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Studies of Nondefective Adenovirus 2-Simian Virus 40 Hybrid Viruses

IX. Template Topography in the Early Region of Simian Virus 40

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The DNAs of the five nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid viruses contain overlapping segments of the early region of wild-type SV40 DNA. The complementary DNA strands of these five viruses have been separated with synthetic polyribonucleotides in isopycnic cesium chloride gradients. The relative amounts of early and late SV40 template in the DNA of each virus were determined by RNA-DNA hybridization with late lytic SV40 RNA, which contains sequences complementary to both templates. From the distribution of early and late templates in the five overlapping SV40 segments, we conclude that either the entire early region of SV40 is symmetrically transcribed in vivo, or, more probably, that the early SV40 templates are not contiguous.

Cells lytically infected with simian virus 40 (SV40) synthesize viral-specific RNA in two phases. Before viral DNA synthesis begins, only the early genes are transcribed (early RNA); after the onset of viral DNA replication, both the early and late templates are transcribed (early-plus-late RNA) (2, 3, 17, 25, 26, 29). Recent studies indicate that the early template appears to occupy about one-third of one strand (the minus strand) of SV40 DNA, whereas the late template occupies about two-thirds of the opposite strand (the plus strand) (12, 13, 21, 28).

In the work reported here, we have attempted to determine template topography in the early region of SV40 by using the DNAs of the five nondefective adenovirus 2 (Ad2)-SV40 hybrid viruses, $Ad2^+ND_1$, $Ad2^+ND_2$, $Ad2^+ND_3$, $Ad2^+ND_4$, and $Ad2^+ND_5$ (18-20). The properties of these hybrid viruses that make them useful for such a study are shown in Table 1. Each virus contains a different amount of SV40 DNA covalently linked to Ad2 DNA (4, 10, 11, 16). Heteroduplex mapping studies have shown that the SV40 segment in each virus is colinear with the others and with SV40 DNA, and that all segments have a common end located 0.11 ± 0.01 SV40 units from the R1 endonuclease cleavage site (Table 1) (11, 23, 24).

Cells lytically infected with each of the nondefective hybrid viruses accumulate SV40-specific RNA complementary only to the minus (early) strand of SV40 DNA. (G. Khoury, A. M. Lewis, Jr., M. Oxman, and A. S. Levine, Nature N. Biol., in press). Three of the viruses induce the synthesis of early SV40 antigens, whereas none induce late SV40 antigens (19, 20). Moreover, cells lytically infected with $Ad2^+ND_4$ (which contains the largest segment of SV40 DNA) accumulate RNA containing all of the sequences present in early SV40 RNA (17) and synthesize all of the early SV40 antigens that have been described (19). Thus, the SV40 segments in the nondefective hybrid viral DNAs comprise a unique series of overlapping portions of the early region of wild-type SV40 DNA.

We have previously shown that the complementary strands of $Ad2^+ND_1$ DNA can be separated by isopycnic banding in the presence of synthetic polyribonucleotides (27). By hybridizing the separate strands of $Ad2^+ND_1$ DNA with early-plus-late SV40 RNA, it was shown that portions of both the early and late SV40 templates were present, suggesting that $Ad2^+ND_1$ contains an SV40 transcription control region. In the present study, we have separated the complementary strands of all five nondefective hybrid viral DNAs and determined the relative amounts of early and late SV40 templates in each. The results suggest that the early region of the SV40 genome may contain two or more early templates separated by late templates. However, an alternative interpretation of the results, requiring extensive

symmetrical transcription in the early region, cannot be excluded.

MATERIALS AND METHODS

Tissue culture and viruses. BSC-1 and Vero lines of African green monkey kidney (AGMK) cells, human embryonic kidney (HEK) cells, and KB cells were maintained as described (19). SV40 (strain 777) stocks were grown by low-multiplicity infection of BSC-1 cells (26). Stocks of the nondefective hybrid viruses Ad2$^+$ND$_1$, Ad2$^+$ND$_2$, Ad2$^+$ND$_3$, and Ad2$^+$ND$_5$, as well as nonhybrid Ad2 (strain adenoid 6), were grown in primary HEK cells. Ad2$^+$ND$_4$ was grown in primary AGMK cells. Ad2 and hybrid virus stocks were demonstrated to be free of adeno-associated virus types 1 through 4; all virus stocks were shown to be free of mycoplasmas.

Radiolabeling of viral DNA and virus purification. Pools of Ad2 and the nondefective hybrid viruses were prepared in KB suspension cultures harvested 48 h after inoculation with 1 to 5 PFU/cell. At the time of infection, cultures were labeled with [2-^{14}C]thymidine (56 mCi/mmol; New England Nuclear Corp.) at 0.02 to 0.08 μCi/ml. The viruses were purified by two bandings in CsCl and dialyzed against 0.01 M Tris (pH 7.8) as previously described (27).

Viral DNA strand separation. Single-stranded DNA was prepared from purified virus by the method of Szybalski et al. (30). The virus was lysed by incubation with Pronase (100 μg/ml; Calbiochem; B grade, nuclease-free) for 30 min at 37 C in 0.001 M EDTA-0.001 M K_2HPO_4, pH 8.6. Sarkosyl (CIBA-Geigy, NL-97) was then added to a concentration of 1% together with poly(uridylic, guanylic) acid [poly(U,G)] [Schwarz/Mann, lot 7001; 2 to 3 μg of poly(U,G) per μg of DNA]. The mixture was heated in boiling water for 5 min, chilled in ice, adjusted to a density of 1.76 g/cm^3 with saturated CsCl in 0.001 M Tris-0.001 M EDTA (pH 7.4), and centrifuged in a Spinco type 50 rotor at 55,000 $\times$ g for 60 h at 8 C (15). Fractions were collected from the bottom of the gradient, and samples of each fraction were counted in Aquasol (New England Nuclear Corp.) in a Packard Tri-Carb scintillation spectrometer. The fractions containing the DNA strands were pooled, and each pool was self-annealed at 65 C for 3 h in 6 M CsCl (30). The self-annealed DNA was either diluted 1:25 in cold 6 $\times$ SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), and collected immediately on nitrocellulose filters, or rebanded in CsCl to remove double-stranded DNA. The second banding was carried out in a Spinco SW50.1 rotor at 84,000 $\times$ g for 45 h at 8 C. The purified single-stranded DNA was self-annealed and collected on nitrocellulose filters as described above. Residual poly(U,G) was not removed from the DNA strands since the poly(U,G)-DNA complexes (which are 15 to 20 nucleotides long) are stable only in high salt concentrations and at low temperatures; these complexes are readily dissociated under the conditions used here for RNA-DNA hybridization (30).

Velocity sedimentation of viral DNA in alkaline sucrose. DNA was dialyzed against 0.001 M Tris-0.001 M EDTA, pH 7.4; 0.2-ml samples were layered on to preformed 5 to 20% linear sucrose gradients in 0.2 M NaOH-0.7 M NaCl-0.15% Sarkosyl and centrifuged in a Spinco SW50.1 rotor at 40,000 rpm for 2 h at 4 C. Tritiated SV40 DNA was included with each gradient as a sedimentation marker.

