55 and 24 on the SV40 genome. However, the major initiation site for *E. coli* RNA polymerase is located approximately 15% of the length of the SV40 genome from one R·R_1 cleavage site (21) in the segment of SV40 sequences which is included in Ad2$^+$ND_1 DNA (22). Thus, when *E. coli* RNA polymerase begins its traverse of SV40 DNA, it must start by heading into the late region of the viral genome, transcribing the E strand.

We have mapped "early" and "late" species of viral RNA against four specific fragments of SV40 DNA, and from these experiments we can piece together the pattern of transcription of stable species of RNA at different times after infection. Before SV40 DNA synthesis begins, stable RNA is transcribed from a set of E strand sequences situated partly in fragment A and partly in fragment C. If the early functions of SV40 are contiguous, the 5′ end of early RNA is located at position 55, and the 3′ end at position 24 on the SV40 map (see Fig. 1). These results are in good agreement with those of Khoury *et al.* (14), who used fragments obtained by cleavage of SV40 DNA with a restriction activity from *H. influenzae* to assign early RNA to the region between positions 57 and 26. Although it is clear that all the early functions of SV40 are coded by this region, it is not clear that all the DNA sequences of the region code for early functions. Khoury *et al.* (14) found that RNA extracted from cells late in infection hybridized to the late (+) strand of *H. influenzae* fragments H and I, which map around position 36 (11). This result may mean that the early genes are divided into two or more segments by short stretches of late genes. Further analysis of the early region using smaller specific fragments is necessary to resolve this problem.

There is no difference in the hybridization pattern of the E strands of fragments A and C to RNA extracted from cells at early and late times after infection. However, at late times a new class of viral RNA appears on polysomes, which is synthesized only from the L strand and contains all or nearly all the sequences of fragments B and D and part of the sequences of fragments A and C. For all fragments, the sum of the stable transcripts of the E and L strands amounts to the equivalent of one strand of the viral DNA. This result suggests that the stable species of early and late viral RNA are transcribed from nonoverlapping regions of the SV40 genome (Table 2).

By contrast to the results obtained with polysomal RNA extracted 28 hr after infection, total RNA extracted from cells late in lytic infection hybridizes to the equivalent of more than one strand of the DNA of each of the four fragments. The origin and fate of these symmetric RNA sequences are not clear. They could indeed be the primary transcription products and the precursors of stable, asymmetric polysomal RNA, as Aloni (23, 24) has suggested, based on experiments that detected short-lived species of virus-specific symmetric RNA. Alternatively, they may arise from transcription of integrated viral genomes (25), and may play no functional role in SV40 lytic infection. Finally, the presence of symmetric RNA sequences late after infection may be no more than a reflection of the degenerate condition of the cells. It is conceivable, therefore, that cells infected with SV40 may contain not only stable informational, but also stable noninformational and transient RNA. It seems to us that the best hope of resolving the problem is to isolate and map discrete RNA species against specific fragments of SV40 DNA.

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