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+ND₁

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Ad2+ND₁, a nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid virus, has been previously shown to contain a small segment of the SV40 genome covalently linked to Ad2 deoxyribonucleic acid (DNA). The SV40 portion of this hybrid virus has been characterized by relating the SV40-specific ribonucleic acid (RNA) sequences transcribed from the Ad2+ND₁ DNA to those transcribed from the DNA of SV40 itself. RNA-DNA hybridization-competition studies indicate that the SV40 component of Ad2+ND₁ consists of some, but not all, of that part of the SV40 genome which is transcribed early, i.e., prior to viral DNA replication, in SV40 lytic infection.

The oncogenic capacity of simian virus 40 (SV40) appears to be closely associated with the activity of one or more of its genes, although the mechanisms involved are presently unknown (1, 14, 16). Identification of the functions of the SV40 genes associated with oncogenicity should contribute to an understanding of this biological phenomenon. Since the SV40 genome is small (3, 12), containing enough information for only 5 to 8 proteins of average size (150 to 200 amino acids each), an analysis of the viral genome is feasible. In an attempt to characterize individual SV40 genes, we have begun to utilize nondefective adenovirus-SV40 (Ad-SV40) hybrid viruses (23, 24) as a source of well-defined segments of the SV40 genome.

Investigations of several Ad-SV40 hybrid viruses have demonstrated that each population contains hybrid virions composed of covalently linked Ad and SV40 deoxyribonucleic acid (DNA) within Ad capsids (4, 21, 30, 33, 36). The first such hybrid viruses described were defective; replication of the hybrid virions required simultaneous infection with nonhybrid (helper) Ad (7, 35). In contrast to these defective hybrid viruses, the recently isolated nondefective Ad2-SV40 hy-

brid virus, Ad2+ND₁, replicates efficiently without the aid of nonhybrid Ad. This nondefective hybrid virus grows to high titer [10^8 to 10^9 plaque-forming units (PFU)/ml] and forms plaques with one-hit kinetics in both human embryonic kidney and primary African green monkey kidney cells (23). Although failing to induce SV40 T antigen, the Ad2+ND₁ virus induces an additional SV40 antigen, the U antigen (23, 24). The SV40 U antigen, like the T antigen, appears early during the replicative cycle of SV40 (prior to synthesis of viral DNA) and is synthesized in the presence of inhibitors of DNA synthesis (24). However, U antigen is heat stable whereas T antigen is not (24). The genome of the Ad2+ND₁ virus is a recombinant molecule with a molecular weight of 22×10^6 to 25×10^6 daltons (13, 21). It is composed of approximately 99% Ad2 DNA and 1% (220,000 to 250,000 daltons) SV40 DNA (21). Thus Ad2+ND₁ is equivalent to a deletion mutant of SV40 which contains only enough SV40 DNA to code for one or two proteins. However, in contrast to the deletion mutants of SV40 itself (41) which cannot replicate without helper SV40 virions, Ad2+ND₁ virions can be propagated and plaque-purified in the ab-

sence of helper viruses. Therefore, genetically homogeneous stocks of this hybrid virus may be obtained, and the techniques of nucleic acid hybridization and hybridization-competition may be utilized to characterize its SV40 genetic information.

This report describes the relationship of the SV40-specific ribonucleic acid (RNA) sequences transcribed from the Ad2⁺ND₁ DNA to those transcribed from the DNA of SV40 itself.

MATERIALS AND METHODS

Tissue culture. The BSC-1 (18) and Vero (40) lines of African green monkey kidney (AGMK) cells were grown in Eagles' minimal essential medium supplemented with penicillin (250 units/ml), streptomycin (250 µg/ml), and 2 mM glutamine (EMEM) plus 10% fetal bovine serum (FBS). Roller bottle cultures were refed biweekly until confluent monolayers were formed, at which time the medium was replaced with EMEM plus 2% FBS. Primary AGMK and human embryonic kidney (HEK) cells were grown in EMEM plus 10% FBS and maintained in EMEM plus 2% agammaglobulinic calf serum (AGCS).

Viruses. A pool of SV40 strain 777 (6) was produced in BSC-1 cells infected at low multiplicity (approximately 10^{-5} PFU/cell) (5). The infected cultures were refed biweekly. When all cells exhibited cytopathic effect (CPE), the combined cells and supernatant fluids were harvested, frozen and thawed three times, clarified by centrifugation at $1,000 \times g$ for 30 min, and stored in portions at -70°C . This SV40 seed pool ($10^{9.3}$ PFU/ml) served as the inoculum for the preparation of all SV40 stocks used to obtain SV40 DNA and to produce SV40-specific RNA. Like the seed pool, these virus stocks ($10^{8.7}$ to $10^{9.3}$ PFU/ml) were produced in BSC-1 cells utilizing low multiplicity infection.

