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Patterns of Simian Virus 40 Deoxyribonucleic Acid Transcription

II. In Transformed Cells

GEORGE KHOURY, JANET C. BYRNE, KENNETH K. TAKEMOTO, AND MALCOLM A. MARTIN

Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

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The pattern of simian virus 40 (SV40) deoxyribonucleic acid transcription has been examined in 11 SV40-transformed cell lines. In all cases, substantial regions of the minus strand (35–75%) appeared to be transcribed. In the lines tested, these regions included the "early" gene sequences. The SV40-specific ribonucleic acid from at least two of the transformed cell lines represented significantly greater portions of the minus strand than are represented in "early" lytic ribonucleic acid. Small regions of the plus strand appeared to be transcribed in only two of the transformed cell lines.

Productive infection of African green monkey kidney cells by simian virus 40 (SV40) is characterized by the presence of virus-specific neoantigens, replicating viral deoxyribonucleic acid (DNA), capsid proteins, and the appearance of progeny virus. In most SV40-transformed cell lines, however, the same neoantigens appear, but no viral DNA synthesis or viral capsid proteins can be detected. Since infectious virus can frequently be rescued from these cells by physical and chemical agents (16) or by fusion with permissive cells (8, 12, 21), the entire viral genome would appear to be present in most of these established transformed lines. The mechanism which restricts the expression of viral genetic material in SV40-transformed cells is poorly understood at present. Transcriptional control was previously suggested from the results of competition-hybridization experiments that demonstrated the presence of certain SV40-specific ribonucleic acid (RNA) sequences in lytically infected cells which were absent in virus-transformed cells (2, 15). However, other studies indicated that a substantial portion of SV40 DNA was transcribed in some transformed lines (14, 17). Until recently, it has been difficult to directly evaluate the pattern of transcription in virus-free transformed cells or in those undergoing lytic infection with SV40. Procedures have now been described for preparatively separating the strands of SV40 DNA by taking advantage of the fact that SV40 complementary RNA (cRNA), made in vitro with *Escherichia coli* RNA polymerase, hybridizes almost exclusively to one strand of SV40 DNA (11, 13, 22). This has enabled us to determine strand orientation as well as the extent of transcription in acutely infected permissive and nonpermissive mammalian cells (10, 11). These experiments have shown that RNA that is present during the early phases of the lytic cycle is transcribed from the same DNA strand (minus strand) as is the SV40 cRNA prepared in vitro with *E. coli* RNA polymerase. The majority of late sequences are the transcription products of the other strand (plus strand) (10, 13). In the present study, we have examined the pattern of transcription in several virus-free SV40-transformed cell lines. Our results show that: (i) in 9 of 11 transformed lines studied, no transcription could be detected from the plus strand of SV40 DNA; in the two cases where transcription from the plus strand was observed, less than 8% of that strand was expressed; (ii) in 9 of the 11 lines, virus-specific RNA formed stable hybrids with 37 to 50% of the minus strand, a value quite similar to or slightly greater than that obtained with "early lytic" RNA; in the two additional lines, approximately 65 to 75% of the minus strand of SV40 DNA hybridized with transformed cell RNA.

MATERIALS AND METHODS

SV40 ^{32}P-DNA. Confluent monolayers of Vero cells (7) were infected with 1 to 3 plaque-forming units of small-plaque SV40 in medium containing

carrier-free ^{32}P-orthophosphate (100 μCi/ml) as previously described (10, 11). Viral DNA was extracted from purified virions and mechanically sheared to an average size of approximately 3×10^5 daltons (10, 11). The DNA preparations used in these studies had specific activities of 0.5×10^6 to 2.2×10^6 counts per min per μg.

In vitro cRNA and infected cellular RNA. The preparation of in vitro SV40 cRNA using *E. coli* DNA-dependent RNA polymerase has been described in detail (10). "Early lytic" RNA from infected Vero cells was prepared 24 to 30 hr after SV40 infection in the presence of arabinosyl cytosine (20 μg/ml) as previously described (10). "Late lytic" RNA was extracted from SV40-infected Vero cells 40 to 60 hr after infection (11).

