JOURNAL OF VIROLOGY, May 1973, p. 672-681
Vol. 11, No. 5

Studies of Nondefective Adenovirus 2-Simian Virus 40 Hybrid Viruses

VII. Characterization of the Simian Virus 40 RNA Species Induced by Five Nondefective Hybrid Viruses

ARTHUR S. LEVINE, MYRON J. LEVIN, MICHAEL N. OXMAN, AND ANDREW M. LEWIS, JR.

Hematology Branch, National Cancer Institute, Bethesda, Maryland 20014, Virus Research Unit, Division of Infectious Diseases, Children's Hospital Medical Center and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115, and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

Received for publication 7 December 1972

Five nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid viruses have been isolated and found to contain segments of SV40 DNA covalently linked to Ad2 DNA. The quantity of SV40 DNA present is a stable characteristic of each hybrid virus, and varies from less than 5% (in $Ad2^+ND_3$) to more than 30% (in $Ad2^+ND_4$) of the SV40 genome. We have characterized the SV40 portions of these hybrids by relating the SV40-specific RNA sequences transcribed in cells infected with each hybrid virus to those transcribed in cells infected with each of the other hybrid viruses and with SV40 itself. RNA-DNA hybridization-competition experiments indicate that the number of unique SV40 RNA sequences transcribed in infected cells is proportional to the size of the SV40 DNA segment contained within each hybrid and, in the case of the three hybrids which induce detectable SV40-specific antigens, to the number of SV40 antigens induced. Furthermore, the SV40-specific RNA sequences transcribed from any one of the hybrids are completely represented in the RNA transcribed from all other hybrids with longer SV40 segments. Thus, the SV40 DNA regions in the five hybrid viruses appear to contain some nucleotide sequences in common. The SV40-specific RNA transcribed from $Ad2^+ND_4$, the hybrid containing the largest SV40 segment, is qualitatively similar to the SV40-specific RNA transcribed early (i.e., prior to viral DNA replication) in SV40 lytic infection. Thus, it appears that no significant amount of late SV40 DNA is transcribed during infection by any of the five nondefective Ad2-SV40 hybrid viruses.

We have described the isolation and characterization of five nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid viruses which differ in their host range and in the SV40 functions which they induce in infected cells (19-21). All five are able to replicate without the assistance of nonhybrid helper virions (19, 20). The genome of each hybrid virus has been shown to contain SV40 DNA covalently linked to Ad2 DNA (5, 10, 16). The size of the SV40 segment in each of the hybrid viruses is different, but even in $Ad2^+ND_4$, which contains the largest amount of SV40 DNA, the SV40 segment is considerably smaller than the whole SV40 genome (10). Thus, with respect to their potential utility in experiments directed at the analysis of the SV40 genome, the nondefective hybrid viruses are the equivalent of independently replicating deletion mutants of SV40.

To effectively utilize these hybrid viruses in the genetic analysis of wild-type SV40, it is necessary to further characterize the SV40 genetic information which they contain. One of the viruses, $Ad2^+ND_1$, has already been the subject of detailed biological and biochemical investigation (5, 16, 21, 26). This hybrid has been found to induce only one detectable SV40 antigen (U antigen) and to contain an amount of SV40 DNA equivalent to approximately 10% of the wild-type SV40 genome. When the technique of RNA-DNA hybridization-competition was employed to examine the SV40-specific RNA transcribed in $Ad2^+ND_1$-infected cells, it was found that this hybrid induced the synthe-

sis of approximately 15% of the total SV40-specific RNA sequences transcribed during lytic infection by wild-type SV40. Thus, in the case of Ad2$^+$ND$_1$, there appears to be a good correspondence between the amount of SV40 DNA in the hybrid virus genome, the number of SV40-specific RNA sequences transcribed in infected cells, and the number of SV40 antigens induced. Since the four remaining hybrids were found to differ in their content of SV40 DNA (10) and in the SV40-specific biological functions which they induce (20), we have analyzed the SV40-specific RNA sequences transcribed in cells infected with each of the five nondefective Ad2-SV40 hybrid viruses by means of reciprocal hybridization-competition reactions. These experiments have allowed us to (i) determine to what extent transcription of SV40-specific RNA sequences accords with the amount of SV40 DNA in each hybrid, and with the number of SV40-specific functions induced; (ii) determine whether the information transcribed from the SV40 DNA of the various hybrids is different or overlapping; and (iii) relate the SV40-specific sequences transcribed from these hybrids to those transcribed during lytic infection by wild-type SV40.

MATERIALS AND METHODS

Tissue culture. The BSC-1 (11), Vero (32) and CV-1 (12) lines of African green monkey kidney (AGMK) cells were grown in Eagle minimal essential medium supplemented with penicillin (250 units/ml), streptomycin (250 μg/ml), and 2 mM glutamine (EMEM) plus 10% fetal bovine serum (EMEM-10). Roller and stationary bottle cultures were refed biweekly until confluent monolayers were formed. Commercially obtained human embryonic kidney (HEK) and primary AGMK cells were grown in EMEM-10. All AGMK cell lines were repeatedly shown to be free of mycoplasmas by anaerobic culture on Hayflick medium (9).

Viruses. 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, whereas Ad2$^+$ND$_4$ was grown in primary AGMK cells. Pools of each virus, representing the second passage after plaque purification, were prepared in cells infected with a multiplicity of 1 to 5 PFU/cell. The derivation and biological characterization of each of these hybrid virus pools are discussed in an accompanying report (20).

Pools of SV40 (strain 777) were produced by low multiplicity (approximately 10^{-5} PFU/cell) of infection using an inoculum of virus from a single seed pool ($10^{9.3}$ PFU/ml) as previously described (26). All pools used to obtain SV40 DNA were grown in BSC-1 cells, as were most pools used to produce SV40-specific RNA. However, an SV40 pool prepared in CV-1 cells ($10^{9.8}$ PFU/ml) was used to induce the early SV40 RNA employed in two of three hybridization-competition experiments involving Ad2$^+$ND$_4$ ^{3}H-RNA.