Preparation of in vivo RNA. To prepare tritiated early-plus-late virus-specific RNAs, confluent cultures of Vero cells were infected with Ad2 or SV40 as previously described (26, 27). After 3 h, [5-^{3}H]uridine, [2,8-^{3}H]adenosine, and [5-^{3}H]cytidine (17 to 35 Ci/mmol, New England Nuclear Corp.) were added, each to a concentration of 20 μCi/ml. Cells infected with Ad2 were harvested 27 h postinfection (p.i.), and those infected with SV40 were harvested at 51 h p.i. Early SV40 RNA was synthesized in Vero cells in the presence of cytosine arabinoside (20 μg/ml) and labeled for 24 h with [5-^{3}H]uridine (100 μCi/ml) added at 4 to 6 h p.i. (26, 27). Uninfected Vero RNA was labeled for 48 h by using radioactive precursors identical with those used to label early-plus-late viral RNA. Radioactive RNA was extracted from whole cells by a hot phenol-sodium dodecyl sulfate (SDS) method (26, 27) and stored at −30 C in 2 $\times$ SSC + 0.05% SDS.

Determination of the specific activities of DNAs and RNAs. Samples of labeled viral DNA and cell RNA were precipitated on membrane filters (Mil-

TABLE 1. *Properties of the five nondefective Ad2-SV40 hybrid viral DNAs, with diagrams illustrating colinearity of the SV40 segments with each other and with SV40 DNA*

Viral DNA	Early SV40 antigens	Size of SV40 insertions (S)[a]	Size of Ad2 deletions (A)[a]	SV40 DNA/hybrid DNA (%)[b]
Ad2$^+$ND3	None	0.065	0.369	0.98
Ad2$^+$ND1	U	0.178	0.376	2.65
Ad2$^+$ND5	None	0.278	0.493	4.15
Ad2$^+$ND2	U,TSTA	0.324	0.425	4.75
Ad2$^+$ND4	U,TSTA,T	0.434	0.310	6.16
SV40(R$_1$)	U,TSTA,T			

[a] S and A are expressed in SV40 units, where one SV40 unit is the length of one complete SV40 genome (11).

[b] The percentage of hybrid DNA that is SV40 is given by the equation 100S/6.92 − A + S, where 6.92 is the length of an Ad2 genome in SV40 units (11). Symbols: ——, SV40 DNA; —~~—, Ad2 DNA.

lipore Corp.) with 5% trichloroacetic acid and counted in a scintillation spectrometer. DNA and RNA concentrations were determined by diphenylamine and orcinol methods, respectively, as described previously (26, 27).

Attempts to determine directly the specific activity of SV40 RNA by hybridization with SV40 DNA (followed by chemical assay) were unsuccessful, since the amount of RNA bound to DNA filters was insufficient for accurate assay. Scaling up the experiment was not feasible due to the excessive amount of RNA required. However, it is evident that the specific activity of the viral component of whole-cell RNA would be greater than that of the whole-cell RNA itself if any of the RNA present at the time the cells were harvested had been synthesized before the addition of radioactive RNA precursors. The specific activity of the viral component can be approximated by the relationship (1/T) (specific activity of whole-cell RNA), where T is the fraction of RNA that turns over (degraded and resynthesized) during the period of infection. For this work, we have attempted to estimate T by measuring the loss (during the period of infection) of label from SV40-infected cell RNA prelabeled with tritiated uridine. The amount of tritiated uridine reutilized for RNA synthesis was determined by hybridizing the RNA with SV40 DNA, using [^{14}C]uridine (added at the time of infection) as an internal control. It is assumed that the specific activity of any RNA (viral or host) synthesized after infection would depend only on its nucleotide content since the precursors of each RNA species are likely to come from the same nucleotide pool. However, this method probably only measures the lower limit of T since the concentration of isotope in the nucleotide pool changes with time. Nevertheless, the values for T obtained in this way were quite reproducible: 0.40 ± 0.02 and 0.60 ± 0.03 for labeling periods of 24 and 48 h, respectively.

Synthesis of in vitro SV40 complementary RNA. Tritiated RNA was asymmetrically transcribed from form I SV40 DNA (cRNA-I) by using *Escherichia coli* DNA-dependent RNA polymerase (containing sigma factor). All four ribonucleotide triphosphate precursors were labeled with tritium. Unlabeled carrier RNA was added to the reaction mixture before final purification as described (27). The form I SV40 DNA used as template had been purified by isopycnic banding with ethidium bromide in CsCl. This DNA sedimented in alkaline sucrose gradients in a single peak at 53*S*. The small amount of symmetrical transcription present in the cRNA-I was estimated by determining the fraction of total RNA rendered RNase resistant (trichloroacetic acid precipitable) after self-annealing at 65 C for 16 h (21).

RNA-DNA hybridization. ^{14}C-labeled viral DNA (either single-stranded or denatured double-stranded) and unlabeled denatured *E. coli* DNA were immobilized on nitrocellulose filters as previously reported (27). Filters were incubated for 18 h at 60 C in 0.2 ml of RNA (10×10^6 to 80×10^6 counts/min) in 2 × SSC + 0.05% SDS. After incubation, filters were washed with 2 × SSC at 60 C and treated with RNase as previously described (27). To determine the distribution of [^{3}H]RNA between the complementary strands of [^{14}C]DNA, two identical filters, impregnated with either heavy or light complementary strands of viral DNA or with 0.1 μg of *E. coli* DNA (the blank), were incubated in separate vials with identical solutions of the appropriate RNA as described above. After being washed, the filters were counted in a scintillation spectrometer for sufficient time to reduce the standard deviation to less than 4% of the net counts per minute by using the same channel settings employed in determining the specific activities of the nucleic acids. The ^{3}H/^{14}C ratio was determined for each filter after correcting for the counts per minute bound to the blank (3×10^{-4} to 5×10^{-4}% of the input counts per minute) and for spillover between channels. Since the specific homology ([^{3}H]RNA/[^{14}C]DNA) is proportional to the ^{3}H/^{14}C ratio on a particular filter, the percentage of hybridizable RNA binding to each strand (homology distribution) is given by the relationship

$$100(^3H/^{14}C)_{H \text{ or } L}/(^3H/^{14}C)_H + (^3H/^{14}C)_L$$

where H and L indicate heavy and light strand, respectively.

RESULTS

Separation of the complementary strands of viral DNA. The distribution of Ad2 DNA-poly(U,G) complexes in an isopycnic CsCl gradient is shown in Fig. 1. After collection of the gradient, fractions were combined to form three DNA pools consisting of heavy strand (H), light strand (L), and the top component (T). Each pool was then self-annealed and rebanded. These gradients demonstrated that, whereas the T pool formed a single peak near the density of native Ad2 DNA, both the H and L pools yielded two peaks (Fig. 2). The two peaks arising from self-annealed H and L pools are presumed to consist of pure single-stranded DNA (banding at the higher density) and sin-

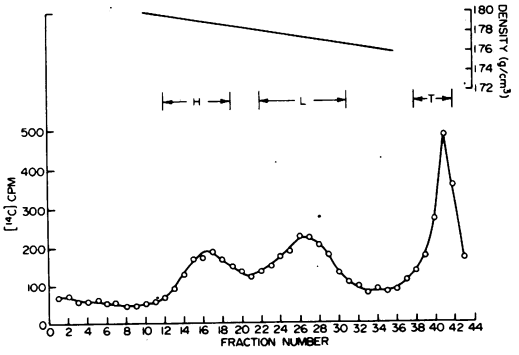

FIG. 1. *Initial separation of the complementary strands of Ad2 DNA in an isopycnic CsCl gradient in the presence of poly(U,G). H, L, and T indicate the fractions combined to form initial pools of heavy strand, light strand, and top component, respectively.*