Ad2⁺ND₁ virus (23) was maintained in primary HEK cells. Pools of virus, representing passages 9 to 13, were grown in monolayer cultures of primary HEK in 32-oz glass prescription bottles (ca. 960 ml). These pools had titers ranging from $10^{8.4}$ to $10^{8.8}$ PFU/ml by plaque assay in HEK cells, and each pool was shown by complement fixation tests to induce SV40 U antigen (24) in tube cultures of either HEK or AGMK cells.

Ad2 (strain Ad6) was maintained in the Laboratory of Viral Diseases, National Institutes of Health, by serial passage in HEK cells.

All virus pools were demonstrated to be free of adeno-associated virus types 1 to 4 by complement fixation tests.

Radiolabeling of viral DNA and virus purification. SV40 labeled with ^{14}C -thymidine was grown as described above except that the multiplicity of infection was approximately 10 PFU per cell. When 5 to 10% of the cells exhibited SV40 CPE (approximately 72 hr after infection), the medium was replaced with 25 ml of EMEM plus 5% FBS per bottle containing 0.5 µCi of thymidine-2- ^{14}C (New England Nuclear, 0.1 mCi/ml, 28 mCi/mM) per ml. When all of the cells

showed SV40 CPE, cells and medium were harvested and stored at -70°C . This procedure resulted in SV40 whose DNA had a specific activity of 4,700 counts per min per µg.

Labeled and unlabeled SV40 were purified by a modification of the technique of Burnett et al. (9). The crude SV40 harvest was centrifuged for 16 hr (Spinco 21 rotor; $40,000 \times g$), and the pellet was frozen and thawed three times and resuspended in 25 ml of the supernatant fluid. Sodium deoxycholate was added to a final concentration of 1%, and the mixture was stirred for 30 min at 37°C . The virus suspension was then centrifuged for 20 min at $20,000 \times g$ in a Sorvall centrifuge (RC2-B; SS-34 rotor), and the resulting supernatant fluid and pellet, both of which contained large amounts of virus, were separately processed.

The supernatant fluid was stirred for 30 min at 37°C with 50 µg of deoxyribonuclease (DPFF, Worthington Biochemical Corp.) per ml, 50 µg of pancreatic ribonuclease (XII-A, Sigma Chemical Co.) per ml, and 0.01 M magnesium acetate. *Crotalus adamanteus* venom (25 µg per ml; K&K Laboratories) was then added, and stirring was continued for an additional 15 min. Alpha-chymotrypsin (25 µg per ml; CDS, Worthington Biochemical Corp.) and subtilisin (25 µg per ml; Nagarse, Enzyme Development Corp.) were then added, and stirring was continued for a final 10 min at 37°C . This processed supernatant fluid was then diluted to 40 ml with EMEM and immediately centrifuged into a 15-ml cushion of saturated KBr (Spinco SW 25.2 rotor; $75,000 \times g$) for 2.5 hr at 4°C .

The pellet was homogenized in 15 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5, with a Ten Broeck homogenizer. The suspension was then digested with enzymes as described for the supernatant fluid, but utilizing twice the concentration of each enzyme. The enzyme-treated material was clarified by centrifugation (Sorvall SS-34 rotor; $20,000 \times g$) for 20 min, diluted to 40 ml with EMEM, and centrifuged into a KBr cushion as described above.

Both the processed supernatant fluid and the supernatant fluid from the processed pellet yielded dense opalescent bands of SV40 approximately one-third of the way into the cushion. These bands were removed, combined, dialyzed against three changes of 0.01 M Tris-0.001 M ethylenediaminetetraacetic acid (EDTA), pH 7.5 at 4°C , diluted to 40 ml, and re-cushioned as above. This band was collected, dialyzed, and then rebanded in CsCl (density = 1.34 g/cm^3) in a Spinco SW39 rotor at $100,000 \times g$ for 24 hr at 4°C . The resulting band (density = 1.34 g/cm^3) was dialyzed and stored at -70°C .

Pools of Ad2⁺ND₁ virus for DNA extraction were grown in HEK cells by inoculating confluent monolayers with 20 to 40 PFU per cell. Infected cultures were maintained in EMEM plus 2% AGCS and harvested by scraping when 75% of the cells exhibited adenovirus CPE. Cells and medium were centrifuged for 15 min at $1,000 \times g$, and the pellet was resuspended in 0.1 M Tris-buffered saline (pH 8.1) containing 0.001

m Ca^{+} and 0.001 M Mg^{+} . The virus in these suspensions was purified by a modification of the method of Burnett (39).