All virus pools were demonstrated to be free of adeno-associated virus types 1 to 4 by complement fixation tests, and to be free of mycoplasmas by anaerobic culture on Hayflick medium (9).

Virus purification and radiolabeling of SV40 DNA. SV40 was purified from cells and medium by a modification (26) of the technique of Burnett et al. (3). Radiolabeled SV40 was prepared as previously described (26) using 0.3 to 0.5 μCi of thymidine-2-^{14}C (New England Nuclear Corp., 20–30 mCi/mmol) per ml of medium.

DNA extraction. Viral DNA was extracted from purified virus by papain digestion (PAP, Worthington Biochemical Corp.) followed by sodium dodecyl sulfate (SDS)-phenol extraction and alcohol precipitation (28), or by Pronase (B grade, nuclease-free, Calbiochem)-SDS digestion, followed by phenol extraction and dialysis (13). Viral DNA was stored at −30 C in 0.1 × SSC (SSC = 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 6.9). The physical properties of SV40 DNA prepared by these methods have been reported previously (5). This SV40 DNA, the product of low multiplicity infection, was free of detectable host cell (monkey) nucleotide sequences as determined by sensitive hybridization assays (18, 26).

Escherichia coli DNA was extracted by the method of Marmur (23). The concentrations of DNA solutions were determined by a modified diphenylamine reaction (4) using calf thymus DNA (Calbiochem) as a standard.

Preparation of radiolabeled RNA. Early and late SV40-specific RNA was prepared in roller bottle cultures of Vero cells as previously described (26).

Hybrid virus-specific RNA was prepared by infecting 32-oz (0.95-liter) bottle cultures of Vero cells with 10 to 50 PFU of hybrid virus per cell suspended in 10 ml of EMEM plus 2% fetal bovine serum (EMEM-2). Vero cells, unlike primary AGMK cells, are permissive for nonhybrid Ad2 and for all five of the nondefective hybrid viruses (20). Three to four hours after infection, 20 ml of EMEM-2 was added to each culture. The cells were harvested by scraping 24 h after infection. Tritiated hybrid virus-specific RNA was prepared by refeeding the cultures 4 h postinfection with 15 ml of EMEM-2 containing 20 to 40 μCi/ml of each of three tritiated nucleotides (New England Nuclear: uridine-5-^3H, >20 Ci/mmol; adenosine-2,8-^3H, 20 to 30 Ci/mmol; and cytidine-5-^3H, 15 to 30 Ci/mmol).

RNA from infected and uninfected Vero cells was extracted by a hot phenol-SDS procedure (17) and stored at −30 C in 2 × SSC plus 0.05% SDS. RNA concentrations were determined by an orcinol reaction (2) with yeast-soluble RNA (Calbiochem) as a standard.

RNA-DNA hybridization and hybridization-competition. The procedure for hybridization of ^{3}H-RNA with single-stranded DNA immobilized on nitrocellulose membrane filters (Millipore Corp., HAWP) was that of Gillespie and Spiegelman (7) with slight modifications (17). Hybridization reactions

were performed at 60 C with 13-mm filters in a volume of 0.25 ml of 2 × SSC plus 0.05% SDS. Unless otherwise stated, an incubation period of 20 h was employed. All RNA-DNA hybrids were washed, treated with pancreatic ribonuclease (XII-A, Sigma; 20 μg/ml) and ribonuclease T_1 (B grade, Calbiochem; 10 units/ml) for 1 h at room temperature, and washed again before scintillation counting.

Hybridization-competition experiments were performed by preincubating the DNA-containing filters with increasing amounts of unlabeled competitor RNA for 12 h at 60 C and then adding a saturating amount (separately determined with the same batch of DNA filters and ^{3}H-RNA) of radioactive RNA. Incubation was then continued for an additional 14 h, after which the filters were washed, ribonuclease treated, and washed again before scintillation counting. The extent of nonspecific competition by cellular RNA was initially determined separately by preincubating SV40 DNA filters with appropriate amounts of unlabeled RNA extracted from uninfected Vero cells (see Fig. 1-5). In subsequent experiments (see Fig. 6-8), unlabeled RNA from uninfected Vero cells was mixed directly with the unlabeled competitor RNA (from virus-infected Vero cells) in varying ratios so that the same total amount of RNA was present at each point in the competition experiment.

The SV40 DNA on the filters was ^{14}C-labeled to permit an accurate determination of the amount of DNA still present at the end of the final hybridization and washing procedures. Filters were counted for sufficient time to achieve a counting accuracy of ±5%, or better, for both ^{3}H and ^{14}C. The ^{3}H counts per minute bound to SV40 DNA filters were corrected for ^{14}C counted in the ^{3}H channel and for nonspecific binding to *E. coli* DNA. The net virus-specific ^{3}H counts per minute were then normalized to the stated amount of SV40 DNA per filter on the basis of the ^{14}C counts per minute.

RESULTS

Relationships between the SV40-specific RNAs induced by the various nondefective hybrid viruses. RNA extracted from Vero cells acutely infected with the five nondefective hybrid viruses was employed in hybridization-competition reactions, so that the SV40 transcription product of each hybrid could be compared with that of the others.