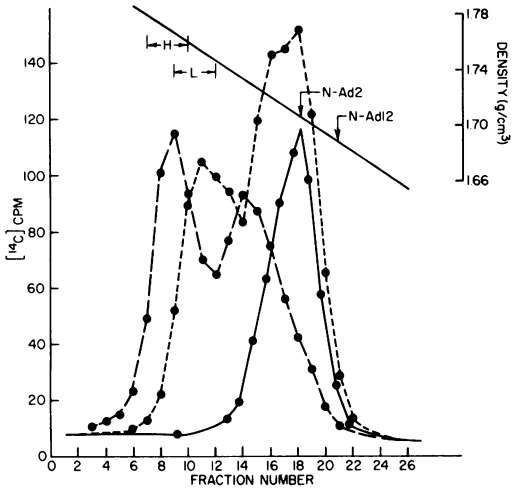

FIG. 2. *Rebanding in isopycnic CsCl gradients of the H, L, and T pools of Ad2 DNA after self-annealing. Symbols: - - -, heavy strand; -----, light strand; and ——, top component. Arrows indicate the densities of native Ad2 DNA (N-Ad2) and native Ad12 DNA (N-Ad12). H and L indicate the fractions combined to form the final pools of heavy and light strands.*

gle-stranded DNA containing varying amounts of DNA-DNA duplexes (banding at the lower density). The DNA-DNA duplexes probably arose from cross-contamination during the initial separation. Since the T pool banded at the density of native DNA, we assume that it is composed of equal amounts of self-annealed H and L strands which cannot hybridize with poly(U,G). The double-stranded nature of the T pool was confirmed by measuring the density shift on melting (0.021 g/cm^3 compared with 0.024 g/cm^3 for unfractionated Ad2 DNA). It should be noted that the amount of DNA in the T pool varied greatly between separations and was nearly zero when the initial separation was carried out with small amounts of DNA (less than 50 μg). Similarly, the amount of cross-contamination in the H and L pools was greatly reduced when small amounts of DNA were used in the initial separation.

When the H or L pools prepared from the second banding (Fig. 2) were again self-annealed and rebanded, the DNAs formed single sharp peaks at densities much higher than that of double-stranded DNA (Fig. 3). In contrast, when equal amounts of H and L DNAs were reannealed together and rebanded, the DNA appeared in a single peak near the density of native Ad2 DNA (Fig. 3). It should be noted that the densities of the single-stranded H and L DNAs in Fig. 3 are higher than the density of denatured Ad2 DNA; this is probably due to residual poly(U,G) which binds only to single-stranded DNA.

From the experiments shown in Fig. 1 through 3, we conclude that the initial strand separation is imperfect and that both the H and L pools are contaminated with varying amounts of DNA from the opposite strand. The cross-contamination can be neutralized for RNA-DNA hybridization purposes by self-annealing the DNA before it is immobilized on nitrocellulose filters; DNA in the duplex form is not available for hybridization with RNA (31). DNA strands, separated in a single gradient (Fig. 1), are useful for qualitative determination of RNA-DNA homology; however, for quantitative purposes it is necessary to remove the DNA-DNA duplexes (Fig. 2). The amounts of DNA recovered in the final H and L strand pools after the second banding (Fig. 2) were always virtually identical, as expected if both pools are composed of complementary single strands free of DNA-DNA duplexes. The separation procedure employed here does, however, give rise to strand fragmentation. When the final H and L pools were dialyzed and sedimented through linear alkaline sucrose gradients, they moved at 27*S* as estimated from SV40 DNA markers in

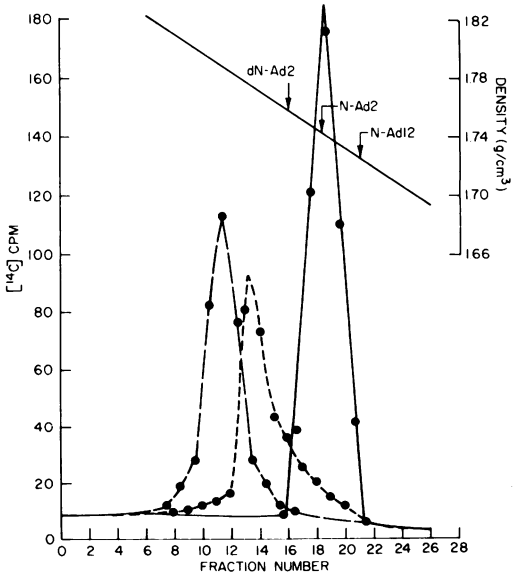

FIG. 3. *Isopycnic CsCl gradients of the self-annealed final pools of Ad2 H and L DNA strands and of H + L reannealed strands. Symbols: - - -, heavy strands; -----, light strand; ——, reannealed heavy plus light strands. Arrows indicate the density of native Ad2 DNA (N-Ad2), native Ad12 DNA (N-Ad12), and denatured Ad2 DNA (dN-Ad2).*

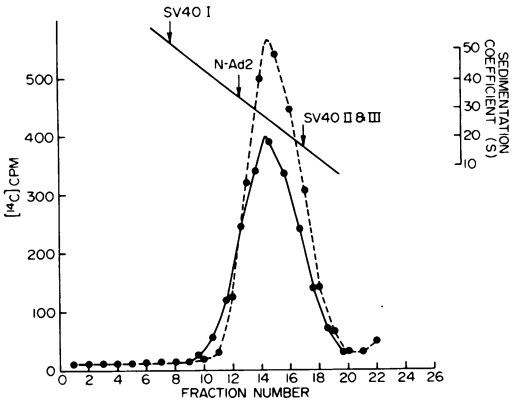

FIG. 4. *Velocity sedimentation of the separated complementary strands of Ad2 DNA in alkaline sucrose. Symbols: ——, heavy strand; -----, light strand. The ordinate on the right indicates the sedimentation coefficient in Svedberg units (S). Arrows indicate the sedimentation coefficients for SV40 DNA-I (53S), SV40 DNA-II + III (17S), and unfractionated Ad2 DNA (34S).*

the same gradients (Fig. 4). This sedimentation coefficient indicates that most of the DNA is about one-half the size of intact Ad2 DNA, which we found to move in identical gradients at 34*S* (15). Thus, the single-stranded viral DNA is approximately 3.5 SV40 units in length, or about eight times the size of the SV40 segment in Ad2$^+$ND$_4$. Since the DNA in the two strand pools is fragmented, it is possible that portions of one or both strands, lacking poly(U,G) binding sites, may be deleted in the separation process. If asymmetrical deletions from one strand only occurred, it would be impossible to determine the template distribution quantitatively from the observed homology distribution of early-plus-late SV40 RNA between the separated strands of hybrid DNA. However, if a segment of one strand only were missing from the strand pools, the density gradient of the reannealed strand pools (Fig. 3) would contain either two peaks at the densities of single- and double-stranded DNA or a single peak at an intermediate density. In fact, the DNA bands in a single sharp peak at the density of native double-stranded DNA. On the other hand, the result shown in Fig. 3 does not preclude the occurrence of symmetrical deletions. Unless the deletion included a portion of the SV40 moiety of the hybrid viral DNA, symmetrical deletions would have no effect on the observed homology distribution. It is unlikely that a deletion would contain any of the SV40 DNA, since the SV40 insertion is always near one end of the hybrid DNA molecule whereas the break in the strand occurs near the middle in most molecules (see Fig. 4).