Transformed cellular RNA. Total cellular RNA was prepared from confluent monolayers of the SV40-transformed cell lines listed in Table 1. The cells were washed with phosphate-buffered saline (*p*H 7.6), suspended in RNA lysing solution (0.1 M NaCl-0.01 M sodium acetate [*p*H 5.3]-bentonite [1 mg/ml]-0.35% sodium dodecyl sulfate), and extracted twice with phenol at 60 C. The RNA was treated with electrophoretically purified pancreatic deoxyribonuclease (Worthington, 100 μg/ml) for 1 hr at 20 C, extracted a final time with phenol, applied to Sephadex G-100 (Pharmacia), and collected in the void volume.

Separation and purification of SV40 DNA strands. To separate the SV40 DNA strands, 125 μg of SV40 cRNA, prepared in vitro with *E. coli* RNA polymerase, was incubated with 0.1 to 0.5 μg of denatured SV40 ^{32}P-DNA fragments in a reaction mixture (10 ml) containing 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, for 1 hr at 68 C. The reaction mixture was applied to a hydroxyapatite (HA) column. Single-stranded DNA was eluted in a small volume of 0.14 M phosphate buffer (PB) at 60 C, and the DNA associated with the cRNA was recovered by washing the column with 0.4 M PB at 60 C (10). SV40 cRNA present in eluates was eliminated by treatment of the fractions with 0.5 N NaOH for 6 hr at 37 C. Both fractions were allowed to "self-associate" at a sodium ion concentration of 1.0 M for 24 to 48 hr at 68 C, and the purified plus- and minus-strand fragments were obtained as a "single-strand eluate" in 0.14 M PB from HA. To show that separation of fragmented, rather than intact, strands of SV40 DNA does not affect the fidelity of strand separation, equal amounts of intact plus strands and sheared plus strands were incubated to a C_0t (4) of 5.5×10^{-4} in 1.1 M NaCl at 68 C. Under these conditions, approximately 60% of unfractionated SV40 DNA fragments will reassociate. Less than 2% of the sheared plus strands of SV40 DNA reannealed with the intact plus strands, indicating that the separation of SV40 fragments results in plus- and minus-strand pieces with a polarity identical to those of separated intact strands.

DNA-RNA hybridization. Reaction mixtures containing 2×10^{-3} to 8×10^{-3} μg of separated DNA strand fragments, varying amounts of transformed cellular RNA, 1.0 M NaCl, and 0.01 M Tris-hydrochloride (*p*H 7.5) in 0.10 to 0.25-ml volumes were incubated for 20 hr at 68 C. Samples were diluted to 5 ml with ice-cold 0.14 M PB and stored at 4 C until analysis. The fraction of DNA fragments in hybrid molecules was analyzed on HA as described (10). Single-stranded DNA molecules were eluted in 0.14 M PB at 60 C, whereas DNA-RNA hybrids were eluted with 0.4 M PB at 60 C.

Evaluation of nucleic acid hybrids with single-strand-specific nuclease, S-1. S-1 nuclease, purified from Takadiastase powder (3, 19), was a gift of E. Scolnick. DNA-RNA hybridization reactions were performed as described in the preceding paragraph. Samples of 0.05 ml containing about 300 counts/min were removed from each reaction mixture for analysis on HA. The remainder of the reaction mixtures (approximately 0.15 ml), diluted 10 times with buffer to give a final concentration of 0.3 M NaCl, 10^{-5} M $ZnSo_4$, 0.03 M sodium acetate buffer (*p*H 4.0), and 30 μg of denatured calf thymus DNA per ml, was divided into three equal portions. One of these served as a control, whereas the two others were treated with 25 μliters or 50 μliters of S-1 nuclease, amounts more than sufficient