Extraction of viral DNA. Viral DNA was extracted from the purified virus preparations by papain (PAP, Worthington Biochemical Corp.) digestion followed by sodium dodecyl sulfate (SDS)-phenol extraction (32). Viral DNA was stored at -30°C in $0.1\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 6.9).

Acute infections cycle of SV40 in Vero cells. One day after reaching confluence, matched monolayer cultures of Vero cells in small roller bottles were changed to EMEM plus 2% AGCS. After 24 hr, they were drained and infected with SV40 at a multiplicity of 50 PFU/cell in 10 ml of EMEM plus 2% AGCS, with or without 10 μg of a DNA inhibitor, cytosine arabinoside (CA), per ml. They were rolled at 37°C for 1.5 hr, drained, washed with the same medium, and refed with 40 ml of EMEM plus 2% AGCS with or without 10 μg of CA per ml. Two hours before each chosen time point, cultures were labeled with uridine- $5\text{-}^3\text{H}$ (final concentration = 50 $\mu\text{Ci/ml}$) or thymidine-methyl- ^3H (final concentration = 30 $\mu\text{Ci/ml}$). These procedures were performed at 37°C , and all media were prewarmed to 37°C . After 4 hr, the cultures were drained and washed, and the cells were harvested by trypsinization (20). The data from these 4-hr labeling periods were expressed at the midpoints. Samples of washed cells were counted in a hemocytometer and fixed directly on glass cover slips for fluorescent-antibody (FA) staining. Harvested cells were also seeded onto glass cover slips in EMEM plus 10% FBS containing 10 μg of CA per ml and, after they had attached and spread (2 to 3 hr), were fixed for FA staining. The remaining cells were pelleted and stored at -70°C for subsequent extraction of DNA or RNA.

Preparation of early and late SV40 RNA and $\text{Ad2}^{+}\text{ND}_1$ RNA. Batches of unlabeled early SV40 RNA (SV40-specific RNA transcribed from the DNA of input virions in the absence of SV40 DNA replication) were prepared by infecting confluent roller bottle cultures of Vero cells with 40 to 60 PFU/cell of SV40 in the presence of CA as described above, except that 20 μg of CA per ml was employed, and the cells were harvested 30 to 36 hr postinfection. Early SV40 ^3H -RNA was prepared identically, except that uridine- $5\text{-}^3\text{H}$ was added (final concentration = 50 $\mu\text{Ci/ml}$) at 6 or 12 hr postinfection.

Unlabeled late SV40 RNA (SV40-specific RNA extracted after SV40 DNA replication has occurred) was prepared in a similar manner, except that a multiplicity of 20 to 30 PFU/cell was employed, CA was omitted, and the cells were harvested at 40 to 44 hr postinfection. Late SV40 ^3H -RNA was prepared identically, except that uridine- $5\text{-}^3\text{H}$ (final concentration = 50 $\mu\text{Ci/ml}$) was added 24 to 28 hr postinfection.

Matched preparations of labeled and unlabeled SV40 RNA were prepared by infecting replicate cultures of the same batch of cells, labeling some but not others, and harvesting labeled and unlabeled cultures in parallel.

$\text{Ad2}^{+}\text{ND}_1$ RNA was prepared by similarly infecting confluent roller bottle cultures of Vero cells with 40 PFU/cell of $\text{Ad2}^{+}\text{ND}_1$ and harvesting the cells 22 to 24 hr after infection. $\text{Ad2}^{+}\text{ND}_1$ ^3H -RNA was identically prepared except that uridine- $5\text{-}^3\text{H}$ (final concentration = 50 $\mu\text{Ci/ml}$) was added 4 to 5 hr postinfection.

Extraction of nucleic acids from infected and uninfected cells. DNA from Vero, BSC-1, and primary hamster cells and from *Escherichia coli* was extracted by the method of Marmur (25). RNA from infected and uninfected cells was extracted by a hot phenol-SDS procedure (20) and stored at -30°C in $2\times$ SSC plus 0.05% SDS. The concentrations of DNA solutions were determined by a modified diphenylamine reaction (10) with calf thymus DNA (Calbiochem) as a standard. RNA concentrations were determined by an orcinol reaction (8) with yeast soluble RNA (Calbiochem) as a standard.