The results of competition against ^{3}H-RNA extracted from cells infected with Ad2$^+$ND$_1$ are shown in Fig. 1. As expected, unlabeled Ad2$^+$ND$_1$ RNA competed efficiently with the Ad2$^+$ND$_1$ ^{3}H-RNA for SV40 DNA binding sites. The RNAs transcribed from each of the three hybrid viruses with a larger content of SV40 DNA than Ad2$^+$ND$_1$ (10) were also effective competitors of Ad2$^+$ND$_1$ ^{3}H-RNA, indicating that these hybrids (Ad2$^+$ND$_2$, Ad2$^+$ND$_4$ and Ad2$^+$ND$_5$) induce all of the SV40-specific sequences induced by Ad2$^+$ND$_1$. RNA from cells infected with Ad2$^+$ND$_3$, the hybrid virus which contains the smallest amount of SV40 DNA (<5% of the SV40 genome [10]), competed with only 39% of the SV40-specific counts per minute in Ad2$^+$ND$_1$ ^{3}H-RNA. It is interesting that Ad2$^+$ND$_5$ appears to induce all of the SV40-specific RNA sequences induced by Ad2$^+$ND$_1$, because Ad2$^+$ND$_5$ does not induce any detectable SV40 antigens (20), whereas Ad2$^+$ND$_1$ induces SV40 U antigen (21).

The results of competition against ^{3}H-RNA from cells infected with Ad2$^+$ND$_2$ are shown in Fig. 2. As expected, unlabeled Ad2$^+$ND$_2$ RNA is an effective competitor of Ad2$^+$ND$_2$ ^{3}H-RNA. RNA from cells infected with Ad2$^+$ND$_4$, the hybrid virus which contains the largest segment of SV40 DNA, is also an effective competitor of Ad2$^+$ND$_2$ ^{3}H-RNA. However, Ad2$^+$ND$_3$ RNA competed with only 15%, Ad2$^+$ND$_1$ RNA with only 44%, and Ad2$^+$ND$_5$ RNA with only 82% of the SV40-specific counts per minute in Ad2$^+$ND$_2$ ^{3}H-RNA. These data indicate that only Ad2$^+$ ND$_4$ induces all of the SV40-specific RNA sequences transcribed in cells infected with Ad2$^+$ND$_2$. The extent of competition observed with the RNA induced by each of the other hybrid viruses appears to be proportional to its relative content of SV40 DNA.

The quantity of SV40-specific RNA induced by Ad2$^+$ND$_3$ is small (20) and thus large amounts of ^{3}H-RNA from Ad2$^+$ND$_3$-infected cells were required to yield sufficient SV40-specific counts per minute for competition experiments. Moreover, even at saturation, Ad2$^+$ND$_3$ ^{3}H-RNA yielded fewer SV40-specific counts per minute bound to filters containing 0.1 μg of SV40 DNA than did ^{3}H-RNA from cells infected with any of the other hybrids, a result which is not unexpected if the RNA induced by Ad2$^+$ND$_3$ is complementary to a smaller portion of the SV40 genome. Consequently, filters containing 1.0 μg of SV40 DNA were used in experiments with Ad2$^+$ND$_3$ ^{3}H-RNA, whereas competition experiments with the other four hybrid virus ^{3}H-RNAs were accomplished with filters containing only 0.1 μg. It is apparent that all of the hybrids induced RNA which competed efficiently with Ad2$^+$ND$_3$ ^{3}H-RNA (Fig. 3). Thus it appears that the SV40-specific RNA sequences induced by Ad2$^+$ND$_3$ are also induced by the other four hybrid viruses.

The relationship between the RNA induced by each of the hybrid viruses and the ^{3}H-RNA induced by Ad2$^+$ND$_4$ is shown in Fig. 4. The extent of competition observed with each hybrid RNA is proportional to the relative amount of SV40 DNA in the hybrid virus genome (Table

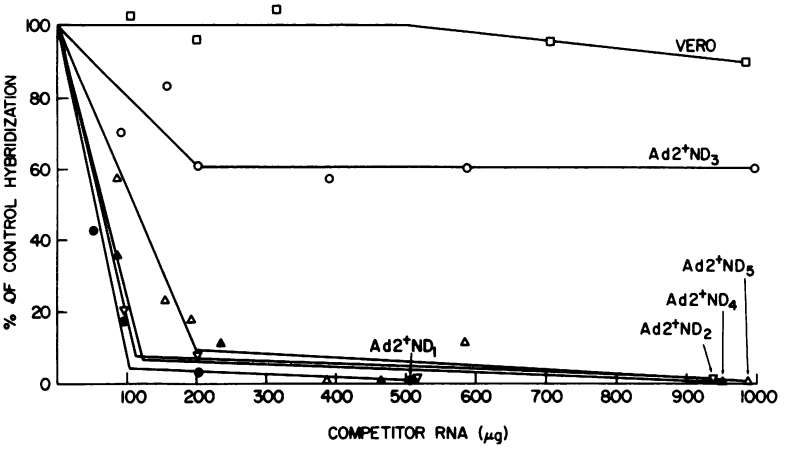

FIG. 1. *Hybridization-competition of unlabeled nondefective hybrid virus-specific RNAs with Ad2⁺ND₁ ³H-RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 0.1 μg of ¹⁴C-SV40 DNA filters as described in the text. Radioactive RNA was added to reaction vials after filter had been preincubated with unlabeled RNA. The control ³H-RNA counts per minute bound (no competitor) are the ³H-counts per minute bound to the SV40 DNA filter minus the counts per minute bound to an appropriate E. coli DNA (blank) filter. The average of duplicate determinations is shown, and the counts per minute bound at other points in the competition curves are plotted as percentage of this control value. Nonspecific competition was separately assayed with unlabeled, uninfected Vero cell RNA. Input of Ad2⁺ND₁ ³H-RNA = 108 μg; 106 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 30. Control ³H-RNA counts per minute bound (no competitor) = 159.*

