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⁺ND₁ Genome

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A nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid virus, Ad2⁺ND₁, has been plaque-isolated from an Ad2-SV40 hybrid population. This virus, unlike the defective Ad-SV40 hybrid populations previously described, replicates without the aid of nonhybrid adenovirus helper. Consequently, the hybrid virus deoxyribonucleic acid (DNA) can be obtained free of nonhybrid adenovirus DNA. The DNA of the Ad2⁺ND₁ virus was shown by ribonucleic acid (RNA)-DNA hybridization to consist of nucleotide sequences complementary to Ad2- and SV40-specific RNA. Techniques of equilibrium density and rate zonal centrifugation were employed to demonstrate that these Ad2 and SV40 nucleotide sequences were linked together in the same DNA molecules by alkali-resistant bonds. Calibration curves were established relating the amount of tritium-labeled SV40-specific RNA (prepared *in vitro* or *in vivo*) bound to given amounts of SV40 DNA in a hybridization reaction, and these curves were employed to determine the equivalent amount of SV40 DNA in the Ad2⁺ND₁ molecule. From the results obtained, it was estimated that 1% of the Ad2⁺ND₁ DNA consists of SV40 nucleotide sequences.

A nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid virus (designated Ad2⁺ND₁) has been plaque-isolated from an Ad2-SV40 hybrid population. The Ad2⁺ND₁ virus, in contrast to the defective Ad-SV40 hybrids (14, 18, 20, 27, 29), replicates efficiently, with one-hit kinetics, in both human embryonic kidney (HEK) and African green monkey kidney (AGMK) cell cultures without the aid of nonhybrid Ad2 virions. During acute cytolytic infection, this virus induces SV40-specific ribonucleic acid (RNA) and a previously unrecognized SV40-specific antigen (SV40 U antigen) which is detectable by immunofluorescence and complement fixation tests with sera from SV40 tumor-bearing hamsters (19, 21). Because the Ad2⁺ND₁ virus is nondefective, it offers a unique opportunity to obtain and study Ad-SV40 hybrid deoxyribonucleic acid (DNA) free of the non-

hybrid adenovirus DNA which is present in DNA extracted from defective Ad-SV40 hybrid populations.

This report provides evidence for the covalent linkage of the Ad2 and SV40 portions of the Ad2⁺ND₁ DNA. The hybrid DNA was studied by equilibrium density centrifugation in neutral and alkaline cesium chloride and by rate zonal centrifugation in alkaline sucrose. Nucleic acid hybridization of the DNA from the resultant fractions indicated that the Ad2- and SV40-specific nucleotide sequences in the Ad2⁺ND₁ DNA could not be physically separated. In addition, since the Ad2⁺ND₁ DNA is free of helper Ad2 DNA, it was possible to determine the relative amounts of Ad2 and SV40 DNA in the Ad2⁺ND₁ hybrid molecule.

MATERIALS AND METHODS

Viruses. Pools of Ad2⁺ND₁ virus representing passages 9 to 13 (19) were prepared in monolayer cultures of primary HEK cells and had titers of 10^{8.4} to 10^{8.8} plaque-forming units (PFU)/ml by plaque assay in HEK cells. Each pool induced SV40 U

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antigen, detectable by complement fixation tests in tube cultures of either HEK or AGMK cells.

Ad2 (strain Ad6) has been maintained in the Laboratory of Viral Diseases by serial passage in HEK cells.

All pools of Ad2+ND₁ and Ad2 used in these experiments were demonstrated to be free of adeno-associated virus types 1 to 4 by complement fixation testing.

SV40 strain 777 (3) was propagated by low-multiplicity infection of BSC-1 cells (13). Titers ranged from $10^{8.7}$ to $10^{9.3}$ PFU/ml in primary AGMK cells.

All virus pools were frozen and thawed three times and, with the exception of the SV40 pools, were clarified by low-speed centrifugation ($500 \times g$ for 10 min) prior to storage at -70°C .

Tissue culture. BSC-1 cells were grown as monolayer cultures in 32-oz glass prescription bottles 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). The Vero line of AGMK cells (35) was similarly propagated. Primary HEK and AGMK cells were purchased commercially and grown in EMEM plus 10% FBS.

Preparation of purified viruses and extraction of virus DNA. Infected HEK or Vero cell packs for purification of Ad2+ND₁ virus were prepared by infecting 32-oz bottles with 20 to 40 PFU/cell in 15 ml of EMEM plus 2% agammaglobulinic calf serum (AGCS). After a 3-hr adsorption period, an additional 30 ml of this medium was added and the infected cultures were incubated at 37°C . Cultures were harvested by scraping when 75% of cells showed typical Ad cytopathic effect (CPE). Ad2 cell packs were prepared in bottles of HEK, employing methods of inoculation and harvest identical to those for Ad2+ND₁.