RNA-DNA hybridization and hybridization-competition. The procedure for hybridization of ^3H -RNA with single-stranded DNA immobilized on nitrocellulose membrane filters was that of Gillespie and Spiegelman (17) with slight modifications (20). Hybridization reactions were performed at 60°C with 13-mm filters in a volume of 0.25 ml of $2\times$ SSC plus 0.05% SDS. Unless otherwise stated, an incubation period of 18 to 20 hr was employed. All RNA-DNA hybrids were washed, treated with pancreatic ribonuclease (20 $\mu\text{g/ml}$) and ribonuclease T_1 (B grade, Calbiochem; 10 units/ml) for 1 hr 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 hr and then adding a saturating amount (separately determined with the same batch of DNA filters and ^3H -RNA) of radioactive RNA. The incubation was then continued for an additional 14 hr before the filters were washed, ribonuclease-treated, and washed again; the tritium counts per minute retained were determined in a scintillation spectrometer. In most experiments, the SV40 DNA on the filters was ^{14}C -labeled to permit an accurate determination of the DNA present on the filters at the end of the final hybridization. Filters were counted for sufficient time to achieve a counting accuracy of $\pm 5\%$, or better, for both ^3H and ^{14}C . The ^3H -counts per minute bound to SV40 DNA filters were corrected for ^{14}C counted in the ^3H channel and for nonspecific binding to *E. coli* DNA. The net virus-specific ^3H -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.

DNA-DNA hybridization. SV40 DNA synthesis was determined by DNA-DNA hybridization utilizing a modification of the method of Denhardt (15). Prior to hybridization, the DNA-containing filters were preincubated for 6 hr at 60°C with $3\times$ SSC plus 0.05% bovine serum albumin (Armour Pharmaceutical). ^3H -labeled DNA extracted from SV40-infected Vero cells was denatured and sonically treated (Branson sonifier, tap 6; chamber deoxygenated with N_2 for 10 min; sonically treated twice for 30 sec). Portions of the

sonically treated DNA (20,000 counts/min; 4.8 to 5.9 μ g of DNA) were incubated with nitrocellulose filters containing single-stranded DNA (1.0 μ g of SV40 or *E. coli* DNA) in a volume of 0.3 ml of 3 $\times$ SSC plus 0.05% bovine serum albumin at 60 C for 24 hr. After hybridization, the filters were washed on each side with 150 ml of 6 $\times$ SSC at 60 C before scintillation counting. All determinations were performed in triplicate, and the counts per minute binding to *E. coli* DNA blanks (<1% of input counts per minute) were subtracted. 3 H-DNA from uninfected Vero cells did not bind to SV40 DNA to an greater extent than to *E. coli* DNA.

In vitro preparation of complementary RNA (cRNA). Tritium-labeled RNA complementary to Ad2⁺ND₁ DNA (cRNA) was synthesized in vitro by a modification (22) of the method of Chamberlain and Berg (11) by using DNA-dependent RNA polymerase (containing sigma factor) kindly supplied by Richard R. Burgess.

SV40 T- and V-antigen assays. SV40 T and V antigens were assayed by an indirect FA procedure (29). SV40 T antigen was detected with a serum pool from hamsters bearing virus-free SV40 tumors. SV40 V antigen was detected with a pool of ascitic fluid from hamsters immunized with purified SV40 virus (38). This pool, at a dilution of 1:5, does not stain SV40 T antigen in T-antigen positive SV40-transformed mouse, hamster, and human cells or in acutely infected monkey cells in which DNA synthesis has been inhibited by CA. It was used at a dilution of 1:20. Methods of quantitation have been previously described (28).

RESULTS

Properties of SV40 DNA utilized. The validity of the experiments to be described depends upon the use of SV40 DNA free of contamination by host (monkey) cell DNA. For this reason, we have described the preparation of the SV40 and its DNA in detail and tested it for host cell contamination as follows. (i) A 1- μ g amount of SV40 DNA was tested by RNA-DNA hybridization by using 20 $\times$ 10⁶ counts/min of Vero cell 3 H-RNA (10,000 counts per min per μ g of RNA). The SV40 DNA did not bind any 3 H-RNA counts per minute in excess of the 30 counts/min bound by 1.0 μ g of *E. coli* DNA.

(ii) SV40 DNA was used as a template for the preparation of SV40-complementary 3 H-cRNA (2.6 $\times$ 10⁶ counts per min per μ g of RNA). This in vitro synthesized SV40 RNA (3 $\times$ 10⁶ counts/min) was then hybridized with 100 μ g of monkey, hamster, and *E. coli* DNA. The 80 counts/min bound by monkey DNA was less than that bound by the control hamster or *E. coli* DNA species. (Each bound 100 counts/min.)