Hybridization of viral DNA with various RNAs. Nitrocellulose filters impregnated with heavy or light DNA strands prepared from Ad2 and the five nondefective hybrid viruses were challenged with RNAs prepared from uninfected Vero cells, early-plus-late SV40 or Ad2 RNA, early SV40 RNA, or SV40 cRNA-I (Table 2). It should be noted that RNA from uninfected Vero cells exhibited no homology with any of the DNAs used in this work, and RNA prepared from SV40-infected Vero cells showed no homology with Ad2 DNA, indicating that nonspecific binding arising, for example, from contaminating host sequences or residual poly(U,G) did not occur. Moreover, the amount of early-plus-late Ad2 RNA that binds to denatured, unfractionated Ad2 DNA is the same as the average amount binding to the two separated complementary Ad2 DNA strands when each filter is normalized to equal amounts of DNA. Similarly, DNA from the T pool of an Ad2$^+$ND$_4$ separation binds an amount of early-plus-late SV40 RNA equivalent to the average bound by separated Ad2$^+$ND$_4$ strands and an amount of Ad2 RNA equivalent to that bound by unfractionated Ad2$^+$ND$_4$, indicating that the T pool is equivalent to a mixture of H and L strands.

The homology distribution (the percentage of hybridizable RNA binding to each strand) observed when once-banded DNA (Fig. 2) was challenged with a variety of RNAs is presented in Table 3. The first column of data shows the homology distribution between the separated DNA strands of Ad2 and three hybrid viruses when challenged with early-plus-late Ad2 RNA. These results indicate that there is little difference between the Ad portions of the hybrid DNAs and Ad2 DNA itself, and are similar to the homology distribution reported by other workers (8) using intact strands of Ad2 DNA. The next two columns present the homology distributions observed between the separated DNA strands of the nondefective hybrid viruses when challenged with SV40 cRNA-I and pure early SV40 RNA; both RNAs bound almost exclusively to the light strand of the hybrid DNAs. In contrast, early-plus-late SV40 RNA bound mainly to the heavy strand (last column). Together, these data show that the complementary DNA strands, or at least the SV40 moieties in both strands, are well separated. Moreover, these results serve to identify the SV40 DNA in the light strand of each hybrid DNA as a portion of the minus strand of SV40 DNA. The identity between the minus strand of

SV40 and the early SV40 template strand is in agreement with the results of other studies (13, 28). The small amount of homology seen on the heavy strand when challenged with cRNA-I probably arises from in vitro symmetrical transcription; as reported by others (21), we found that about 8.5% of our cRNA-I was transcribed from the plus strand of SV40.

To measure quantitatively the relative amounts of early and late SV40 template in each hybrid viral DNA, it is desirable that the amount of RNA in the challenge be sufficient to saturate all of the SV40 DNA sequences. Saturation curves for the separated strands of $Ad2^+ND_4$ DNA challenged with early-plus-late SV40 RNA are shown in Fig. 5, where it is seen that maximal hybrization is achieved with an input of 50×10^6 counts/min of RNA per vial. Since the SV40 DNA sequences of the other four hybrids are entirely contained within $Ad2^+ND_4$, an amount of RNA (0.5 mg) sufficient to saturate $Ad2^+ND_4$ was used to challenge the DNAs of the other hybrid viruses. It will be noted in Fig. 5 that the ratio of the specific homology ($^3H/\mu g$ of hybrid DNA) on the light strand of Ad^+ND_4 to that on the heavy strand does not vary greatly even with subsaturating RNA challenges.

In addition to using saturating amounts of RNA, the quantitative determination of homology distribution requires the use of pure single-stranded DNA free of DNA-DNA duplexes; accordingly, for such experiments, only twice-banded, single-stranded DNA (Fig. 2) was employed. The first column of data in Table 4 shows that the distribution of SV40 cRNA-I between purified strands of hybrid viral DNAs is very similar to that shown in Table 3 for

TABLE 2. *Hybridization of separated DNA strands of Ad2 and the nondefective hybrid viruses with various RNAs*[a]

Viral DNA strand[b]	Ad2 RNA (early + late)	SV40 RNA			Vero RNA (uninfected)
		cRNA-I	Early	Early + late	
Ad2					
H	372 (0.11)	NT[c]	NT	−1 (0.03)	−17 (0.12)
L	3,817 (0.11)	NT	NT	0 (0.04)	−4 (0.12)
U	1,860 (0.09)	NT	NT	−5 (0.10)	3 (0.09)
$Ad2^+ND_1$					
H	1,696 (0.10)	318 (0.13)	−10 (0.10)	408 (0.12)	−9 (0.12)
L	17,576 (0.16)	8.918 (0.15)	52 (0.20)	311 (0.11)	4 (0.12)
$Ad2^+ND_2$					
H	2,045 (0.12)	174 (0.08)	5 (0.10)	345 (0.07)	−13 (0.08)
L	11,770 (0.12)	5,065 (0.07)	233 (0.18)	256 (0.06)	−7 (0.08)
$Ad2^+ND_3$					
H	NT	102 (0.27)	NT	316 (0.20)	−25 (0.21)
L	NT	2,407 (0.26)	NT	91 (0.18)	2 (0.21)
$Ad2^+ND_4$					
H	2,047 (0.17)	2,055 (0.12)	0 (0.15)	484 (0.08)	−6 (0.11)
L	8,012 (0.15)	20,311 (0.16)	1,125 (0.10)	350 (0.08)	7 (0.08)
U	480 (0.015)	NT	NT	NT	NT
T	2,836 (0.08)	NT	NT	350 (0.07)	NT
$Ad2^+ND_5$					
H	NT	409 (0.14)	−25 (0.19)	678 (0.14)	−13 (0.14)
L	NT	12,109 (0.17)	84 (0.12)	310 (0.13)	−1 (0.13)

[a] Typical single experiments were done as described in Materials and Methods. Data shown are the average net tritium counts per minute for duplicate filters. The numbers in parentheses are the average amounts of viral DNA (in micrograms) for two filters. The specific activities of the [^{14}C]DNAs ranged from 200 to 2,000 counts per min per μg; the specific activities of the [^3H]RNAs ranged from 10^5 to 2×10^5 counts per min per μg. The range of RNA inputs and the amount of nonspecific binding to *E. coli* blanks are given in Materials and Methods.

[b] The letters H, L, U, and T indicate heavy strand, light strand, unfractionated DNA, and top component (see Fig. 1), respectively.

[c] NT, Not tested.

nonpurified DNA strands, indicating that cross-contaminating DNA is not available for hybridization. The once-banded DNA used to obtain the data shown in Table 3 contained as much as 10 to 15% cross-contamination, most of which was removed from the DNA used for the experiments shown in Table 4. It should be noted that the heavy strand of $Ad2^+ND_4$ consistently bound a greater proportion of cRNA-I than did the heavy strands of the other hybrid DNAs. This finding may indicate that in vitro symmetrical transcription occurs at two or more loci in the early region of SV40, one locus being common to all of the hybrid DNAs and another present only in $Ad2^+ND_4$. The other two columns in Table 4 show the homology distributions observed when purified hybrid DNA strands were saturated with pure early and early-plus-late SV40 RNA; again the results were similar to those shown in Table 3.