To prepare radiolabeled Ad2+ND₁ DNA, Vero cell monolayers were infected as described above. Twenty-four hours after infection, when about 25% of the cells demonstrated Ad CPE, the medium was decanted and replaced with EMEM plus 2% AGCS containing 0.2 μCi of thymidine-2- ^{14}C (Amersham/Searle, 56.2 mCi/mm) per ml. The cultures were reincubated for 6 hr at 37°C before further labeling was interrupted by the addition of a 100-fold excess of unlabeled thymidine phosphate. The cultures were harvested by scraping when 75% of the cells showed Ad CPE. This procedure yielded Ad2+ND₁ DNA with a specific activity of 3,200 counts per min per μg of DNA.

In each case, harvested cells and fluids were centrifuged at $500 \times g$ for 15 min, and the cell packs were suspended in 0.01 M tris (hydroxymethyl)-aminomethane (Tris)-buffered saline (TBS), pH 8.1, containing 10^{-3} M Ca^{2+} and 10^{-3} M Mg^{2+} prior to storage at -70°C .

Cell packs of Vero cells dually infected with SV40 and Ad2 were prepared by inoculating cultures with SV40 virus at a multiplicity of 10 PFU/cell. After 24 hr at 37°C , Ad2 (10 PFU/cell) was inoculated into the same bottles. The time interval between infections was selected on the basis of the finding of O'Connor et al. (25) that the maximum number of dually in-

fected AGMK cells occurred when SV40 infection preceded Ad infection by 24 hr. Cultures were incubated at 37°C until 75% of cells showed Ad CPE and were then harvested by scraping. The cells were pelleted at $500 \times g$ for 15 min, and the supernatant was centrifuged again for 3 hr at 20,000 rev/min (Spinco model L ultracentrifuge, no. 21 rotor). Both pellets were combined, suspended in 0.01 M TBS, and frozen at -70°C .

Unlabeled SV40 was prepared by infecting confluent monolayer cultures of BSC-1 cells in large glass roller bottles (Bellco Glass Co., no. 7013) with SV40 at low multiplicity (approximately 100 PFU/bottle) in EMEM plus 2% FBS. The medium was changed twice weekly until 1 to 5% of the cells exhibited SV40 CPE. The cultures were then given a final change of medium, and the cells and medium were harvested when 100% of the cells exhibited CPE.

SV40 virus labeled with thymidine-2- ^{14}C was prepared in a similar manner except that the multiplicity of infection was approximately 10 PFU/cell. When 5 to 10% of the cells exhibited SV40 CPE, the medium was replaced with 25 ml of fresh medium containing 0.5 μCi of thymidine-2- ^{14}C per ml. The labeled cultures were harvested by scraping when all cells showed SV40 CPE. This procedure resulted in SV40 DNA with a specific activity of 4,700 counts per min per μg . The crude harvests of labeled and unlabeled SV40 were stored at -70°C .

For purification of Ad2+ND₁ and Ad2, the cell packs were subjected to enzyme digestion followed by equilibrium density gradient centrifugation in CsCl (5, 34).

SV40 was purified by a modification (Oxman et al., *in preparation*) of the technique of Burnett (5). Crude SV40 harvests were centrifuged at $60,000 \times g$ to sediment cells and virions. The pellets were suspended and sequentially treated at 37°C with 1% sodium deoxycholate, deoxyribonuclease and ribonuclease, *crotalus adamanteus* venom, α -chymotrypsin, and subtilisin. The enzyme-treated crude virus was then twice cushioned into saturated KBr and banded in CsCl (density = 1.34 g/cm³) in a Spinco SW 39 rotor at 35,000 rev/min for 24 hr at 4°C . The banded virus was dialyzed and stored at -70°C . The harvest of the mixed infection of Vero cells with SV40 and Ad2 was purified in a similar manner.

DNA was extracted from the purified virus preparations by papain digestion followed by sodium dodecyl sulfate (SDS)-phenol extraction (28); viral DNA was stored at -30°C in $0.1 \times \text{SSC}$ ($\text{SSC} = 0.15$ M NaCl + 0.015 M sodium citrate, pH 6.9).