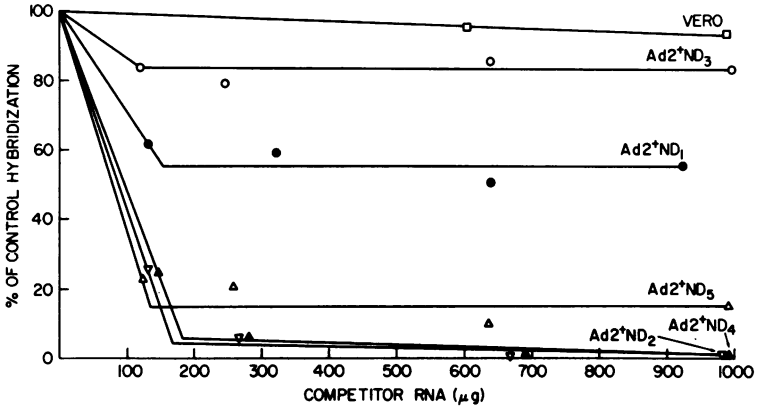

FIG. 2. *Hybridization-competition of unlabeled nondefective hybrid virus-specific RNAs with Ad2⁺ND₂ ³H-RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 0.1 μg of ¹⁴C-SV40 DNA filters. Other conditions as indicated in the legend to Fig. 1. Input of Ad2⁺ND₂ ³H-RNA = 132 μg; 91 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 30. Control ³H-RNA counts per minute bound (no competitor) = 203.*

1). Thus Ad2⁺ND₁, which contains 29% as much SV40 DNA as Ad2⁺ND₄, induces RNA which competes with 30% of the SV40-specific counts per minute in Ad2⁺ND₄ ³H-RNA; Ad2⁺ND₂, which contains 75% as much SV40 DNA as Ad2⁺ND₄, competes with 72% of the SV40-specific counts per minute in Ad2⁺ND₄ ³H-RNA; Ad2⁺ND₃, which contains 6% as much SV40 DNA as Ad2⁺ND₄, competes with 8% of the SV40-specific counts per minute in Ad2⁺ND₄ ³H-RNA; and Ad2⁺ND₅, which contains 63% as much SV40 DNA as Ad2⁺ND₄, competes with 58% of the SV40-specific counts per minute in Ad2⁺ND₄ ³H-RNA.

The results of competition against ³H-RNA from cells infected with Ad2⁺ND₅ are shown in Fig. 5. Ad2⁺ND₂ and Ad2⁺ND₄ both induce RNA with all of the SV40 sequences found in Ad2⁺ND₅ ³H-RNA, whereas the RNA induced by Ad2⁺ND₁ competed with only 55% and that

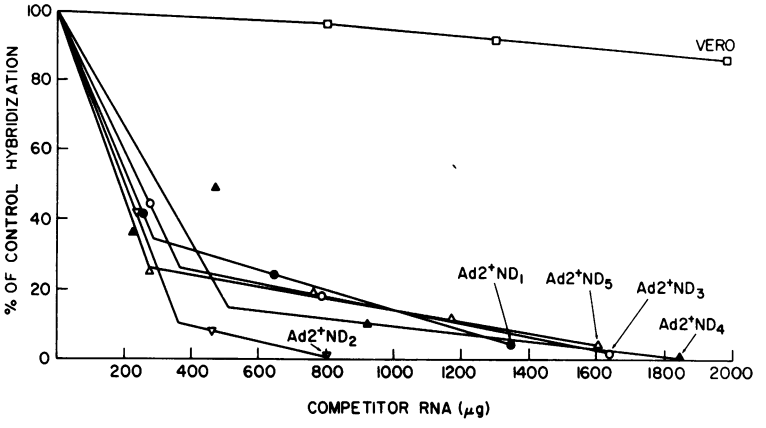

FIG. 3. *Hybridization-competition of unlabeled nondefective hybrid virus-specific RNAs with Ad2⁺ND₃ ³H-RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 1.0 μg of ¹⁴C-SV40 DNA filters. Other conditions as indicated in the legend to Fig. 1. Input of Ad2⁺ND₃ ³H-RNA = 256 μg; 25 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 1.0 μg of E. coli DNA filters = 9. Control ³H-RNA counts per minute bound (no competitor) = 80.*

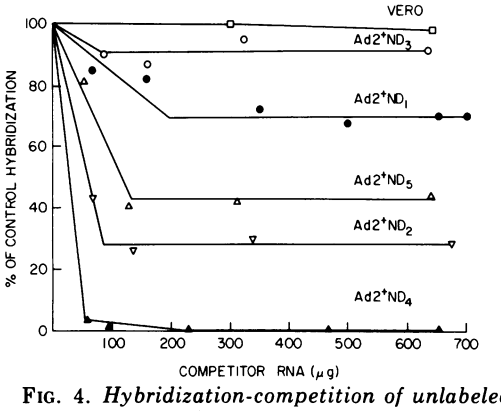

FIG. 4. *Hybridization-competition of unlabeled nondefective hybrid virus-specific RNAs with Ad2⁺ND₄ ³H-RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 0.1 μg of ¹⁴C-SV40 DNA filters. Other conditions as indicated in the legend to Fig. 1. Input of Ad2⁺ND₄ ³H-RNA = 66 μg; 38 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 6. Control ³H-RNA counts per minute bound (no competitor) = 158.*

induced by Ad2⁺ND₃ with only 24% of the SV40-specific counts per minute in Ad2⁺ND₅ ³H-RNA. Thus, in spite of its failure to induce detectable SV40 U antigen, Ad2⁺ND₅ induces SV40-specific RNA sequences corresponding to a larger fraction of the SV40 genome than does the U antigen-inducing hybrid, Ad2⁺ND₁.