(iii) SV40 DNA filters (1 μ g) were utilized for RNA-DNA hybridization with early SV40 3 H-RNA (65,000 count per min per μ g of RNA). The hybridized 3 H-RNA was then eluted (0.1 $\times$

SSC, 90 C, 15 min) with approximately 85% efficiency (less than 5% of the DNA was eluted) and rehybridized with both SV40 DNA and monkey (BSC-1) cell DNA filters (1 μ g). Forty-seven per cent of the eluted 3 H-RNA rehybridized with SV40 DNA, whereas only 1.7% was bound to the filters containing BSC-1 DNA. Thus, in spite of the fact that most of the 3 H-RNA in preparations of early SV40 3 H-RNA is host (monkey) cell RNA, nearly all of the 3 H-RNA bound to the SV40 DNA filters during the initial hybridization reaction was complementary to SV40, rather than to monkey cell DNA.

The results of these three types of experiments indicate that the SV40 DNA used was free of detectable host cell sequences. The physical properties of this SV40 DNA have already been described (13).

Efficacy of CA. Under the conditions employed in these experiments, 10 μ g of CA per ml inhibited SV40 DNA synthesis by more than 99% and prevented the formation of SV40 V antigen (Table 1). SV40 T-antigen formation was not inhibited. In addition, samples of the cells harvested for each preparation of early SV40 RNA were tested for V antigen. In no case did they contain detectable SV40 V antigen.

Acute infectious cycle of SV40 in Vero cells. The time course of SV40 infection was studied in Vero cells in the presence or absence of an inhibitor of DNA synthesis, CA. SV40 T antigen and SV40-specific RNA were detected as early as 6 hr postinfection, both in the presence and in the absence of CA, and reached maximal levels between 24 and 30 hr (Fig. 1). In contrast, CA effectively prevented the synthesis of SV40 DNA and V antigen, both of which were detectable at 12 hr postinfection in untreated cultures. These observations indicate that inhibitors of DNA replication are required to obtain appreciable quantities of early SV40 RNA free of late SV40 RNA contamination. We have also observed the appearance of SV40 V antigen in BSC-1 and primary AGMK cells (Fig. 2) at 12 hr postinfection, indicating that inhibitors of DNA replication are needed for the preparation of early SV40 RNA in these cells as well. The time course of T-antigen synthesis was not altered by the presence of CA (Fig. 1). The SV40-specific RNA measured at the plateau in CA-treated Vero cells (early SV40 RNA) represented approximately 2% of the amount of SV40-specific RNA synthesized in the absence of CA.

Characterization of early and late SV40 RNA by hybridization-competition. As shown in Fig. 3A, unlabeled late SV40 RNA competed efficiently (more than 90%) with late SV40 3 H-RNA.

TABLE 1. *Effect of cytosine arabinoside (CA) on total and simian virus 40 (SV40) DNA synthesis in SV40-infected Vero cells^a*

Treatment	Per cent cells positive		Total extracted DNA		Total SV40 DNA	
	T antigen	V antigen	³ H-counts/min incorporated per culture	Per cent inhibition	³ H-counts/min hybridized per culture	Per cent inhibition
Control...	88	46	1.3 × 10 ⁷		5.8 × 10 ⁶	
CA.....	91	0	1.9 × 10 ⁶	98.6	2.6 × 10 ⁴	99.5

^a Vero cells infected with SV40 at a multiplicity of 50 plaque-forming units per cell in the presence and absence of 10 μg of CA per ml were labeled with thymidine-methyl-³H 24 hr postinfection and harvested at 28 hr. DNA was extracted as described in the text and acid-precipitable ³H-counts per minute were determined. Samples of the extracted DNA were sonically treated and hybridized with 1-μg SV40 DNA filters as described in the text. Determinations were done in triplicate, and ³H-counts per minute bound to 1-μg *E. coli* DNA filters (<1% of input counts per minute) were subtracted. Sonically treated ³H-DNA extracted from uninfected Vero cells was bound equally to SV40 and *E. coli* DNA filters. SV40 T and V antigens were detected by fluorescent-antibody staining.

FIG. 1. *SV40 infection of Vero cells in the presence and absence of cytosine arabinoside (CA). Vero cells were infected with SV40 in the presence or absence of CA as detailed in the text. SV40 RNA synthesis during each labeling period was determined by RNA-DNA hybridization of extracted ³H-RNA. SV40 DNA synthesis was determined by DNA-DNA hybridization of extracted ³H-DNA. SV40 T- and V-antigen synthesis was measured by fluorescent-antibody staining. Symbols: SV40 T antigen with CA (○), without CA (●); SV40 V antigen with CA (△), without CA (▲); SV40 RNA with CA (□), without CA (■); SV40 DNA without CA (▨).*

Similarly, unlabeled early SV40 RNA was an effective competitor of early SV40 ³H-RNA (Fig. 3B). Unlabeled late SV40 RNA also competed with more than 95% of the early ³H-RNA when 1,050 μg of unlabeled competitor RNA was employed (*experiment not shown*). However, the converse was not true since unlabeled early SV40

RNA competed with only 35% of the late SV40 ³H-RNA (Fig. 3A). These findings are in agreement with those previously published (2, 26, 27, 37).