Size and topography of the SV40 templates in $Ad2^+ND_4$. By using data from the same experiments in which quantitative homology distributions were obtained (Table 4), the sizes of the SV40 segments in each hybrid DNA were calculated and expressed as the percentage of hybrid viral DNA that is SV40 (Table 5). The reconstructed segment size in the light strand of each hybrid DNA (column 2) was computed by dividing the specific homology observed on the light strand of each hybrid DNA (column 1) by the specific homology observed on denatured, linear SV40 DNA when both were challenged with equal amounts of SV40 cRNA-I. The quotient was divided by two, since the SV40 DNA filters contained both complementary strands, and was corrected for the fraction of RNA that was symmetrically transcribed. The reconstructed template sizes on both strands of all five hybrid DNAs saturated with early-plus-late SV40 RNA, and on the light strand of $Ad2^+ND_4$ saturated with pure early SV40 RNA, are presented in column 4. These values were obtained by dividing the specific homology observed on each strand (column 3) by the specific activity of the viral component of the RNA challenge; the numbers were corrected for the difference between the average molecular

TABLE 3. *Homology distribution of various RNAs between the separated complementary DNA strands (once-banded) of Ad2 and the nondefective hybrid viruses*[a]

Viral DNA strand[b]	Ad2 RNA (early + late)	SV40 RNA		
		cRNA-I	Early	Early + late
Ad2				
H	12.2 ± 2.9 (4)	NT[c]	NT	0
L	87.8 ± 2.9 (4)	NT	NT	0
$Ad2^+ND_1$				
H	15.8 ± 3.5 (2)	2.7 ± 0.5 (4)	0 (2)	63.8 ± 5.3 (5)
L	84.2 ± 3.5 (2)	97.3 ± 0.5 (4)	100 (2)	36.2 ± 5.3 (5)
$Ad2^+ND_2$				
H	15.4 (1)	3.8 ± 0.9 (3)	2 (1)	62.9 ± 9.1 (4)
L	84.6 (1)	96.2 ± 0.9 (3)	98 (1)	37.1 ± 9.1 (4)
$Ad2^+ND_3$				
H	NT	10.2 (1)	NT	86.7 (1)
L	NT	89.8 (1)	NT	13.3 (1)
$Ad2^+ND_4$				
H	18.3 (1)	9.9 ± 1.0 (4)	0 (1)	58.1 ± 2.2 (3)
L	81.7 (1)	90.1 ± 1.0 (4)	100 (1)	41.9 ± 2.2 (3)
$Ad2^+ND_5$				
H	NT	2.9 ± 0.4 (2)	0 (1)	64.5 (1)
L	NT	97.1 ± 0.4 (2)	100 (1)	35.5 (1)

[a] DNA strands were prepared by a single banding in CsCl (Fig. 1) and immobilized on filters after self-annealing as described in Materials and Methods. Data are expressed as the percentage of hybridizable RNA binding to each strand ± standard deviation; the numbers in parentheses indicate the number of determinations with duplicate filters.

[b] The letters H and L indicate heavy and light strand, respectively.

[c] NT, Not tested.

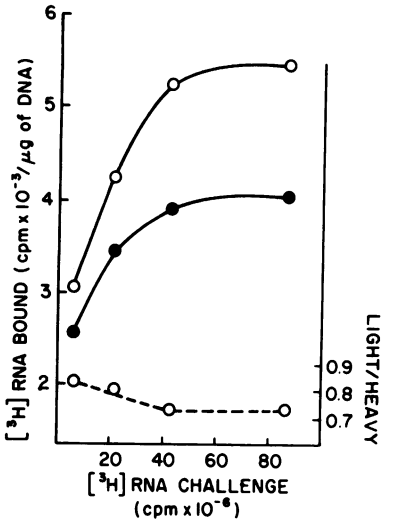

FIG. 5. *Hybridization of separated strands of Ad2⁺ND₄ DNA with increasing inputs of early-plus-late SV40 RNA. Symbols:* O, *heavy strand;* ●, *light strand. The dashed line represents the ratio:* ^3H *counts per minute per microgram of DNA hybridized to the light strand/*^3H *counts per minute per microgram of DNA hybridized to the heavy strand. The specific activity of the viral component of the challenging* [^3H]*RNA was 1.5 × 10⁵ counts per min per μg. The specific activity of the* [^{14}C]*DNA was 1,600 counts per min per μg.*

weight of ribonucleotides and deoxyribonucleotides. The specific activity of the viral component of whole-cell RNA was estimated from the measured specific activity by dividing the latter by the fraction (T) of RNA turning over during the labeling period (see Materials and Methods). It should be noted that the SV40 template sizes calculated for the light strand of Ad2⁺ND₄ using either pure early or early-plus-late SV40 RNA were nearly the same. Moreover, the sum of the reconstructed template sizes on the two complementary strands (column 5) was approximately the same as the segment sizes reconstructed from cRNA hybridization (column 2) and the segment sizes obtained from electron micrographs (Table 1, column 4).

If transcription from the SV40 genome is entirely asymmetric (i.e., only one of the two complementary strands is transcribed at any locus), and if the early-plus-late SV40 RNA homology distribution between the separated strands of hybrid DNA is an accurate measure of the relative amount of early and late SV40 template in each hybrid DNA, it is possible to compute the size of the SV40 template in each strand by multiplying the SV40 segment sizes shown in Table 1, column 2 by the appropriate value of the homology distribution (Table 4, column 3); these values are shown in the last column of Table 5. It should be noted that the computed sizes of the SV40 templates on the heavy strands of Ad2⁺ND₅ and Ad2⁺ND₂ are nearly identical.

Given that the SV40 template on the heavy strand of the hybrid viral DNA is a portion of the late SV40 template and that the template on the light strand is early, template maps of the SV40 moiety in each hybrid DNA can be constructed by using the template sizes shown in Table 5, column 6. Since the SV40 segments in each hybrid DNA are colinear and have a common end (Table 1), we started with Ad2⁺ND₃, and increments of early and late template were added so as to minimize the number of discontinuities (thereby maximizing the size of each template); the result for Ad2⁺ND₄ is shown in Fig. 6A. Model B was constructed by assuming that the early template is continuous (comprising about one-third of the SV40 genome) and is transcribed symmetrically; the two ends of the early template

TABLE 4. *Homology distribution of various RNAs between the separated complementary DNA strands (twice-banded) of the nondefective hybrid viruses*[a]

Viral DNA strand[b]	SV40 RNA		
	cRNA-I	Early	Early + late
Ad2⁺ND₁			
H	3.9 (1)	NT[c]	56.6 ± 1.6 (2)
L	96.1 (1)	NT	43.4 ± 1.6 (2)
Ad2⁺ND₂			
H	3.0 (1)	NT	53.9 ± 1.2 (2)
L	97.0 (1)	NT	46.1 ± 1.2 (2)
Ad2⁺ND₃			
H	3.9 (1)	NT	71.3 ± 1.3 (2)
L	96.1 (1)	NT	28.7 ± 1.3 (2)
Ad2⁺ND₄			
H	9.9 ± 3.8 (2)	0 (2)	57.7 ± 0.3 (3)
L	90.1 ± 3.8 (2)	100 (2)	42.3 ± 0.3 (3)
Ad2⁺ND₅			
H	3.9 (1)	NT	63.0 ± 2.9 (3)
L	96.1 (1)	NT	37.0 ± 2.9 (3)

[a] DNA strands were prepared by rebanding self-annealed DNA strands (Fig. 2) as described in Materials and Methods. Data are expressed as the percentage of hybridizable RNA binding to each strand ± standard deviation; the numbers in parentheses indicate the number of determinations with duplicate filters.