Radiolabeling of RNA. To prepare ^3H -labeled Vero cell RNA, confluent roller-bottle cultures were refed with 40 ml of EMEM plus 2% AGCS. After a 20 to 24-hr interval, 2 ml of medium was removed and replaced with 2 ml of uridine-5- ^3H (Amersham/Searle, 24 to 30.7 Ci/mm) to yield a final concentration of 50 $\mu\text{Ci}/\text{ml}$. The cells were incubated for 10 hr and harvested as previously described (16). The ^3H -labeled HEK RNA was prepared in 32-oz bottles by a similar technique (19).

^3H -RNA from cells lytically infected with SV40 (late SV40 RNA) was prepared by infecting roller-

bottle cultures of Vero cells with SV40 at a multiplicity of 30 to 70 PFU/cell. Twenty-four hours after infection, at a time when SV40 DNA synthesis had already commenced, the cultures were labeled with uridine-5- ^3H (final concentration; 50 $\mu\text{Ci/ml}$). The cells were harvested by trypsinization 16 hr later (16).

Extraction of cellular RNA and DNA and techniques of RNA-DNA hybridization. The radiolabeled RNA was extracted by a hot phenol-SDS procedure (16) as modified from the methods of Scherrer and Darnell (32) and Kirby (15). Cellular DNA was extracted by the method of Marmur (22). The procedure for hybridization of RNA with single-stranded DNA immobilized on nitrocellulose membrane filters was that of Gillespie and Spiegelman (11) with slight modifications (16). Hybridization reactions were performed with 13-mm filters in a volume of 250 μliters of $2\times\text{SSC}$ plus 0.05% SDS for 16 hr at 60 C. All RNA-DNA hybrids were treated with pancreatic ribonuclease (Sigma, type XII-A; 20 $\mu\text{g/ml}$) and ribonuclease T_1 (Calbiochem, B grade; 10 units/ml) for 1 hr at room temperature before scintillation counting (16).

The concentrations of the viral and cellular DNA solutions were determined by a modified diphenylamine reaction (6) with calf thymus DNA (Calbiochem) as a standard. RNA concentrations were determined by the orcinol reaction (4) with soluble RNA from yeast (Calbiochem) as a standard.

In vitro preparations of virus-specific RNA (cRNA). Tritium-labeled complementary RNA (cRNA) complementary to Ad2, SV40, or Ad2 $^+\text{ND}_1$ DNA was synthesized in vitro by a modification (17) of the method of Chamberlain and Berg (7) and kindly supplied by A. S. Levine.

Equilibrium density centrifugation of viral DNA in neutral and alkaline cesium chloride. CsCl (Harshaw Chemical Corp., optical grade) was dissolved in 0.01 M Tris buffer (pH 8.1), and the density of the solution was determined from its refractive index (Abbe L refractometer). Native viral DNA preparations (16 to 20 μg) were mixed with 4.0 ml of CsCl solution (final density = 1.710 g/cm^3) and centrifuged in a Spinco model L ultracentrifuge, no. 40 rotor, at 33,000 rev/min for 62 to 66 hr at 25 C (10). The contents of each tube were collected in 0.125-ml fractions through the bottom of the tube by using a constant volume displacement device, and the OD_{260} and refractive index were determined on alternate fractions. Each fraction was diluted with 3 ml of $0.01\times\text{SSC}$; the DNA was denatured by boiling for 15 min, followed by immediate cooling in an ice-salt mixture. A 7-ml amount of $6\times\text{SSC}$ was added to each sample, and the denatured DNA was then immobilized on nitrocellulose membrane filters.

Alkaline density gradients were prepared by dissolving CsCl in 0.08 M phosphate buffer, pH 12.55 (glass electrode; Beckman Zeromatic pH meter). DNA (20 to 25 μg) was dissolved in 0.3 ml of 0.1 M NaOH at room temperature and added to the CsCl solution; the pH was adjusted to 12.6 prior to centrifugation. Since alkali denaturation of DNA with a content of guanine plus cytosine similar to that of Ad2 DNA increases its buoyant density by 0.06 g/cm^3 (33), the final density of the CsCl solution was

adjusted gravimetrically to 1.760 g/cm^3 . Adherence of denatured DNA to the walls of the polyallomer tubes was avoided by pretreating the tubes overnight at 4 C with a solution of 0.25% recrystallized bovine serum albumin (Armour Pharmaceutical Co.). The centrifugation was carried out as described above for native DNA, the fractions were collected in 0.1 ml of 0.1 M citric acid, and the OD_{260} and the density were measured on alternate fractions. Seven milliliters of $6\times\text{SSC}$ was added to the denatured DNA fractions which were then immobilized on nitrocellulose filters.