In each experiment, unlabeled, uninfected Vero cell RNA was employed to measure nonspecific competition. The results, which are indicated in the legend to each figure, demonstrated that even with large amounts of added RNA nonspecific competition was generally limited to 10% or less of the ³H-RNA bound in the absence of competitor. No corrections for this nonspecific competition were made in the values for "specific" competition.

Early and late SV40-specific sequences in Ad2+ND₁ ³H-RNA. Hybridization-competition of unlabeled Ad2+ND₁ RNA with Ad2+ND₁ ³H-RNA revealed complete competition (Fig. 4A). Furthermore, both early and late SV40 RNA competed 85% or more with the Ad2+ND₁ ³H-

FIG. 2. *Time course of SV40 T- and V-antigen synthesis in Vero, BSC-1, and primary AGMK cells. Monolayer cultures on glass cover slips were infected with approximately 40 PFU/cell of SV40. Cover slips were harvested at the indicated times, fixed, and fluorescent-antibody stained for SV40 T and V antigens. Symbols: Vero T antigen (■), V antigen (□); BSC-1 T antigen (▲), V antigen (△); primary AGMK T antigen (●), V antigen (○).*

FIG. 3. Characterization of early and late SV40 RNA by hybridization-competition reactions. Hybridization-competition experiments were performed with a saturating amount of ^3H -RNA and SV40 DNA filters as described in the text. 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 a 0.03- μg *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 per cent of this "control" value. Nonspecific competition with 360 μg of unlabeled Vero cell RNA = 0%; with 780 μg = 5%; with 1,320 μg = 9%. This is not subtracted. (A) Unlabeled late SV40 RNA competing with late SV40 ^3H -RNA (1.6×10^6 counts/min added; 26×10^3 counts per min per μg of RNA) with 0.03- μg SV40 DNA filters. Average counts per minute bound to 0.03- μg *E. coli* DNA filters = 2. Control ^3H -RNA counts per minute bound (no competitor) = 405. Unlabeled early SV40 RNA competing with late SV40 ^3H -RNA (1.1×10^6 counts/min added; 14.8×10^3 counts per min per μg of RNA) with 0.03- μg SV40 DNA filters. Average counts per minute bound to 0.03- μg *E. coli* DNA filters = 3. Control ^3H -RNA counts per minute bound (no competitor) = 88. (B) Unlabeled early SV40 RNA competing with early SV40 ^3H -RNA (2×10^6 counts/min added; 7.1×10^3 counts per min per μg of RNA) with 0.03- μg SV40 DNA filters. Average counts per minute bound to 0.03- μg *E. coli* DNA filters = 8. Control ^3H -RNA counts per minute bound (no competitor) = 105.

RNA (Fig. 4B, C). A comparison of the slopes of the competition curves obtained with early and late SV40 RNA species (Fig. 4B, C) suggests that the SV40-specific sequences in Ad2+ND₁ ^3H -RNA are present in much higher concentrations in the late SV40 RNA competitor than in the early SV40 RNA competitor. This is not unexpected since the concentration of early sequences in late SV40 RNA preparations is usually 10- to 30-fold higher than in early SV40 RNA preparations (2).

To determine further the extent to which Ad2+ND₁ RNA shares nucleotide sequences with early and late SV40 ^3H -RNA, unlabeled Ad2+ND₁ RNA was used as a competitor. Ad2+ND₁ RNA competed with 40% of the early SV40 ^3H -RNA; 150 μg produced maximum competition, and further addition of competitor Ad2+ND₁ RNA did not increase the extent of competition (Fig. 5). In contrast, this same preparation of unlabeled Ad2+ND₁ RNA competed with only 15% of late SV40 ^3H -RNA even when the amount of competitor was eight times larger than that required to produce maximum competition with early

SV40 ^3H -RNA (1,200 versus 150 μg). However, nonspecific competition (10% at the equivalent RNA concentration) represents such a large fraction of the total that no estimate can be made of the Ad2+ND₁ nucleotide sequences present in late SV40 RNA.