Relationship between the SV40-specific RNA induced by Ad2⁺ND₄ and the early and late RNA induced by wild-type SV40. Ad2⁺ND₄ RNA appears to contain all of the SV40-specific nucleotide sequences induced by the other four nondefective hybrid viruses. Consequently, we compared the SV40-specific RNA sequences induced by Ad2⁺ND₄ with those transcribed early and late in SV40 lytic infection. We have previously shown that in Vero cells, 10 μg of cytosine arabinoside (CA) per ml inhibits SV40 DNA synthesis by more than 99% and prevents the formation of SV40 capsid (V) antigen without preventing the formation of SV40 tumor (T) antigen. Early SV40 RNA (produced in the presence of CA) was found to compete with only 35% of the SV40-specific counts per minute in late SV40 ³H-RNA (RNA synthesized after the onset of viral DNA replication), indicating that early genes (i.e., genes expressed prior to viral DNA replication) comprise only a fraction of the SV40 genome (1, 24–26, 30). Since all three SV40-specific antigens induced by Ad2⁺ND₄ appear to represent the expression of early SV40 genes (6, 8, 21, 27), it was particularly interesting to compare the SV40-specific RNA sequences in Ad2⁺ND₄ RNA with those in early SV40 RNA.

The results of competition of unlabeled Ad2⁺ND₄ RNA with early SV40 ³H-RNA are shown in Fig. 6. The RNA induced by Ad2⁺ND₄ RNA is an effective competitor of early SV40 RNA, and thus it appears that Ad2⁺ND₄ induces SV40-specific RNA sequences corresponding to all of the early SV40 genes.

The inverse experiment, i.e., competition of unlabeled early SV40 RNA with Ad2⁺ND₄ ³H-RNA, is technically more difficult. The concentration of SV40-specific RNA sequences

TABLE 1. *Summary of biological and biochemical properties of the nondefective Ad2-SV40 hybrid viruses*

Hybrid virus	Host range	SV40 antigen induction				Relative amount of SV40 DNA[a]	Extent of competition by unlabeled hybrid virus-induced RNA[b] with SV40-specific ^{3}H-RNA induced by:					
		T	U	TSTA	V		Ad2+ND$_4$	Ad2+ND$_2$	Ad2+ND$_5$	Ad2+ND$_1$	Ad2+ND$_3$	Early SV40 infection
Ad2+ND$_4$	AGMK HEK	+	+	+		1.00	100	100	100	100	100	100
Ad2+ND$_2$	AGMK HEK		+	+		0.75	72	100	100	100	100	—[c]
Ad2+ND$_5$	HEK					0.63	58	82	100	100	100	—
Ad2+ND$_1$	AGMK HEK		+			0.29	30	44	55	100	100	40[d]
Ad2+ND$_3$	HEK					0.06	8	15	24	39	100	—

[a] Calculated from data in Fig. 5, reference 10.

[b] Expressed as the percentage of inhibition of the control (i.e., no specific competitor present) hybridization reaction. Values for nonspecific competition are shown in Fig. 1–8 and are not subtracted.

[c] Not done.

[d] Reference 26.

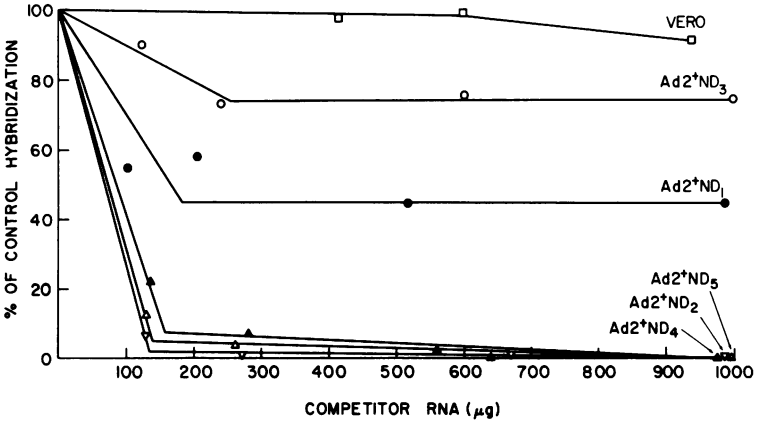

FIG. 5. *Hybridization-competition of unlabeled nondefective hybrid virus-specific RNAs with Ad2+ND$_5$ ^{3}H-RNA. Hybridization-competition experiments were performed with a saturating amount of ^{3}H-RNA and 0.1 μg of ^{14}C-SV40 DNA filters. Other conditions as indicated in the legend to Fig. 1. Input of Ad2+ND$_5$ ^{3}H-RNA = 120 μg; 78 × 10^3 counts per minute per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 25. Control ^{3}H-RNA counts per minute bound (no competitor) = 173.*

present early in SV40 infection is very low (1, 22, 24–26, 30), and consequently very large amounts of this competitor are required. Since early SV40 RNA produced in the presence of CA is transcribed exclusively from the DNA of incoming virions, the multiplicity of infection was increased to more than 300 PFU per cell in an attempt to increase the concentration of SV40 sequences (there was no detectable breakthrough of late RNA synthesis at this multiplicity, see Fig. 8). Nevertheless, even with the addition of more than 2 mg of competitor prepared in this manner (resulting in a total RNA concentration of 10 mg/ml in the hybridization reaction mixture), early SV40 RNA could be shown to compete with only 92% of the SV40-specific counts per minute in Ad2+ND$_4$ ^{3}H-RNA (Fig. 7). Yet in spite of the large amount of competitor employed, it does not appear that a plateau of competition was

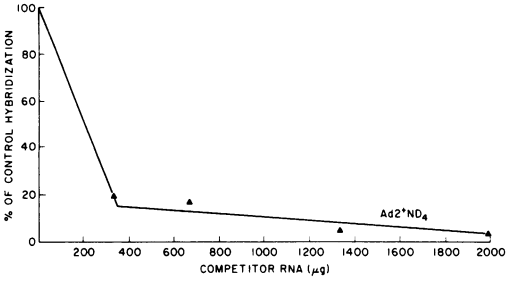

FIG. 6. *Hybridization-competition of unlabeled Ad2⁺ND₄ RNA with ³H-SV40 early RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 0.01 μg of SV40 DNA filters as described in the text. Nonspecific competition was internally controlled by mixing unlabeled, uninfected Vero cell RNA with unlabeled competitor RNA in varying ratios, so that a constant total amount of unlabeled RNA was present at each point in the competition experiment. Other conditions as indicated in the legend to Fig. 1. Input of ³H-SV40 early RNA = 277 μg; 180 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 0.005 μg of E. coli DNA filters = 63. Control ³H-RNA counts per minute bound (only uninfected, unlabeled Vero cell RNA present) = 263.*