Zone sedimentation of viral DNA in alkaline sucrose. Linear 12-ml gradients were prepared in albumin-coated tubes from 5 and 20% sucrose solutions in 0.02 M trisodium phosphate (final pH, 12.0). A 2- μg amount of ^{14}C -Ad2 $^+\text{ND}_1$ DNA (3,200 counts per min per μg) was denatured for 30 min in 0.25 M NaOH at room temperature. The denatured DNA was layered onto the gradient and centrifuged at 39,000 rev/min for 12 hr at 6 C in a Spinco SW40 rotor. Fractions (0.4 ml) were collected with an ISCO (model D) density gradient fractionator. From the odd-numbered fractions, 50 μliters was dissolved in 0.5 ml of water plus 5.0 ml of Scintisol Complete (Isolab, Inc.) for determination of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. The DNA from the remainder of the fractions was immobilized on nitrocellulose filters as described above.

RESULTS

Demonstration of Ad2 and SV40 DNA in the Ad2 $^+\text{ND}_1$ genome. In a previous publication, it was reported that the plaque-isolated non-defective Ad2 $^+\text{ND}_1$ virus induced both Ad2- and SV40-specific RNA during acute cytolysis infection in HEK or Vero cells (19), indicating that the virus contained segments of both Ad2 and SV40 DNA. To demonstrate this directly, denatured Ad2 $^+\text{ND}_1$ DNA was immobilized on nitrocellulose filters and hybridized with radioactive Ad2 and SV40 cRNA synthesized in vitro.

The Ad2 $^+\text{ND}_1$ DNA hybridized extensively with both SV40 ^3H -cRNA and Ad2 ^3H -cRNA (Table 1). Radioactive RNA from Vero cells, the cell line in which the virus was grown, did not react with the Ad2 $^+\text{ND}_1$ DNA, thus demonstrating that the viral DNA was free of contaminating cellular DNA. In contrast, nonhybrid Ad2 DNA reacted with Ad2 ^3H -cRNA but not with SV40 ^3H -cRNA, and SV40 DNA hybridized with SV40 ^3H -cRNA but not with Ad2 ^3H -cRNA. However, Ad2 $^+\text{ND}_1$ ^3H -cRNA, prepared in vitro from an Ad2 $^+\text{ND}_1$ DNA template, hybridized with both SV40 and Ad2 DNA.

The Ad2 and SV40 DNA species were free of contaminating cellular DNA as demonstrated by the absence of any reaction with HEK or Vero cell ^3H -RNA. This was further substantiated by the absence of hybridization between human

TABLE 1. *Hybridization reaction of viral ^3H -complementary ribonucleic acid (cRNA) and cellular ^3H -RNA with viral and cellular DNA*

Source of DNA on filter	DNA on filter (μg)	Type of tritiated RNA ^a	RNA input ^b	Net counts/min retained on filter ^c
Ad2+ND ₁	0.5	Ad2	2.5	2,070
		SV40	2.7	230
		Vero ^d	5.9	0
Ad2	0.25	Ad2	2.5	1,040
		Ad2+ND ₁	2.5	4,700
		SV40	1.0	0
		HEK	10.0	0
SV40	0.03	Ad2	1.3	0
		Ad2+ND ₁	2.5	540
		SV40	2.7	470
		Vero	10.0	0
KB ^e	0.5	Ad2	2.5	0
		Ad2+ND ₁	1.0	0
		SV40	2.7	0
		HEK	10.0	187
Vero	0.5	Ad2	1.3	0
		Ad2+ND ₁	2.5	0
		SV40	1.0	0
		Vero	10.0	248

^a Specific activity: adenovirus 2 (Ad2) cRNA, 3.6×10^5 counts per min per μg ; simian virus 40 (SV40) cRNA, 3.4×10^5 counts per min per μg ; nondefective Ad2-SV40 hybrid (Ad2+ND₁) cRNA, 2.6×10^6 counts per min per μg ; Vero cell RNA, 3.0×10^4 counts per min per μg ; human embryonic kidney (HEK) cell RNA, 2.0×10^4 counts per min per μg .

^b Values expressed as counts/minute $\times 10^4$.

^c Virus-specific counts/minute retained on the DNA filter were determined in duplicate after a 20-hr hybridization period. Amount of background counts/minute bound to equal amounts (0.03 to 0.5 μg) of *Escherichia coli* DNA ($<2 \times 10^{-4}\%$ of input) has been subtracted.

^d ^3H -Vero RNA and ^3H -HEK RNA were extracted from Vero or HEK cells labeled with ^3H -uridine, as described in text.