To compare further the SV40-specific nucleotide sequences in Ad2+ND₁ with those of early SV40 (Fig. 4) under conditions requiring less total RNA in the hybridization-competition reactions, the purified hybrid virus DNA was employed as a template for the *in vitro* synthesis of radiolabeled complementary RNA (Ad2+ND₁ ^3H -cRNA). When the DNA of SV40 itself was used as template in an identical polymerase reaction, the complementary RNA transcribed contained all of the SV40 nucleotide sequences present in SV40-infected cells (21). Competition experiments performed with this Ad2+ND₁ ^3H -cRNA and early SV40 RNA (Fig. 6B) confirmed the observation that early SV40 RNA contains most (if not all) of the SV40 nucleotide sequences present in Ad2+ND₁ RNA (cf. Fig. 4C). However, these shared nucleotide sequences are present in a

FIG. 4. Hybridization-competition of unlabeled $Ad2^{+}ND_1$ RNA, late SV40 RNA, and early SV40 RNA with $Ad2^{+}ND_1$ 3H -RNA. Hybridization-competition experiments were performed with a saturating amount of 3H -RNA and SV40 DNA filters as described in the text. 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 per cent of this "control" value. Nonspecific competition with 100 μg of unlabeled Vero cell RNA = 0%; with 1,000 μg = 10%; with 3,000 μg = 11%. This is not subtracted. (A) Unlabeled $Ad2^{+}ND_1$ RNA competing with $Ad2^{+}ND_1$ 3H -RNA (0.72×10^6 counts/min added; 6.7×10^3 counts per min per μg of RNA) with 0.11- μg SV40 DNA filters. Average counts per minute bound to 0.11- μg *E. coli* DNA filters = 5. Control 3H -RNA counts per minute bound (no competitor) = 98. (B) Unlabeled late SV40 RNA competing with $Ad2^{+}ND_1$ 3H -RNA (6.5×10^6 counts/min added; 25×10^3 counts per min per μg of RNA) with 0.03- μg SV40 DNA filters. Average counts per minute bound to 0.03- μg *E. coli* DNA filters = 5. Control 3H -RNA counts per minute bound (no competitor) = 94. (C) Unlabeled early SV40 RNA competing with $Ad2^{+}ND_1$ 3H -RNA (0.72×10^6 counts/min added; 6.7×10^3 counts per min per μg of RNA) with 0.11- μg SV40 DNA filters. Average counts per minute bound to 0.11- μg *E. coli* DNA filters = 6. Control 3H -RNA counts per minute bound (no competitor) = 108.

lower concentration (per microgram of total extracted RNA) in early SV40 RNA than in $Ad2^{+}ND_1$ RNA; equivalent competition required larger amounts of competitor early SV40 RNA than $Ad2^{+}ND_1$ RNA (cf. Fig. 6A and 6B).

DISCUSSION

The replication of SV40 appears to be characterized by an orderly sequence of synthetic events which permits a functional division of the viral genome. Transcription of early genes begins prior to the onset of DNA replication, whereas late genes are not transcribed until viral DNA synthesis commences. We therefore sought to prepare early and late SV40 RNA for comparison with the RNA transcribed from SV40 genes present in the nondefective $Ad2$ -SV40 hybrid virus, $Ad2^{+}ND_1$. For such studies, it is necessary to prepare early SV40 RNA preparations free of late SV40 RNA. SV40 DNA replication, measured by de novo viral DNA synthesis or indicated by the appearance of capsid (V) antigen, was detected by the 12 hr midpoint after infection

in Vero, BSC-1, and primary AGMK cells. Thus RNA prepared from cells harvested 12 hr or more after infection probably contains significant quantities of late SV40 RNA. Consequently, to prepare early SV40 RNA, CA was utilized to block viral DNA synthesis.

The early and late SV40 RNA employed in these experiments had properties similar to those previously reported (2, 26, 27, 37). Unlabeled late SV40 RNA competed efficiently with early as well as with late SV40 3H -RNA, indicating that the late RNA contained all of the SV40 nucleotide sequences present in early RNA. Although unlabeled early SV40 RNA competed efficiently with early SV40 3H -RNA, it competed with only 35% of late SV40 3H -RNA (Fig. 3A). Thus, provided that all of the SV40 RNA molecules in late SV40 3H -RNA have similar specific activities, we may conclude that early SV40 RNA is transcribed from only one-third of the SV40 genome. Since prereplicative SV40 RNA comprises only 2% of the SV40 RNA synthesized after the onset of viral DNA replication (Fig. 1), SV40 RNA

FIG. 5. Hybridization-competition of unlabeled Ad2+ND₁ RNA with early and late SV40 ³H-RNA. Hybridization-competition experiments were performed with a saturating amount of ³H-RNA and SV40 DNA filters as described in the text. 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 a 0.03-μg *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 per cent of this "control" value. Nonspecific competition with 1,200 μg of unlabeled Vero cell RNA = 10%. This is not subtracted. Unlabeled Ad2+ND₁ RNA competing with early SV40 ³H-RNA (2 × 10⁶ counts/min added; 7.1 × 10³ counts per min per μg of RNA) with 0.025-μg SV40 DNA filters. Average counts per minute bound to 0.03-μg *E. coli* DNA filters = 0. Control ³H-RNA counts per minute bound (no competitor) = 151. Unlabeled Ad2+ND₁ RNA competing with late SV40 ³H-RNA (0.45 × 10⁶ counts/min added; 1.7 × 10³ counts per min per μg of RNA) with 0.025-μg SV40 DNA filters. Average counts per minute bound to 0.03-μg *E. coli* DNA filters = 4. Control ³H-RNA counts per minute bound (no competitor) = 106.