[b] The letters H and L indicate heavy and light strand, respectively.

[c] NT, Not tested.

were fixed by using the observed homology distributions for $Ad2^+ND_3$ and $Ad2^+ND_4$ (Table 4). In Table 6, the two models are compared with respect to the two parameters measured in this work. The homology distribution is expressed as the ratio of early to late template (E/L), and the reconstructed segment size is expressed as (E + L)/S, where S is the segment size as measured by electron microscopy (Table 1). It will be noted that the ratios of E/L in both models agree well with the observed values, except that in model B the ratio of E/L for $Ad2^+ND_5$ is about 50% higher than the observed value. The observed ratio of (E + L)/S for all viruses fits only model A.

DISCUSSION

The experiments reported here show unambiguously that early-plus-late SV40 RNA contains more sequences homologous with the plus strand of SV40 DNA in $Ad2^+ND_4$ (late or anti-early template) than with the minus strand. These results lead to the conclusion that either the early template is discontinuous as shown in Fig. 6A or the early region is transcribed symmetrically in late lytic SV40 infec-

TABLE 5. *Size of the SV40 templates in the complementary DNA strands of the nondefective hybrid viruses as reconstructed from hybridization data with various SV40 RNAs*

Viral DNA[a]	SV40 cRNA-I		SV40 early-plus-late and early RNAs			
	Specific homology[b] ($\times 10^{-4}$)	SV40 DNA[c]/ hybrid DNA (%)	Specific homology ($\times 10^{-3}$)	SV40 DNA[d]/ hybrid DNA (%)	Total SV40 template on both strands (%)	Length of SV40 template (SV40 units)[e]
$Ad2^+ND_3$						
H	NA[f]	NA	1.18	0.71	0.99	0.046 ± 0.001
L	0.91	0.67	0.47	0.28		0.019 ± 0.001
$Ad2^+ND_1$						
H	NA	NA	4.41	1.72	3.02	0.101 ± 0.003
L	6.12	2.68	3.33	1.30		0.077 ± 0.003
$Ad2^+ND_5$						
H	NA	NA	6.01	2.35	3.75	0.175 ± 0.008
L	7.27	3.18	3.59	1.40		0.103 ± 0.008
$Ad2^+ND_2$						
H	NA	NA	4.41	2.65	4.91	0.175 ± 0.004
L	7.23	5.34	3.77	2.26		0.149 ± 0.004
$Ad2^+ND_4$						
H	NA	NA	5.30	3.18	5.51	0.250 ± 0.001
L	13.26	6.84	3.88	2.33		0.184 ± 0.001
L	NA	NA	9.16[g]	2.19	NA	

[a] The letters H and L indicate heavy and light strand, respectively.

[b] Specific homology = net tritium counts per minute per microgram of DNA.

[c] The percentage of hybrid DNA (light strand) that is SV40 DNA is estimated from hybridization with SV40 cRNA-I by the equation: 100 (specific homology on hybrid DNA strand)/(specific homology of SV40 DNA) (2) (0.915), where the specific homology on denatured SV40 DNA was: 12.5×10^5 for $Ad2^+ND_1$ and $Ad2^+ND_5$; 7.4×10^5 for $Ad2^+ND_2$ and $Ad2^+ND_3$; and 10.6×10^5 for $Ad2^+ND_4$. The fraction of cRNA-I complementary to the minus strand of SV40 is 0.915.

[d] The percentage of hybrid DNA that is SV40 template is estimated from hybridization with early-plus-late SV40 RNA by the equation: 100 (specific homology on hybrid DNA strand) (0.95)/(1/T) (specific activity of tritiated RNA), where the specific activity of the tritiated SV40 early-plus-late RNA was: 1.46×10^5 for $Ad2^+ND_1$ and $Ad2^+ND_5$; and 0.95×10^5 for $Ad2^+ND_2$, $Ad2^+ND_3$, and $Ad2^+ND_4$. The specific activity of the SV40 early RNA was 1.59×10^5. The value of T was 0.6 for early-plus-late RNA and 0.4 for early RNA (see Materials and Methods).

[e] The length of SV40 template in each DNA strand ± standard deviation was calculated by multiplying the SV40 segment size (Table 1, column 2) by the appropriate value of the early-plus-late RNA homology distribution (Table 4).

[f] NA, Not applicable.

[g] Obtained with pure early SV40 RNA.

tion as shown in model B. In both models, the location of the left end of the early template at 0.15 SV40 units from the R1 cleavage site is supported by the work of Dhar et al. (R. Dhar, S. Zain, S. M. Weissman, J. Pan, and K. Subramanian, Proc. Nat. Acad. Sci. U.S.A., in press), who have established an identity between the early SV40 RNA complementary to $Ad2^+ND_1$ DNA and the cRNA-I transcribed in vitro near an initiation site located within the G fragment of SV40 DNA (see Fig. 6). Model A was constructed to fit the observed values for the homology distribution and assumed no symmetrical transcription, as suggested by the reconstruction calculations (Table 6). However, the sum of the two early templates in model A comprises only 18.4% of the SV40 genome. Since the entire early SV40 template is generally believed to be contained within $Ad2^+ND_4$ (14, 17; J. Sambrook, B. Sugden, W. Keller, and P. Sharp, Proc. Nat. Acad. Sci. U.S.A., in press), model A leads to the conclusion that the early template is 50% smaller than observed by other methods (2, 3, 12, 13, 17, 21, 25, 26, 28, 29). Moreover, the two early templates shown in model A would predict RNA and protein products that are much smaller than have been suggested by other workers (7, 32, 34). Model B was constructed with the early template comprising one-third of the SV40 genome, as is generally believed. However, to approximate the observed homology distributions, it was necessary to assume further that either the entire early region is transcribed symmetrically or the anti-early region is homologous with RNA transcribed from regions of the SV40 genome not contained within $Ad2^+ND_4$ and expressed late in infection; neither of these situations is supported by our reconstruction calculations (Table 6).