^e KB DNA was obtained from KB cells maintained in spinner cultures of Eagle's media supplemented with 5% horse serum.

(KB) and monkey (Vero) cell DNA species and any of the viral cRNA species employed.

Linkage of Ad2 and SV40 DNA. Ad2+ND₁ DNA was centrifuged to equilibrium in a neutral CsCl density gradient in a fixed angle rotor, and the fractions were collected. The DNA from the

fractions was fixed on nitrocellulose filters and hybridized with Ad2 and SV40 ^3H -cRNA prepared in vitro (Fig. 1A). The peak representing binding of Ad2 cRNA to the various fractions of Ad2+ND₁ DNA coincides with the peak of binding of SV40 cRNA, indicating that DNA complementary to both Ad2 cRNA and SV40 cRNA is concentrated at the same point in the gradient, corresponding to a mean density of 1.715 g/cm^3 .

FIG. 1. *Equilibrium density gradient centrifugation of native DNA in cesium chloride. The counts/minute of tritiated SV40 and Ad2 cRNA bound by DNA filters, prepared from 0.125-ml fractions of the gradient, are shown. Buoyant densities obtained for Ad2 and SV40 peaks are indicated. (A) Ad2+ND₁ DNA (16 μg): starting density = 1.700 g/cm^3 , pH 8.2 (B) Mixture of Ad2 DNA (5.8 μg) and SV40 DNA (5.2 μg): starting density = 1.700 g/cm^3 , pH 8.2 (C) DNA (15 μg) obtained from virions propagated in mixed infection of Vero cells with SV40 and Ad2: starting density = 1.700 g/cm^3 , pH 8.2. Each reaction mixture contained 0.25 ml of $2 \times \text{SSC}$, filters prepared from the fractions of centrifuged DNA, and 10,000 counts/min of Ad2 ^3H -cRNA (specific activity, 1.5×10^6 counts per min per μg) or SV40 ^3H -cRNA (specific activity, 2.3×10^6 counts per min per μg). Some vials also contained filters with 0.03 μg of *E. coli* DNA as blanks. The quantity of ^3H -cRNA bound to *E. coli* DNA filters (10 to 20 counts/min) has been subtracted from the amount bound to centrifuged DNA. Symbols: Ad2 ^3H -cRNA bound ($\bullet$); SV40 ^3H -cRNA bound ($\circ$).*

Mixtures of DNA from separately grown and purified Ad2 and SV40 were readily separated in similar gradients (Fig. 1B). DNA extracted from virions propagated in Vero cells doubly infected with Ad2 and SV40 also yielded separate Ad2 and SV40 peaks (Fig. 1C). In both of these control experiments, the buoyant densities of the two viral DNA species corresponded to those previously published [Ad2 = 1.716 (12); SV40 = 1.700 (8)].

It can be seen (Fig. 1A) that the curves of SV40 and Ad2 hybridizable DNA have identical band widths. Comparison of these curves with those in Fig. 1B and 1C shows that the densities and band widths of Ad2+ND₁ DNA and Ad2 DNA are similar. In contrast, the band widths of Ad2 (molecular weight = 23×10^6) and SV40 (2.25×10^6) DNA species in Fig. 1B and 1C differ considerably. This is expected since band width in density gradients varies inversely with the square root of the molecular weight (24). These data, taken together, indicate that the SV40 and the Ad2 nucleotide sequences present in Ad2+ND₁ DNA reside in molecules of the same size and density, that the SV40 nucleotide sequences present in Ad2+ND₁ DNA preparations reside in molecules which are considerably larger than molecules of SV40 viral DNA, and that the molecular weight of the Ad2+ND₁ DNA is similar to that of Ad2.

The gradients run with alkali-denatured DNA (Fig. 2) also indicate that the Ad2 and SV40 components of (denatured) Ad2+ND₁ DNA are not separable (Fig. 2A). The peaks of the curves representing the hybridization of Ad2 ³H-cRNA and SV40 ³H-cRNA with the fractions from the Ad2+ND₁ DNA gradient coincide at a density of 1.764 g/cm³. This shift, under alkaline conditions, from a buoyant density of 1.716 g/cm³ corresponds to that expected (33) for a DNA with the guanine and cytosine content of Ad2 (G + C = 56%). The denatured Ad2 DNA in the control (Fig. 2B) exhibits one peak at 1.764 g/cm³ and a broad, less dense peak which probably represents incompletely denatured Ad2 DNA. The curve representing SV40 DNA was separated from the completely denatured Ad2 DNA peak.