synthesized during the labeling period employed for late SV40 ³H-RNA will not be significantly diluted by unlabeled early RNA. Therefore, the specific activity of the uridine in early and late SV40 RNA sequences in the late SV40 ³H-RNA preparations should be approximately the same. However, if one considers the reported differences in uridine content of early (19% uridine) and late (27% uridine) SV40 RNA (2), the ob-

served plateau of 35% competition of unlabeled early with late ³H-RNA might correspond to as much as 50% of the total SV40 nucleotide sequences (i.e., approximately three or four genes).

Early and late SV40 RNA were compared with SV40 nucleotide sequences present in RNA extracted from cells infected with Ad2+ND₁. Both early and late SV40 RNA were effective competitors (Fig. 4B, C). The extent of competition by unlabeled early SV40 RNA suggests that it contained all of the SV40 RNA sequences transcribed in Ad2+ND₁-infected cells. Comparable results were obtained when early SV40 RNA was competed with Ad2+ND₁ cRNA synthesized *in vitro* (Fig. 6). Thus, the SV40 portion of this hybrid Ad2+ND₁ DNA molecule apparently originated from a portion of SV40 DNA which is transcribed prior to DNA replication in SV40 lytic infection. Furthermore, unlabeled Ad2+ND₁ RNA competed with only 40% of the early SV40 ³H-RNA (Fig. 5), suggesting that only one-third to one-half of the early SV40 genes are present in the Ad2+ND₁ genome. Therefore, the SV40 portion of the Ad2+ND₁ genome probably consists of one or two early SV40 genes.

These findings are in agreement with immunofluorescent studies which indicated that SV40 U antigen is synthesized early during the replication of SV40 (24) and with results obtained by the examination of the Ad2+ND₁ DNA itself, which indicated that the covalently linked hybrid DNA molecule contains 220,000 to 250,000 daltons of SV40 DNA (the equivalent of one or two average-size SV40 genes; references 13, 21). This small amount of early SV40 genetic information in Ad2+ND₁ appears to induce the synthesis of at least one SV40 antigen, the U antigen. Ad2+ND₁ thus contains significantly less SV40 genetic information than does a previously studied defective Ad7-SV40 hybrid virus, E46⁺, which contains a greater proportion (or all) of the early SV40 genes (27) and which induces the synthesis of SV40 T (19, 31, 34) as well as SV40 U antigen (24).

Four additional nondefective Ad2-SV40 hybrid viruses currently under investigation contain the information for different, though overlapping, sets of SV40 biological functions (A. M. Lewis, Jr., et al., *in preparation*). They also induce the synthesis in infected cells of different, although overlapping, species of SV40 RNA (A. S. Levine et al., *in preparation*). Thus, the comparison of the SV40-specific RNA transcribed from the DNA of each of these hybrid viruses with early and late SV40 RNA, as well as with the SV40-specific RNA transcribed from the DNA of each of the others, should permit the assignment of specific SV40 biological functions to identifiable

FIG. 6. Hybridization-competition of unlabeled early SV40 RNA and Ad2+ND₁ ³H-cRNA. Hybridization-competition experiments were performed with a saturating amount of in vitro synthesized Ad2+ND₁ ³H-cRNA (3.44×10^8 counts/min; 2.6×10^6 counts per min per μg of RNA) and 0.005-μg SV40 DNA filters as described in the text. 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

SV40 nucleotide sequences. Hopefully, this type of analysis will eventually lead to the identification of the segment(s) of the SV40 genome which is associated with its oncogenic capacity.

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per minute bound to a 0.005-μg *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 per cent of this "control" value. Nonspecific competition with 105 μg of unlabeled Vero cell RNA = 0%; with 700 μg = 7%; with 1,400 μg = 13%. This is not subtracted. (A) Unlabeled Ad2+ND₁ RNA competing with Ad2+ND₁ ³H-cRNA. Average counts per minute bound to 0.005-μg *E. coli* DNA filters = 98. Control ³H-cRNA counts per minute bound (no competitor) = 272. (B) Unlabeled early SV40 RNA competing with Ad2+ND₁ ³H-cRNA. Average counts per minute bound to 0.005-μg *E. coli* DNA filters = 84. Control ³H-cRNA counts per minute bound (no competitor) = 288.

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