The currently accepted notion that the early template in SV40 comprises one-third (28 to 40%) of the viral genome is mainly based on experiments utilizing RNA-DNA hybridization competition (2, 3, 17, 25, 26, 29) and hydroxyapatite chromatography of hybrids formed between SV40 RNA and random fragments of separated strands of SV40 DNA (12, 13, 28). Both methods inherently lead to overestimates, the former due to the difficulty of controlling nonspecific competition (22) and the latter arising from the fact that partial hybrids are indistinguishable from intact hybrids by the techniques usually employed (28). Moreover, the overestimate inherent in the hydroxyapatite method would probably be exaggerated if the

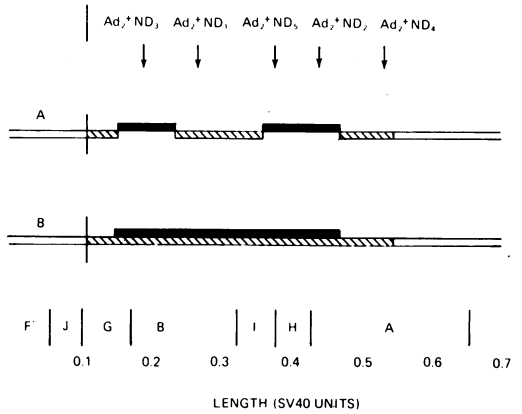

FIG. 6. *Template models for $Ad2^+ND_4$. Symbols: ▬, early SV40 template; ▨, late SV40 template. The scale (abscissa) is in SV40 units with the origin at the E. coli R1 endonuclease cleavage site. The letters on the abscissa indicate the location of the cleavage fragments produced by digestion with the Haemophilus influenzae restriction endonuclease (5, 6). The R1 endonuclease cleavage site is in the F fragment; F' is the portion of F that is contiguous with the J fragment. The arrows indicate the extent of the SV40 segments in each nondefective hybrid DNA, starting from the common end on the left (11; P. Liebowitz et al., Proc. Nat. Acad. Sci. U.S.A., in press).*

TABLE 6. *Comparison of SV40 template models with observed values for homology distribution and reconstructed SV40 segment size*

Hybrid viral DNA	Observed		Model A		Model B	
	(E/L)[a]	(E + L)/S[b]	(E/L)	(E + L)/S[c]	(E/L)	(E + L)/S[c]
$Ad2^+ND_3$	0.413	1.010	0.413	1.000	0.415	1.415
$Ad2^+ND_1$	0.762	1.140	0.762	1.000	0.786	1.786
$Ad2^+ND_5$	0.588	0.904	0.588	1.000	0.863	1.863
$Ad2^+ND_2$	0.851	1.034	0.851	1.000	0.883	1.883
$Ad2^+ND_4$	0.736	0.894	0.736	1.000	0.735	1.735

[a] E, Early template; L, late template. The observed values of E and L are from Table 5, column 6.

[b] S, Segment size from Table 1, column 4. The observed values for E and L used in the ratio (E + L)/S are from Table 5, column 4.

[c] S, Segment size from Table 1, column 2. Values for E and L were obtained from the models shown in Fig. 6.

DNA contained regions where the early and late templates were interspersed as proposed in model A. More recent work using nonrandom fragments of SV40 DNA produced by endonuclease cleavage suggests that either there is late template in the region of the H and I fragments (see Fig. 6) or there is early template on the plus strand of these fragments (14). Our finding that the heavy strand of $Ad2^+ND_4$ (which contains a segment of the plus strand of SV40) is not complementary to early SV40 RNA (Tables 3 and 4) indicates that the former is the case, thus supporting model A. One factor which could influence our data is the specific activity of the individual viral RNA species present in the RNA challenge. Although the specific activities of the various RNA species homologous with $Ad2^+ND_4$ are not known, the compositions of early (19*S*) and late (16*S*) SV40 RNA have been reported (33). Using these data together with the specific activities of the nucleoside precursors used in labeling the RNA, we conclude that the specific activity of the early sequences in the early-plus-late SV40 RNA is about 6% less than that of the late sequences; taking this difference into account increases our estimate of the total early template in model A to only 19.6% of the SV40 genome.

A second factor that might vitiate model A relates to the leaching of RNA-DNA hybrids from nitrocellulose filters when the DNA sequences are saturated (9). Such leaching would lead to low estimates of specific homology, and the effect might be exaggerated with the system used here since no more than 6% of the DNA is ever available for hybridization. On the other hand, we have shown that, at the time of immobilization on filters, the size of the DNA used in this work was about eight times the size of the largest SV40 segment, that in $Ad2^+ND_4$. Thus, at saturation about 90% of the DNA is still single-stranded, which should be sufficient to retain the hybridized portion on the filter. That this is so is supported by the saturation curve for $Ad2^+ND_4$ (Fig. 5); if leaching were occurring, the specific homology would approach zero at complete saturation.

Though the discussion presented above reflects our bias in favor of model A, model B cannot be entirely dismissed. The construction of both models is based on the observed values for homology distribution and reconstructed segment size (Table 6); of these two parameters, the latter is the weakest since it would reflect errors in measurement of specific activities of both the RNA and the DNA (such errors cancel out in determining the homology distribution from the same empirical data). In order for the homology distribution to be an accurate measure of the relative amounts of early and late template in the two complementary DNA strands, it is only necessary that the strand separation method yield a symmetrical separation of the DNA strands in that portion of the hybrid viral DNA containing the SV40 moiety, and that no SV40 DNA be lost during hybridization. On the other hand, in order for the reconstructed segment size to be an accurate measure of the amount of SV40 DNA in each hybrid DNA, it is necessary, in addition to the above, that the DNA strands be intact, that transcription be completely asymmetric, and that the specific activity of the viral RNA be precisely known. To obtain the specific activity of the viral component of the whole-cell RNA, it was necessary to determine the fraction of RNA (T) turning over during the labeling period; the observed values for T, though quite reproducible, are probably no more than estimates for the lower limit of T, since there is no certainty that all RNA species labeled under the same conditions would have the same specific activity. Nevertheless, although the scatter in the reconstructed segment sizes could easily conceal a small amount of symmetrical transcription, the observed agreement between the reconstructed segment sizes and those measured by electron microscopy appears too close to be fortuitous. On the other hand, symmetrical transcription has been demonstrated in SV40-infected cells. When such cells are pulse labeled for short periods (30 min), much of the SV40-specific label is rendered RNase resistant by self-annealing the RNA (1). Though this effect is not detectable in longer pulses, as used in our investigation, it is reasonable to propose that a small evanescent population of symmetrical transcripts is always present, and these might be detected by our methods. In fact, Sambrook et al. (J. Sambrook, B. Sugden, W. Keller, and P. Sharp, Proc. Nat. Acad. Sci. U.S.A., in press) have suggested that the total RNA (nuclear and cytoplasmic) from SV40-infected cells does contain symmetrical transcripts from both early and late templates, amounting to about 20% of the SV40 genome, whereas polysomal RNA harvested at 28 h p.i. appears free of symmetrical transcripts.

In other work, investigators using random fragments of SV40 DNA together with hydroxyapatite chromatography reported that no more than 75% of the plus strand and 35% of the minus strand entered hybrid formation (12, 28). If model B obtains, all of the plus strand should enter hybrid formation, assuming that all of the plus strand outside of $Ad2^+ND_4$ is transcribed.

To summarize, we believe that although more definitive experiments are needed to establish template topography in the early region of SV40, the best interpretation of the data presented here leads to the conclusion that the early region of SV40 consists of at least two templates separated by late templates. Furthermore, this interpretation indicates that the total early region is smaller than currently believed. If these findings are confirmed, they raise important questions about the size and nature of the early transcription and protein products of this oncogenic virus.