Furthermore, the Ad2 and SV40 nucleotide sequences in Ad2+ND₁ DNA could not be separated by velocity sedimentation through an alkaline sucrose gradient (Fig. 3). The curves corresponding to hybridization of Ad2 ³H-cRNA and SV40 ³H-cRNA with the DNA from the gradient fractions coincide, and both also coincide with the curve representing the distribution of the Ad2+ND₁ DNA itself (¹⁴C-labeled) in the gradient.

FIG. 2. Equilibrium density gradient centrifugation of alkali-denatured DNA in alkaline cesium chloride. The counts/minute of tritiated SV40 and Ad2 cRNA bound by DNA filters, prepared from 0.125-ml fractions of the gradient, are shown. All gradients were prepared in polyallomer tubes coated with bovine serum albumin. Buoyant densities for peaks of Ad2+ND₁, Ad2, and SV40 DNA are indicated: (A) Ad2+ND₁ DNA (23.1 μ g): starting density = 1.763 g/cm³, pH 12.60; (B) mixture of Ad2 DNA (29.5 μ g) and SV40 DNA (24 μ g): starting density = 1.759 g/cm³, pH 12.55. Each reaction mixture was identical to that described under Fig. 1. The quantity of ³H-cRNA bound to *E. coli* DNA filters (10 to 20 counts/min) has been subtracted from the amount bound to centrifuged DNA. Symbols: Ad2 ³H-cRNA bound (●); SV40 ³H-cRNA bound (○).

Per cent of SV40 DNA in the Ad2+ND₁ DNA.

To determine the amount of SV40 DNA present in the Ad2+ND₁ DNA molecules, a calibration curve was first established relating the amount of in vitro synthesized SV40 ³H-cRNA bound to the quantity of SV40 DNA present in hybridization reactions (Fig. 4). Filters for this calibration experiment were prepared with various amounts of ¹⁴C-SV40 DNA (0.0025 to 0.0115 μ g) in the presence of a constant amount of unlabeled Ad2 DNA (0.5 μ g/filter). These filters were then hybridized with a saturating amount (previously determined with identical DNA filters and ³H-cRNA) of tritium-labeled SV40 cRNA. The counts/minute bound were observed to be proportional to the amount of SV40 DNA on the filter. The counts/minute of the same preparation of SV40 ³H-cRNA bound to the Ad2+ND₁ DNA were then determined, and the quantity of SV40

FIG. 3. Rate zonal sedimentation of $\text{Ad2}^+\text{ND}_1$ DNA in alkaline sucrose. The counts/minute of tritiated SV40 and Ad2 cRNA bound by DNA filters, prepared from 0.4-ml fractions of the gradient, are shown. The ^{14}C - $\text{Ad2}^+\text{ND}_1$ DNA present in each fraction is also depicted. A 2- μg amount of ^{14}C - $\text{Ad2}^+\text{ND}_1$ DNA (3,200 counts per min per μg) was denatured for 30 min in 0.25 M NaOH and layered on top of a 5 to 20% sucrose gradient (pH 12.0). Hybridization reactions were performed as described under Fig. 1. Symbols: Ad2 ^3H -cRNA bound ($\bullet$); SV40 ^3H -cRNA bound ($\circ$); ^{14}C - $\text{Ad2}^+\text{ND}_1$ DNA ($\blacktriangle$).

DNA that this represented was estimated from the calibration curve. The SV40 and $\text{Ad2}^+\text{ND}_1$ DNA on the filters was ^{14}C -labeled so that the exact amount of DNA present on the filter could be determined at the end of the hybridization reaction. This experiment was performed three times with different quantities of $\text{Ad2}^+\text{ND}_1$ DNA. The results of the three experiments giving the proportion of SV40 DNA in the $\text{Ad2}^+\text{ND}_1$ DNA are shown in Table 2. The average of these three determinations indicates that 1.0% of the $\text{Ad2}^+\text{ND}_1$ DNA consists of SV40 nucleotide sequences.