TABLE 1. *Origin and properties of the SV40-transformed cell lines*

Transformed line	Origin of cells	Transforming SV40 virus	T-antigen	Virus rescue
SV-L-AL/N	Mouse embryo (AL/N)	Large plaque	+	+
WI-18-Va2	Human embryonic lung	Wild type	+	+
SV-S-AL/N	Mouse embryo (AL/N)	Small plaque	+	+
SV-S-Thy	Sheep thyroid	Small plaque	+	ND[a]
SV-S-Tes	Sheep testes	Small plaque	+	+
SV-OM/N	Rat embryo (OM/N)	Small plaque	+	+
SV-34	Hamster tumor (established in culture)	Small plaque	+	+
SV-Macro	Mouse macrophage (Balb/C)	Small plaque	+	+
SV-Py-11[b]	Mouse embryo (Swiss, 3T3)	Wild type	+	+
SV-UV-15, C1-1	Mouse embryo (Swiss, 3T3)	Small plaque[c]	+	−
11-A-8	Mouse embryo (Balb, 3T3)	Small plaque	+	+

[a] Not done.
[b] SV40-polyoma double transformant.
[c] Ultraviolet-irradiated virus.

to degrade the single-stranded DNA in the absence of hybridization. Digestion was performed at 50 C for 75 min, and the nucleic acids were precipitated with cold 5% trichloroacetic acid in the presence of carrier yeast RNA (100 μg/ml). The proportion of DNA resistant to the nuclease digestion was the average of the two assays (which did not vary by more than 10%) compared to the control reaction.

RESULTS

Strand orientation and extent of SV40 DNA transcription were examined by incubating increasing amounts of unlabeled, transformed cell RNA with small amounts of ^{32}P-labeled, fragmented, SV40 DNA plus strands and minus strands as previously described (10). Since the concentrations of SV40-specific sequences in total SV40-transformed cell RNA are low, a high RNA/DNA ratio (10^6 to 4×10^6) was used.

There was essentially no hybridization between a high concentration (1.5 mg/ml) of normal mouse embryo RNA and either the plus (<1%) or minus (2%) strand of DNA. We have previously reported that late lytic SV40 RNA reacted with approximately 65% of the plus-strand DNA fragments (10). However, when increasing amounts of transformed cell RNA were incubated with the plus strand of SV40 DNA, virtually no reaction was observed (Table 2; Fig. 1C). In only 2 of the 11 transformed cell lines examined was there any significant hybridization of RNA to the plus strand (line SV-S-AL/N = 4%; line SV-S-Thy = 7%).

All of the 11 transformed cell RNAs reacted with a large portion of the minus strand of SV40 DNA (Fig. 1A and B; Table 2). Under conditions of RNA excess, 35 to 75% of the minus strand formed stable hybrids with the various cellular RNAs. Although several of these reactions did not reach completion, probably due to the varying amounts of virus-specific RNA in each of the lines tested, there seem to be at least two basic transcription patterns. In most of the transformed lines examined, about 35 to 50% of the minus strand is transcribed, a value similar to that observed when RNA is prepared during the early phases of a lytic infection of green monkey kidney cells (10, 13). The RNA from lines SV-L-AL/N and WI-18-Va2, however, reacted with 65 to 75% of the minus strand. This proportion of the minus strand is considerably greater than that which is expressed early or late in a productive infection (ca. 35%) (10, 13). In these lines, a species of virus-specific RNA is present that is absent from cells undergoing a lytic infection and which must be partially complementary to the late lytic RNA.