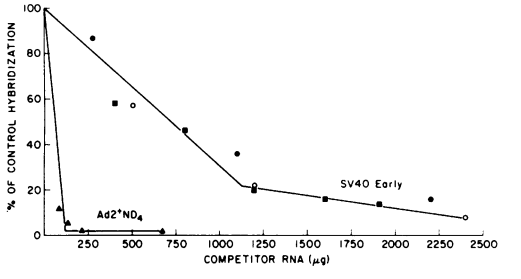

FIG. 7. *Hybridization-competition of unlabeled SV40 early RNA with Ad2⁺ND₄ ³H-RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 0.1 μg of ¹⁴C-SV40 DNA filters as described in the text. All conditions as indicated in the legend to Fig. 6. Input of Ad2⁺ND₄ ³H-RNA = 73 μg; 63 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 10. Control ³H-RNA counts per minute bound (only uninfected, unlabeled Vero cell RNA present) = 229. Three different batches of unlabeled SV40 early RNA were employed, as indicated by the different symbols.*

reached. This failure to reach a plateau is not due to nonspecific competition, for that is internally controlled by the addition of unlabeled RNA from uninfected Vero cells so that the total amount of RNA present is the same for every point on the curve. Thus the 92% competition that was demonstrated almost certainly represents an underestimate. In any case, it is apparent (compare Fig. 4 and 7) that early SV40 RNA contains a significantly greater proportion of the SV40-specific sequences induced by Ad2⁺ND₄ than does the RNA induced by any of the other hybrid viruses. These observations suggest that there is little, if any, transcription of late SV40 genetic information in Ad2⁺ND₄-infected Vero cells. Furthermore, unlabeled Ad2⁺ND₄ RNA was found to compete with a maximum of 43%, and unlabeled early SV40 RNA with 38%, of the SV40-specific counts per minute in late SV40 ³H-RNA (Fig. 8). Since Ad2⁺ND₄ RNA appears to contain all of the SV40 nucleotide sequences present in early SV40 RNA (Fig. 6), these data also suggest that if any late sequences are transcribed in Ad2⁺ND₄-infected cells, they must correspond to a very small percentage of the total genetic information in late SV40 RNA.

DISCUSSION

The reciprocal competition experiments (Fig. 1–5) demonstrate that certain SV40 nucleotide sequences are common to all five of the nondefective Ad2-SV40 hybrid viruses. Thus the SV40 RNA induced by AD2⁺ND₃ (the hybrid with the smallest portion of SV40 DNA) competes with some of the SV40-specific RNA sequences induced by each of the other hybrid viruses. Furthermore, since the SV40 RNA induced by each of the other hybrids competes with 100% of the SV40-specific counts per minute in Ad2⁺ND₃ ³H-RNA (Fig. 3), it is clear that all of the SV40 RNA sequences induced by AD2⁺ND₃ are also induced by each of the other hybrid viruses.

When biological and biochemical data from

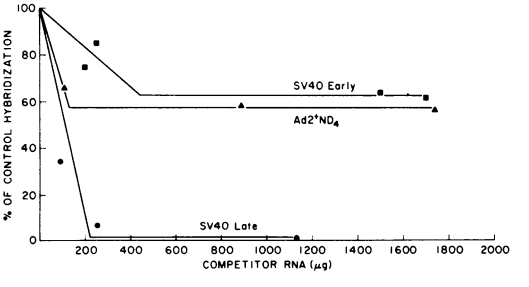

FIG. 8. *Hybridization-competition of unlabeled SV40 early RNA and Ad2⁺ND₄ RNA with ³H-SV40 late RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and 0.1 μg of ¹⁴C-SV40 DNA filters as described in the text. All conditions as indicated in the legend to Fig. 6. Input of ³H-SV40 late RNA = 93 μg; 86 × 10³ counts per minute per μg of RNA. Average counts per minute bound to 0.1 μg of E. coli DNA filters = 10. Control ³H-RNA counts per minute bound (only uninfected, unlabeled Vero cell RNA present) = 214.*

this and the two accompanying reports (10, 20) are summarized (see Table 1), it is apparent that the relative number of SV40-specific nucleotide sequences transcribed in cells infected with each of the five hybrid viruses is proportional to the amount of its SV40 DNA. Moreover, no SV40-specific RNA sequences are detected in cells infected with any one hybrid virus which are not also present in cells infected with each hybrid that contains a larger amount of SV40 DNA. For example, Ad2$^+$ND$_4$ (the hybrid with the largest SV40 DNA region) induces all of the SV40-specific RNA sequences detected in cells infected with each of the other hybrid viruses. AD2$^+$ND$_2$ (the hybrid with the second largest SV40 DNA region) induces all of the SV40-specific RNA sequences induced by Ad2$^+$ND$_1$, Ad2$^+$ND$_3$ and Ad2$^+$ND$_5$, but only 72% of those induced by Ad2$^+$ND$_4$ (Table 1). Thus, at least with respect to those nucleotide sequences which are transcribed in infected cells, the SV40 portions of all five hybrid viruses appear to overlap one another, with the genetic information in the smaller SV40 segments entirely represented in the larger ones. These observations suggest that the SV40 DNA contained in each of the five nondefective Ad2-SV40 hybrid viruses is derived from the same region of the SV40 genome.