This result was confirmed by hybridizing $\text{Ad2}^+\text{ND}_1$ DNA with a saturating amount of tritium-labeled SV40-specific RNA obtained from cells productively infected with SV40 (late SV40 RNA). This late SV40 RNA, extracted from cells 40 hr after infection, is presumed to contain all RNA species transcribed during the lytic cycle of SV40 (1, 23, 26, 30). A calibration curve was again established, relating the amount of late SV40 ^3H -RNA bound to the quantity of SV40 DNA present on the filter. The counts/minute bound were again shown to be proportional to the amount of SV40 DNA on the filter (Fig. 5). The counts/minute of late SV40 ^3H -RNA bound to $\text{Ad2}^+\text{ND}_1$ DNA were then deter-

FIG. 4. Calibration curve for counts/minute of SV40 ^3H -cRNA bound to increasing amounts of ^{14}C -SV40 DNA immobilized on nitrocellulose filters. Each reaction vial contained 0.25 ml of $2\times$ SSC, a filter containing 0.5 μg of unlabeled Ad2 DNA plus a specified amount of ^{14}C -SV40 DNA (specific activity, 4,700 counts per min per μg), a 0.5 μg *E. coli* DNA filter as a blank, and a saturating amount (3.75×10^6 counts/min of SV40 ^3H -cRNA (specific activity, 2.6×10^6 counts per min per μg). The counts/minute bound to the *E. coli* DNA filter (0.04% of input) have been subtracted. The counts/minute of ^3H -cRNA bound to known amounts (μg) of ^{14}C - $\text{Ad2}^+\text{ND}_1$ DNA (specific activity, 3,200 counts per min per μg) are indicated on the figure and extrapolated to the equivalent amount of SV40 DNA. All filters were counted for a sufficient time to yield a counting accuracy of $\pm 1\%$.

mined in two experiments employing different quantities of $\text{Ad2}^+\text{ND}_1$ DNA. The net counts/minute bound to the $\text{Ad2}^+\text{ND}_1$ DNA are indicated in Fig. 5. The proportion of SV40 nucleotide sequences in the $\text{Ad2}^+\text{ND}_1$ DNA estimated from these two experiments was 0.99 and 1.03% (Table 2), in agreement with the results obtained using *in vitro* prepared ^3H -cRNA (1.0%).

It was possible to show that the SV40 cRNA prepared *in vitro* contained all RNA sequences transcribed during SV40 lytic infection. Unlabeled SV40 cRNA was employed as a competitor in the hybridization reaction between tritium labeled late SV40 RNA and SV40 DNA (Fig. 6). The unlabeled SV40 cRNA (synthesized *in vitro*) competed efficiently with late SV40 ^3H -RNA (from infected Vero cells) for SV40 DNA sites. Saturating amounts of late SV40 RNA have been shown to hybridize with 50% of SV40 DNA (23) presumably indicating transcription from one DNA strand.

DISCUSSION

The Ad2- and SV40-specific nucleotide sequences in the $\text{Ad2}^+\text{ND}_1$ DNA preparation could not be separated by alkali denaturation and equilibrium centrifugation in CsCl or by rate zonal sedimentation in alkaline sucrose. These

TABLE 2. Calculation of per cent of simian virus 40 (SV40) DNA in nondefective adenovirus 2-SV40 hybrid (AD2+ND₁) DNA^a

Type of SV40 RNA	Total Ad2+ND ₁ DNA on filter (μg)	Net counts/min of ³ H RNA	SV40 DNA in the Ad2+ND ₁ DNA on filter ^b (μg)	Per cent of SV40 DNA in Ad2+ND ₁ DNA ^c
³ H-cRNA	0.308	6,235	0.003	0.98
	0.475	9,550	0.005	1.05
	0.500	9,800	0.005	1.00
				1.01 ± 0.05 ^d
Late ³ H-RNA	0.303	20	0.003	0.99
	0.483	29	0.005	1.03

^a Hybridization of ¹⁴C-Ad2+ND₁ DNA with SV40 ³H-complementary RNA (cRNA) and late-SV40 ³H-RNA.

^b Amount of SV40 DNA in Ad2+ND₁ was estimated from counts/minute of ³H-cRNA or late ³H-RNA bound to Ad2+ND₁, utilizing the calibration curves presented in Fig. 4 and 5.

^c Per cent = [SV40 DNA (μg, column 4)/Ad2+ND₁ DNA (μg, column 2)] × 100.

^d Average per cent.

FIG. 5. Calibration curve for counts/minute of late SV40 ³H-RNA bound to increasing amounts of ¹⁴C-SV40 DNA immobilized on nitrocellulose filters. Each reaction vial contained 0.25 ml of 2× SSC, a filter containing 0.5 μg of unlabeled Ad2 DNA plus a specified amount of ¹⁴C-SV40 DNA (specific activity, 4,700 counts per min per μg), a 0.5-μg E. coli DNA filter as a blank, and a saturating amount (1.3×10^6 counts/min of late SV40 ³H-RNA (specific activity, 10^4 counts per min per μg). The counts/minute bound to the E. coli filter (3 to 5) have been subtracted. The counts/minute of late SV40 ³H-RNA bound to known amounts (μg) of ¹⁴C-Ad2+ND₁ DNA (specific activity, 3,200 counts per min per μg) were determined in triplicate. The averages are indicated on the figure and extrapolated to the equivalent amount of SV40 DNA. All filters were counted for a sufficient time to yield a counting accuracy of ±2.5%.

data indicate that the Ad2+ND₁ genome is a recombinant composed of Ad2 and SV40 DNA linked together in the same molecule by alkali-resistant, presumably covalent, bonds.