It is quite likely that some of these values represent an overestimate of the proportion of the minus strand which reacts with transformed cell RNA since we have previously shown that HA cannot distinguish between partial and complete DNA-RNA hybrid molecules (11). Consequently, we have monitored the reaction between transformed cell RNA and ^{32}P-labeled SV40 DNA with the single-strand-specific nuclease (S-1) derived from *Aspergillus oryzae* (3, 19). To evaluate the specificity of this enzyme reaction, several control experiments were performed (Table 2). In the absence of RNA, single-strand (minus) SV40 DNA was almost entirely degraded by the enzyme, whereas the hybrids formed between minus-strand DNA and SV40 cRNA were largely resistant to degradation. The hybridization product of minus-strand DNA fragments and early lytic SV40 RNA was 38% resistant to the enzyme. Results of these control experiments were in good agreement with the data obtained by HA chromatography. In subsequent experiments described in Table 2, an excess of RNA from transformed cells was incubated with labeled viral DNA; part of each reaction mixture was analyzed on HA, and the remainder was exposed to the S-1 nuclease. The val-

TABLE 2. *Hybridization of RNA to the separated strand fragments of SV40 DNA*

RNA annealed	Analysis of DNA-RNA hybrid molecules		
	HA (% duplex molecules)[a]		S-1 nuclease (% resistance)
	(+) Strand DNA	(−) Strand DNA	(−) Strand DNA
	<1	2	1
SV-40 cRNA	2	99	96
"Early" lytic	<1	46	38
SV-L-AL/N	1	75	59
WI-18-Va2	<1	77	55
SV-S-AL/N	4	53	42
SV-S-Thy	7	45	40
SV-S-Tes	<1	42[b]	37
SV-S-OM/N	2	50	39
SV-34	1	45	31
SV-Macro	<1	55[b]	34
SV-Py-11	<1	35[b]	20
SV-UV-15, C1-1	<1	48[b]	23
11-A-8	1	50	30

[a] Values represent data in Fig. 1; 3% background for reaction of either strand in the absence of any RNA has been subtracted from each value.

[b] Definite plateau not obtained.