All three of the SV40-specific antigens detected in cells infected with these hybrid viruses are early SV40 antigens (6, 8, 21, 27). Moreover, none of the hybrids induce detectable SV40 V antigen, which is synthesized late in SV40 lytic infection (1, 22, 24–27, 30). Thus, the SV40 DNA in these hybrids must originate from a region of the SV40 genome which contains early SV40 genes. To further characterize the SV40 genetic information in these five nondefective Ad2-SV40 hybrid viruses, the RNA induced by Ad2$^+$ND$_4$ (which contains all of the SV40-specific nucleotide sequences induced by the other four hybrids) was compared with the RNA synthesized early and late in wild-type SV40 lytic infection. We have previously shown that Ad2$^+$ND$_1$, the first of these hybrid viruses to be isolated and characterized (5, 16, 19), induces approximately 40% of the SV40 RNA sequences in early SV40 RNA and does not appear to induce late SV40 RNA sequences (26). In the present study, the RNA induced by Ad2$^+$ND$_4$ has been shown to compete efficiently with all of the SV40-specific RNA sequences present in early SV40 ^{3}H-RNA (Fig. 6). However, in the reciprocal reaction, we were not able to demonstrate that unlabeled early SV40 RNA competed with all of the SV40-specific RNA sequences present in Ad2$^+$ND$_4$ ^{3}H-RNA (Fig. 7). Thus, it is possible that, in addition to inducing the full complement of early SV40 RNA sequences, Ad2$^+$ND$_4$ also induces a small amount of late SV40 RNA.

The results of two additional experiments bear on the question of the transcription of late SV40 genes in Ad2$^+$ND$_4$ infected cells. (i) Early SV40 genes continue to be transcribed late in SV40 lytic infection and, as a consequence, unlabeled early SV40 RNA competes with 30 to 40% of the SV40-specific counts per minute in late SV40 ^{3}H-RNA (Fig. 8; 1, 22, 24–26, 30). Since Ad2$^+$ND$_4$ RNA appears to contain all of the SV40 nucleotide sequences in early SV40 RNA, the fact that it competes with a maximum of only 43% of the SV40-specific counts per minute in late SV40 ^{3}H-RNA (Fig. 8) suggests that if Ad2$^+$ND$_4$ does induce any late SV40 RNA sequences, they must correspond to a very small fraction of the SV40 genome.

(ii) Westphal's demonstration that transcription of form I SV40 viral DNA in vitro by the *E. coli* DNA-dependent RNA polymerase is highly asymmetric (31) has led to the physical separation of the SV40 DNA strands (14, 15, 29). Only one of the viral DNA strands (the E or "minus" strand) is transcribed in vitro by the *E. coli* polymerase. Moreover, early SV40 RNA (from acutely infected Vero cells) also appears to be transcribed exclusively from nucleotide sequences in the minus strand. The other DNA strand (the L or "plus" strand) apparently carries only late SV40 genetic information (14, 15, 29). Since these properties suggested that separated SV40 DNA strands might be effectively employed to differentiate early and late SV40 RNA, RNA from Ad2$^+$ND$_4$-infected Vero cells was tested for its ability to hybridize with the minus and plus strands of SV40 DNA. The Ad2$^+$ND$_4$ RNA was found to hybridize exclusively with the minus strand of SV40, behaving in a manner indistinguishable from that of early SV40 RNA (G. Khoury et al., manuscript in preparation). In contrast, late SV40 RNA (which contains a mixture of early and late SV40 RNA sequences) hybridizes with both the minus and plus strands. Thus the results of this experiment also suggest that Ad2$^+$ND$_4$ does not induce detectable amounts of late SV40 RNA. However, none of the experimental results described herein exclude the possibility that a small number of SV40 anti-late sequences are transcribed from the hybrid virus genomes.

The biological activities of these hybrid viruses reflect the amount and the overlapping nature of the SV40 genetic information which they contain (Table 1). Ad2$^+$ND$_4$, which con-

tains the largest SV40 DNA segment and induces the greatest number of SV40-specific RNA sequences, induces three SV40-specific antigens: T antigen, U antigen, and tumor-specific transplantation antigen (TSTA; A. M. Lewis, Jr., and W. P. Rowe, manuscript in preparation). $Ad2^+ND_2$, which contains 75% as much SV40 DNA and induces 72% as many SV40-specific RNA sequences as $Ad2^+ND_4$, induces two SV40-specific antigens—U antigen and TSTA. $Ad2^+ND_1$, which contains 29% as much SV40 DNA and induces 30% as many SV40-specific RNA sequences as $Ad2^+ND_4$, induces one SV40-specific antigen—U antigen.

The biological behavior of $Ad2^+ND_3$ and $Ad2^+ND_5$ is quite puzzling. Although these hybrids contain SV40 DNA and induce SV40-specific RNA, they both fail to induce detectable SV40 antigens and they both fail to replicate efficiently in AGMK cells. $Ad2^+ND_3$, which contains only 6% as much SV40 DNA and induces only 8% as many SV40-specific RNA sequences as $Ad2^+ND_4$, may simply contain too little SV40 genetic information to code for a functional SV40 protein. However, this explanation cannot apply to $Ad2^+ND_5$, which contains more SV40 DNA than $Ad2^+ND_1$ and which appears to induce all of the SV40-specific RNA sequences detected in $Ad2^+ND_1$-infected cells. Perhaps some information required for the translation of the SV40-specific RNA is missing from either the Ad or the SV40 component of the $Ad2^+ND_5$ genome, or small changes in nucleotide sequences (undetectable by nucleic acid hybridization-competition experiments) have led to the synthesis of proteins with altered antigenicity. It is also possible that some SV40 RNA species are rapidly degraded in the nuclei of infected cells and therefore are not detectable in the experiments reported herein, which employ RNA labeled for 20 h. More precise information about transcription, particularly of value in the case of $Ad2^+ND_5$, might be obtained with briefly labeled nuclear RNA of high specific activity.