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LITERATURE CITED

1. Aloni, Y. 1972. Extensive symmetrical transcription of simian virus 40 DNA in virus-yielding cells. Proc. Nat. Acad. Sci. U.S.A. **69**:2404-2409.
2. Aloni, Y., E. Winocour, and L. Sachs. 1968. Characterization of the simian virus 40-specific RNA in virus-yielding and transformed cells. J. Mol. Biol. **31**:415-429.
3. Carp, R. I., G. Sauer, and F. Sokol. 1969. The effect of actinomycin D on the transcription and replication of simian virus 40 deoxyribonucleic acid. Virology **37**:214-226.
4. Crumpacker, C. S., P. H. Henry, T. Kakefuda, W. P. Rowe, M. J. Levin, and A. M. Lewis, Jr. 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. III. Base composition, molecular weight, and conformation of the $Ad2^+ND_1$ genome. J. Virol. **7**:352-358.
5. Danna, K., G. Sack, and D. Nathans. 1973. Studies of simian virus 40 DNA. VII. A cleavage map of the SV40 genome. J. Mol. Biol. **78**:363-376.
6. Danna, K. J., and D. Nathans. 1972. Bidirectional replication of simian virus 40 DNA. Proc. Nat. Acad. Sci. U.S.A. **69**:3097-3100.
7. Del Villano, B. C., and V. Defendi. 1973. Characterization of the SV40 T antigen. Virology **51**:34-46.
8. Green, M., J. Parsons, M. Pina, K. Fujinaga, H. Caffier, and I. Landgraf-Leurs. 1970. Transcription of adenovirus genes in productively infected and in transformed cells. Cold Spring Harbor Symp. Quant. Biol. **35**:803-818.
9. Haas, M., M. Vogt, and R. Dulbecco. 1972. Loss of simian virus 40 DNA-RNA hybrids from nitrocellulose membranes; implications for the study of virus-host DNA interactions. Proc. Nat. Acad. Sci. U.S.A. **69**:2160-2164.
10. Henry, P. H., L. E. Schnipper, R. J. Samaha, C. S. Crumpacker, A. M. Lewis, Jr., and A. S. Levine. 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VI. Characterization of the deoxyribonucleic acid from five nondefective hybrid viruses. J. Virol. **11**:665-671.
11. Kelly, T. J., Jr., and A. M. Lewis, Jr. 1973. The use of nondefective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. J. Virol. **12**:643-652.
12. Khoury, G., J. C. Byrne, and M. A. Martin. 1972. Patterns of simian virus 40 DNA transcription after acute infection of permissive and nonpermissive cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1925-1928.
13. Khoury, G., and M. A. Martin. 1972. Comparison of SV40 DNA transcription *in vivo* and *in vitro*. Nature N. Biol. **238**:4-6.
14. Khoury, G., M. Martin, T. Lee, K. Danna, and D. Nathans. 1973. A map of SV40 transcription sites expressed in productively infected cells. J. Mol. Biol. **78**:377-389.
15. Landgraf-Leurs, M., and M. Green. 1971. Adenovirus DNA. III. Separation of the complementary strands of adenovirus types 2, 7 and 12 DNA molecules. J. Mol. Biol. **60**:185-202.
16. Levin, M. J., C. S. Crumpacker, A. M. Lewis, Jr., M. N. Oxman, P. H. Henry, and W. P. Rowe. 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. II. Relationship of adenovirus 2 deoxyribonucleic acid and simian virus 40 deoxyribonucleic acid in the $Ad2^+ND_1$ genome. J. Virol. **7**:343-351.
17. Levine, A. S., M. J. Levin, M. N. Oxman, and A. M. Lewis, Jr. 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. VII. Characterization of the simian virus 40 ribonucleic acid species induced by five nondefective hybrid viruses. J. Virol. **11**:672-681.
18. Lewis, A. M., Jr., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry. 1969. A non-defective (competent) adenovirus-SV40 hybrid isolated from the AD2-SV40 hybrid population. Proc. Nat. Acad. Sci. U.S.A. **63**:1128-1135.
19. Lewis, A. M., Jr., A. S. Levine, C. S. Crumpacker, M. J. Levin, R. J. Samaha, and P. H. Henry. 1973. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Isolation of additional hybrids which differ in their simian virus 40-specific biological properties. J. Virol. **11**:655-664.
20. Lewis, A. M., Jr., and W. P. Rowe. 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. I. A newly characterized simian virus 40 antigen induced by the $Ad2^+ND_1$ virus. J. Virol. **7**:189-197.
21. Lindstrom, D. M., and R. Dulbecco. 1971. Strand orientation of simian virus 40 transcription in productively infected cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1517-1520.
22. Lucas, J. J., and H. S. Ginsberg. 1971. Synthesis of virus-specific ribonucleic acid in KB cells infected with type 2 adenovirus. J. Virol. **8**:204-213.
23. Morrow, J. F., and P. Berg. 1972. Cleavage of simian virus 40 DNA at a unique site by a bacterial restriction enzyme. Proc. Nat. Acad. Sci. U.S.A. **69**:3365-3369.
24. Morrow, J. F., P. Berg, T. J. Kelly, Jr., and A. M. Lewis, Jr. 1973. Mapping of simian virus 40 early functions on the viral chromosome. J. Virol. **12**:653-658.
25. Oda, K., and R. Dulbecco. 1968. Regulation of transcription of the SV40 DNA in productively infected and in transformed cells. Proc. Nat. Acad. Sci. U.S.A. **60**:525-532.
26. Oxman, M. N., A. S. Levine, C. S. Crumpacker, M. J. Levin, P. H. Henry, and A. M. Lewis, Jr. 1971. Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. V. Characterization of the simian virus 40 ribonucleic acid species induced by wild-type simian virus 40 and by the nondefective hybrid virus $Ad2^+ND_1$. J. Virol. **8**:215-224.
27. Patch, C. T., A. M. Lewis, Jr., and A. S. Levine. 1972. Evidence for a transcription-control region of simian virus 40 in the adenovirus 2-simian virus 40 hybrid, $Ad2^+ND_1$. Proc. Nat. Acad. Sci. U.S.A. **69**:3375-3379.
28. Sambrook, J., 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.
29. Sauer, G., and J. R. Kidwai. 1968. The transcription of

the SV40 genome in productively infected and transformed cells. Proc. Nat. Acad. Sci. U.S.A. **61**:1256-1263.

30. Szybalski, W., H. Kubinski, Z. Hradecna, and W. Summers. 1971. Analytical and preparative separation of the complementary DNA strands, p. 383-413. *In* L. Grossman and K. Moldave (ed.), Methods in enzymology, vol. 21, part D. Academic Press Inc., New York.
31. Taylor, K., Z. Hradecna, and W. Szybalski. 1967. Asymmetric distribution of the transcribing regions of the complementary strands of coliphage DNA. Proc. Nat. Acad. Sci. U.S.A. **57**:1618-1625.
32. Tonegawa, S., G. Walter, A. Bernardini, and R. Dulbecco. 1970. Transcription of the SV40 genome in transformed cells and during lytic infection. Cold Spring Harbor Symp. Quant. Biol. **35**:823-831.
33. Warnaar, S., and A. deMol. 1973. Characterization of two simian virus 40 specific RNA molecules from infected BSC-1 cells. J. Virol. **12**:124-129.
34. Weinberg, R. A., S. O. Warnaar, and E. Winocour. 1972. Isolation and characterization of simian virus 40 ribonucleic acid. J. Virol. **10**:193-201.