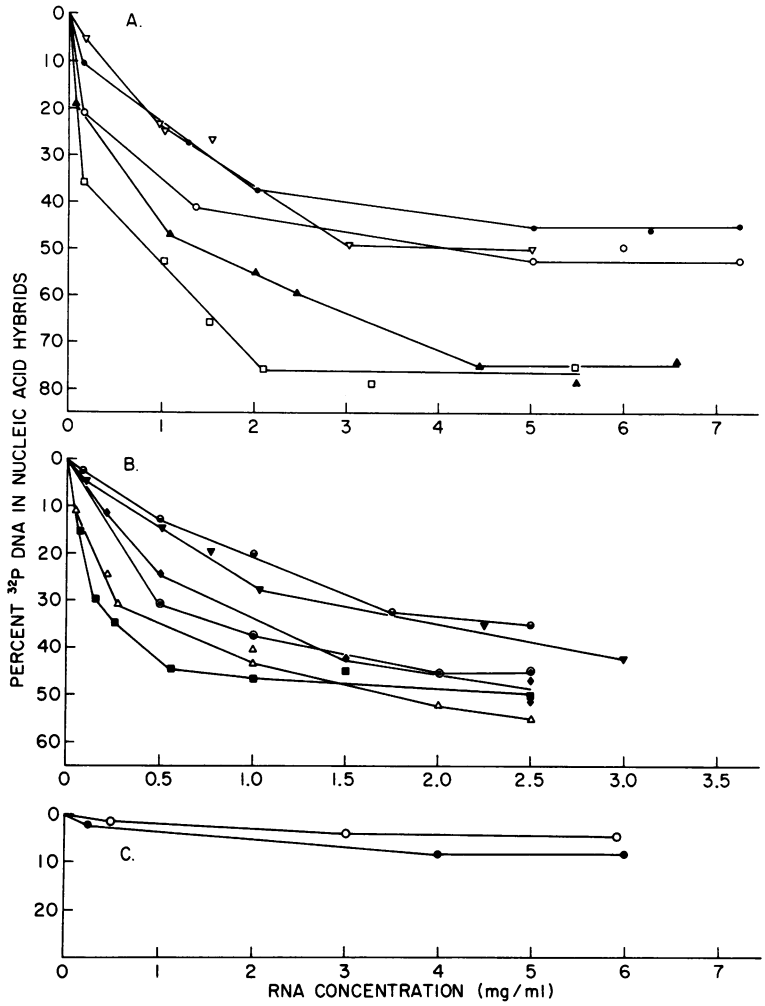

FIG. 1. *Hybridization of SV40-transformed cellular RNA to the plus strands and minus strands of ^{32}P-labeled fragmented SV40 DNA. Reaction mixtures containing increasing concentrations of transformed cellular RNA and SV40 DNA were incubated for 20 hr at 68 C and analyzed for percent DNA fragments in hybrid molecules as described in the text. A, Minus-strand fragments with RNA from line: WI-18-Va2* (□); *SV-L-AL/N* (▲); *SV-S-AL/N* (○); *11-A-8* (▽); *SV-S-Thy* (●). *B, Minus-strand fragments with RNA from line; SV-Macro* (△); *SV-OM/N* (■); *SV-UV-15* (◆); *SV-34* (⊙); *SV-S-Tes* (▼); *SV-Py-11* (◒). *C, Plus-strand fragments with RNA from line: SV-S-Thy* (●); *SV-S-AL/N* (○).

ues obtained with the enzyme assay are on the average 10 to 15% lower than the HA results, as might be expected for a population containing partial hybrid molecules. Both assay systems clearly show that a greater proportion of the minus strand of SV40 DNA is transcribed in lines WI-18-Va2 and SV-L-AL/N than during productive infection of monkey kidney cells.

Comparison of early lytic RNA and transformed cellular RNA. To determine whether the same regions of the minus strand are transcribed early in the lytic cycle and in SV40-transformed cells, several summation-hybrization experiments were performed. In these studies, labeled minus-strand fragments were incubated in a reaction mixture which contained saturating amounts of both early lytic and a transformed cell RNA. The results in Table 3 indicate that for the four transformed lines examined similar fractions of the minus strand react in the presence and absence of early RNA.

Evaluation of the regions of the minus strand transcribed in transformed cells. Although a similar fraction of the minus strand

TABLE 3. *Comparison of SV40-specific RNA present in transformed cells and at early times during lytic infection*

Transformed cell RNA	Percent minus-strand fragments in duplex molecules after hybridization[a]	
	Transformed cell RNA alone	Plus "early" lytic RNA[b]
SV-L-AL/N	75	75
SV-S-AL/N	53	50
SV-S-Thy	45	52
SV-S-OM/N	50	54

[a] Determination by HA as described in text.

[b] Concentrations of transformed cell RNA in the summation hybridization reactions with "early" lytic RNA were equivalent to those which resulted in plateau levels with the transformed cell RNA alone. The concentration of "early" lytic RNA was 1.8 mg/ml.

reacts with 9 of 11 transformed cell RNAs, these experiments do not indicate whether a transcript of the same region of the viral genome is present in each cell line. Therefore, we carried out a series of summation-hybridization experiments similar to those described in the preceding paragraph by utilizing various combinations of transformed cell RNAs and ^{32}P-labeled minus-strand fragments of SV40 DNA (Table 4). In each case, the proportion of the minus strand reacting did not exceed the higher value observed when the transformed cell RNAs were tested individually. This result suggests that similar overlapping polynucleotide sequences in the minus strand of SV40 DNA are transcribed in the cell lines tested.

DISCUSSION

We have now studied the pattern of SV40 DNA transcription in three different biological situations. We have previously shown that in African green monkey cells undergoing productive infection about 35 to 40% of the minus strand of SV40 DNA is transcribed prior to the onset of viral DNA synthesis. During the later stages of the lytic cycle, transcription from the minus strand is accompanied by transcription from approximately 60 to 65% of the plus strand of viral DNA. Although SV40 is unable to replicate in murine cells, virus-specific antigens are synthesized, and many of the infected cells become transformed. The pattern of viral DNA transcription in these acutely infected mouse cells is quite similar to that seen late in the lytic cycle, including the expression of the same plus-strand polynucleotide sequences. In the current study involving virus-free transformed cell lines, the transcription pattern is different. In most all of the cell lines examined, transcription was limited to the minus strand of viral DNA encompassing regions previously designated as early lytic (Fig. 1; Tables 2, 3). This finding is in agreement with previous competition-hybridization studies which showed that transformed cell RNAs could effectively inhibit the reaction of labeled early lytic RNA and SV40 DNA (2, 15). It is also compatible with the presence of the so-called "early viral functions" (represented by T, U, and transplantation antigens) in transformed cells.