The presence of all of the early SV40 genetic information within the small segment of SV40 DNA contained in $Ad2^+ND_4$ suggests that the early genes of wild-type SV40 are localized in one portion of the wild-type SV40 DNA molecule. Furthermore, the data in Table 1 suggest that the U and TSTA functions are adjacent in the SV40 DNA segment of $Ad2^+ND_2$. Thus it seems likely that these two early genes are also adjacent in the wild-type SV40 genome. The SV40 DNA segment of $Ad2^+ND_4$ contains the U, TSTA, and T functions, but the arrangement of the three detectable early SV40 functions (U, TSTA, T, or T, U, TSTA) cannot be determined from the data presented here. Electron microscope investigation of heteroduplexes formed between the nondefective hybrid DNAs (T. J. Kelly, Jr. and A. M. Lewis, Jr., manuscript in preparation) has confirmed the results of the RNA-DNA reciprocal competition experiments. These heteroduplex mapping studies have shown that the linear SV40 DNA segments within the hybrids form an overlapping series beginning at a common origin on the Ad2 DNA molecule.

ACKNOWLEDGMENTS

We thank Wallace P. Rowe and John F. Enders for their constant advice and encouragement during the course of these studies and acknowledge the excellent technical assistance of Anna Kantonis, Helen M. Eliot, John Jones, Margareta Simpson, and Aurella G. Krezlewicz.

This investigation was aided by Public Health Service grants CA-12557 from the National Cancer Institute and AI-01992 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. 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.
2. Brown, A. H. 1946. Determination of pentose in the presence of large quantities of glucose. Arch. Biochem. **11**:269-278.
3. Burnett, J. P., A. O. Summers, S. A. Harrington, and A. C. Dwyer. 1968. Production of highly labeled adenoviruses. Appl. Microbiol. **16**:1245-1250.
4. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**:315-323.
5. 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.
6. Gilden, R. V., R. I. Carp, F. Taguchi, and V. Defendi. 1965. The nature and localization of the SV40-induced complement-fixing antigen. Proc. Nat. Acad. Sci. U.S.A. **53**:684-692.
7. Gillespie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. J. Mol. Biol. **12**:829-842.
8. Girardi, A. J., and V. Defendi. 1970. Induction of SV40 transplantation antigen (Tr Ag) during the lytic cycle. Virology **42**:688-698.
9. Hayflick, L., 1965. Tissue culture and mycoplasma. Tex. Rep. Biol. Med. **23**:285-303.
10. Henry, P. H., L. E. Schnipper, R. J. Samaha, C. S. Crumpacker, A. M. Lewis, Jr., and A. S. Levine. 1973. Studies of nonfective adenovirus 2-simian virus 40 hybrid viruses. VI. Characterization of the deoxyribonucleic acid from five nondefective hybrid viruses. J. Virol. **11**:665-671.
11. Hopps, H., B. C. Bernheim, A. Nisalak, J. H. Tjio, and J.

E. Smadel. 1963. Biologic characteristics of a continuous kidney cell line derived from the African green monkey. J. Immunol. **91**:416–424.
12. Jensen, F. C., A. J. Girardi, R. V. Gilden, and H. Koprowski. 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. Proc. Nat. Acad. Sci. U.S.A. **52**:53–59.
13. Kelly, T. J., Jr., and J. A. Rose. 1971. Simian virus 40 integration site in an adenovirus 7-simian virus 40 hybrid DNA molecule. Proc. Nat. Acad. Sci. U.S.A. **68**:1037–1041.
14. 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.
15. Khoury, G. and M. A. Martin. 1972. Comparison of SV40 DNA transcription in vivo and in vitro. Nature N. Biol. **238**:4–6.
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. Levin, M. J., M. N. Oxman, G. T. Diamandopoulos, A. S. Levine, P. H. Henry, and J. F. Enders. 1969. Virus-specific nucleic acids in SV40 exposed hamster embryo cell lines: correlation with S and T antigens. Proc. Nat. Acad. Sci. U.S.A. **62**:589–596.
18. Levine, A. S., M. N. Oxman, M. J. Levin, P. H. Henry, G. T. Diamandopoulos, and J. F. Enders. 1970. Virus-specific deoxyribonucleic acid in simian virus 40-exposed hamster cells: correlation with S and T antigens. J. Virol. **6**:199–207.
19. Lewis, A. M., Jr., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry. 1969. A nondefective (competent) adenovirus-SV40 hybrid isolated from the Ad2-SV40 hybrid population. Proc. Nat. Acad. Sci. U.S.A. **63**:1128–1135.
20. 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 five hybrids which differ in their simian virus 40-specific biological properties. J. Virol. **11**:655–664.
21. 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.
22. Lindstrom, D. M., and R. Dulbecco. 1972. Strand orientation of simian virus 40 transcription in productively infected cells. Proc. Nat. Acad. Sci. U.S.A. **69**:1517–1520.
23. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
24. Martin, M. A., and D. Axelrod. 1969. The transcription of the SV40 genome in productively infected and transformed cells. Proc. Nat. Acad. Sci. U.S.A. **64**:1203–1210.
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. IV. 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. Rapp, F., J. L. Melnick, and T. Kitahara. 1965. Tumor and virus antigens of simian virus 40: differential inhibition of synthesis by cytosine arabinoside. Science **147**:625–627.
28. Rose, J. A., P. R. Reich, and S. M. Weissman. 1965. RNA production in adenovirus-infected KB cells. Virology **27**:571–579.
29. 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.
30. 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.
31. Westphal, H. 1970. SV40 DNA strand selection by Escherichia coli RNA polymerase. J. Mol. Biol. **50**:407–420.
32. Yasumura, P., and Y. Kawakita. 1963. Studies on SV40 by tissue culture methods: preliminary report of transformation studies in vitro. Nippon Rinsho **21**:1201–1219.