FIG. 6. Competition of unlabeled SV40 cRNA synthesized in vitro with the reaction between late SV40 ³H-RNA and SV40 DNA. Each reaction vial contained 0.25 ml of 2× SSC, one filter with 0.03 μg of ¹⁴C-SV40 DNA, one filter with 0.03 μg of E. coli DNA, a saturating amount (1.3×10^6 counts/min) of late SV40 ³H-RNA (specific activity, 10^4 counts per min per μg of RNA), and increasing amounts of unlabeled competitor SV40 cRNA (●). In some reaction mixtures, unlabeled Vero cell RNA was substituted as a measure of nonspecific competition (○). The amount of ³H-RNA bound to SV40 DNA, at saturation, in the absence of unlabeled RNA (control) was 4,845 counts per min per μg of DNA. The counts/minute of ³H-RNA bound to E. coli DNA filters ($< 4 \times 10^{-4}\%$ of input) have been subtracted from the amount bound to SV40 DNA filters.

Alternative explanations for this association of the Ad2 and SV40 components of Ad2+ND₁ DNA in the CsCl density gradients might be considered. A preparative artifact seems unlikely

since similar results were obtained with Ad2+ND₁, grown in either HEK or Vero cells, and it was possible to demonstrate separation of the Ad2 and SV40 DNA species even when they were obtained from virions propagated in a mixed infection.

Another possible explanation of the results is that the Ad2 and SV40 components are not really linked but are found in the same density region of the gradient because the SV40 component in Ad2+ND₁ DNA is a small segment of SV40 DNA having a density identical to that of Ad2 DNA. If this were the case, we would expect that the band width of this piece of low-molecular-weight ($< 2.25 \times 10^6$) SV40 DNA would be wider than that of Ad2 DNA (molecular weight = 20×10^6). The SV40 component of Ad2+ND₁ DNA, however, had a band width identical to that of the Ad2 (Fig. 1A) and smaller than that of SV40 viral DNA in both the neutral and alkaline gradients (see Fig. 1B, 1C, and 2B). Furthermore, no peak of low-molecular-weight SV40 DNA was detected in the alkaline sucrose velocity gradient (Fig. 3).

Covalent linkage has been demonstrated previously between the Ad7 and SV40 DNA in the hybrid genome of the defective Ad7-SV40 hybrid population (2). However, only with the availability of a nondefective Ad-SV40 hybrid virus, such as Ad2+ND₁, has it been possible to determine the relative amounts of Ad and SV40 DNA in such a hybrid molecule. The calibration data indicate that 1% of the Ad2+ND₁ DNA molecule consists of SV40 nucleotide sequences. The ³H-cRNA used for these experiments was prepared in vitro with *Escherichia coli* RNA polymerase which could conceivably fail to transcribe certain regions of SV40 DNA which are normally transcribed in vivo. However, this SV40 cRNA synthesized in vitro competed completely in the hybridization reaction between late SV40 ³H-RNA and SV40 DNA (Fig. 6). Late SV40 ³H-RNA contains all SV40-specific RNA sequences present late in the SV40 lytic cycle (23). Furthermore, the results obtained with this SV40 ³H-cRNA prepared in vitro were confirmed by using late SV40 ³H-RNA itself (Fig. 5). However, the possibility that Ad2+ND₁ DNA might contain, in addition, SV40 nucleotide sequences which are not transcribed either in vivo or in vitro from SV40 DNA templates has not been excluded.

These results demonstrate that the genome of the Ad2+ND₁ virus is a recombinant containing 99% Ad2 DNA and 1% SV40 DNA. The very similar buoyant density and molecular weight of the DNA of Ad2+ND₁ and Ad2 (9) provide further evidence for the small size of the SV40

component of this Ad-SV40 hybrid virus. The existence of a number of non-defective Ad2-SV40 hybrid viruses containing different SV40 DNA segments will provide reagents for the genetic analysis of SV40 (Oxman et al., *in preparation*).

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