In at least two of the transformed lines examined (Fig. 1A), RNA was detected that was complementary to a greater portion of the minus strand (ca. 75%) than might be expected if only the early sequences were represented. Since most, if not all, of the late functions are represented by RNA from the opposite (plus) strand, this additional RNA product might be expected to contain sequences which are complementary to a portion of the late viral RNA. Whether this region codes for a gene product or represents nonsense is not presently known.

Since previous reports suggested extensive transcription of "late" functions (14, 17), it should be noted that the portion of the transcript from the minus strand which is complementary to late lytic RNA would be proportionally represented in saturation-hy-

TABLE 4. *Comparison of the SV40-specific sequences in RNA from various transformed cell lines*

Transformed cell RNA[a]	Percent minus-strand fragments in duplex molecules after hybridization[b]
WI-18-Va2	77
SV-S-AL/N	53
SV-S-Thy	45
SV-S-OM/N	50
SV-S-AL/N + SV-S-Thy	46
WI-18-Va2 + SV-S-AL/N	70
WI-18-Va2 + SV-S-Thy	68
SV-S-OM/N + SV-S-Thy	49

[a] Concentrations of transformed cell RNA in the summation hybridization reactions were equivalent to those which resulted in plateau levels with each transformed cell RNA alone.

[b] Determination by HA as described in text.

bridization experiments. Furthermore, these sequences would be able to compete against late SV40 RNA in a one-step competition-hybridization study by combining with the late lytic RNA and preventing it from reacting with the viral DNA immobilized on filters. These possibilities are presently being investigated.

The method we employ to characterize SV40-specific RNA in acutely infected or transformed cells measures only the stable forms of viral RNA. RNA molecules that may be rapidly synthesized and degraded would not be present in sufficient concentrations to interact with our labeled DNA probe. For this reason we do not know whether the stable forms of SV40-specific RNA detected in mammalian cells represent the original transcript or reflect post-transcriptional modification. In a recent report, Aloni pulse-labeled green monkey cells with ^{3}H-uridine 48 hr after infection and detected virus-specific RNA that could hybridize with extensive regions of both strands of SV40 DNA (1). These RNA molecules labeled during a short pulse might represent the initial gene product. Subsequent processing would result in the stable RNA forms present in the green monkey and murine cells we have examined. The unexpected finding of stable RNA molecules corresponding to late lytic RNA in abortively infected mouse cells may also reflect post-transcriptional regulation of gene expression with the control exerted at a translational level; the RNA molecules coding for early functions may be preferentially translated.

Although we cannot exclude the possibility that transcription of late functions occurs only transiently at low levels or that the late transcripts in SV40-transformed cell lines are specifically degraded, one interpretation of our data is that late regions, located on the plus strand, may not be transcribed. Transcription of these regions may require that the DNA template be in a "free" rather than an integrated state. This possibility is perhaps supported by the exclusive transcription of the minus strand of SV40 during the lytic cycle of $Ad2^+ND_1$ (C. T. Patch et al., Proc. Nat. Acad. Sci. U.S.A., *in press*) and $Ad2^+ND_4$ hybrid viruses (G. Khoury et al., *unpublished results*), which contain a portion of the SV40 genome integrated within adenovirus 2 DNA. Both of these adeno-SV40 hybrids probably contain late SV40 DNA sequences. Another possibility is the transcription of late SV40 DNA sequences by a specific polymerase (or polymerase modifier) not present in transformed cells, similar to that reported for phage T7 infection of *E. coli* (5, 18).

If the expression of the integrated viral genome in the transformed cells examined in this study is regulated at the transcriptional level, a possible model for the observed pattern of RNA synthesis is illustrated in Fig. 2. During the process of inserting the viral genetic material into the mammalian cell genome, the circular SV40 DNA is probably converted to a linear molecule. If the interruption of the circular form occurs between early and late genes at a point near the "terminator" for early RNA (Fig. 2, site 1) and if transcription begins at the SV40 promoter for early functions, (Fig. 2., I), it might be expected that "early DNA" sequences on the minus strand would be transcribed with a concomitant extension into adjacent host sequences. Such RNA molecules containing both SV40 and mammalian cell genetic information have been previously described (9, 20). If, on the other hand, the circular form of SV40 DNA is cleaved in the middle of the late region (Fig. 2., site 2) and the early termination signal is not recognized, transcription would be initiated at the early "promoter" and continue along the minus strand, resulting in virus-specific RNA that is complementary to late RNA. These results could also be explained by a similar model which involves a specific termination signal for transcription of early SV40 RNA. Transcription would then begin in adjacent host sequences and would involve the minus strand of integrated viral DNA up to and including those regions which code for early RNA. This latter model places a restriction on the integration of SV40 in that the minus strand of viral DNA must be covalently linked to the strand of mammalian DNA which is transcribed by the cellular RNA polymerase. For the present, this model makes no attempt to deal with the small regions of the plus strand which appear to be transcribed in certain transformed cell lines. At present, however, we do not know whether expression of SV40 genetic information is regulated at the transcriptional, post-transcriptional or at both levels in acutely infected green monkey and mouse cells or in virus-free transformed cells.

The data obtained in these studies suggest no obvious correlation between the extent of transcription and the organ or species from which the transformed cells were derived. For example, there seems to be a greater proportion of the genome represented in the SV-L-AL/N mouse line than in the mouse macrophage or the other mouse embryo lines. Although the extent of transcription may be

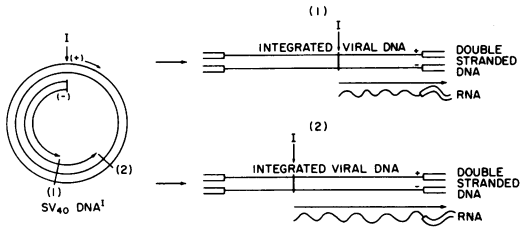

FIG. 2. *A model for the transcription of integrated SV40 DNA. I, Site of initiation of transcription; 1 and 2, theoretical cleavage sites for integration of SV40 DNA into the host chromsome. See text.*

greater in those lines transformed with large-plaque SV40 (SV-L-AL/N) than in cell lines transformed by the small-plaque virus, there is not enough data at present to draw any definite conclusions concerning the factors which might affect the extent of transcription in various transformed cell lines.

None of our experiments delineate topographically those regions of the SV40 DNA involved in the synthesis of early lytic RNA or the virus-specific RNA found in transformed cells. Consequently, we are unable to state whether the regions of the minus strand of viral DNA expressed in these cell lines are contiguous or scattered throughout the molecule. Studies similar to those described above, carried out with discrete fragments of SV40 DNA produced, for example, by digestion with the *Hemophilus influenza* restriction enzyme (6) should provide some of the answers to these questions.

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ADDENDUM IN PROOF

A report which appeared after this manuscript was submitted (J. Sambrook, P. A. Sharp, and W. Keller. 1972. Transcription of simian virus 40. I. Separation of the strands of SV40 DNA and hybridization of the separated strands to RNA extracted from lytically infected and transformed cells. J. Mol. Biol. **70**:57-71.) confirms the previous findings for the pattern of transcription in lytically infected cells (10, 13). In addition, the authors present data, for the transcription pattern of a transformed cell line (SV-3T3), which are in general agreement with our findings.

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