Studies of Nondefective Adenovirus 2-Simian Virus 40 Hybrid Viruses.

VI. Characterization of the DNA from Five Nondefective Hybrid Viruses

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Certain biophysical characteristics of the DNA from each of the five nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid viruses (Ad2⁺ND₁, Ad2⁺ND₂, Ad2⁺ND₃, Ad2⁺ND₄, Ad2⁺ND₅) have been determined. The guanine plus cytosine content varied from 55 to 57% and was not significantly different from that of nonhybrid Ad2 (56%), and the hybrid DNA molecules had mean molecular lengths which were similar to that of the standard, Ad2. The Ad2 and SV40 components of each hybrid were linked by alkali-resistant, presumably covalent bonds. The percentage of SV40 DNA in each hybrid virus was determined by hybridization with SV40 complementary RNA in a calibrated system. The results indicate that each hybrid virus DNA contains a different percentage of SV40 nucleotide sequences. The estimated size of the SV40 DNA component varies from 48,000 daltons for Ad2⁺ND₂ to 840,000 daltons for Ad2⁺ND₄, the latter being equivalent to between one-fourth and one-third of the SV40 genome.

The isolation and characterization of a nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid virus, designated Ad2⁺ND₁, has been reported (15). This virus replicates without the assistance of nonhybrid Ad2 helper virions and induces a heat-stable antigen, the SV40 U antigen (16). The DNA of the Ad2⁺ND₁ hybrid has been shown to consist of covalently linked Ad2 and SV40 DNA (11). The SV40 DNA segment of this virus was estimated to comprise 1% of the hybrid virus DNA molecule and to have a molecular weight of approximately 240,000, corresponding to about 1/10 the molecular weight of an entire SV40 genome. The guanine plus cytosine content and the molecular weight of the Ad2⁺ND₁ DNA molecule were not significantly different from those of nonhybrid Ad2 (3, 11).

In an accompanying report, Lewis et al. (14) have described four additional nondefective Ad2-SV40 hybrid viruses designated Ad2⁺ND₂, Ad2⁺ND₃, Ad2⁺ND₄, and Ad2⁺ND₅. Each of these four new viruses induces the synthesis of SV40-specific RNA during infection, thus in-

dicating the presence of SV40 genetic material in all of these nondefective hybrids. Two of these viruses (Ad2⁺ND₂ and Ad2⁺ND₄) appear to induce the SV40 U and tumor-specific transplantation antigens (A. M. Lewis, Jr. and W. P. Rowe, manuscript in preparation); Ad2⁺ND₃ induces the SV40 tumor antigen as well. In contrast, Ad2⁺ND₅ and Ad2⁺ND₁ do not induce any SV40 antigens detectable by immunofluorescence or complement fixation. A distinguishing factor between Ad2⁺ND₃ and Ad2⁺ND₅ is that the latter induces significantly more hybridizable SV40-specific RNA during acute infection than does Ad2⁺ND₃. These observations (14) suggest that the five nondefective Ad2-SV40 hybrid viruses contain varying portions of the SV40 genome. In this report, we characterize the DNA of each of the four new hybrids and compare each one with the DNA of nonhybrid Ad2 and with the DNA of the prototype nondefective Ad2-SV40 hybrid, Ad2⁺ND₁.

MATERIALS AND METHODS

Viruses, cells, radiolabeling of viral DNA, and virus purification. The strains of Ad2, Ad2⁺ND₁, Ad2⁺ND₂, Ad2⁺ND₃, Ad2⁺ND₄, Ad2⁺ND₅, and

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SV40 employed in these experiments and the procedures for radiolabeling viral DNA with ^{14}C -thymidine have been described (11, 14, 15, 19).

Ad2 and the nondefective Ad2-SV40 hybrid viruses were grown in 32-oz (0.95-liter) bottle cultures of either human embryonic kidney cells (HEK) or Vero cells (a continuous line of African green monkey kidney cells), or in suspension cultures of human (KB) cells. Bottle cultures were infected with 10 to 50 PFU of virus per cell, suspended in 10 ml of Eagle minimal essential medium containing 250 units of penicillin per ml and 250 μg of streptomycin per ml, and 2 mM glutamine supplemented with 2% agammaglobulinemic calf serum (EMEM-2). After a 2- to 4-h adsorption period, 20 ml of EMEM-2 was added to each bottle. When 50 to 75% of the cells exhibited cytopathic effects, cells and fluids were harvested by scraping. Cells were pelleted at $500 \times g$, suspended in 60 ml of Tris-buffered saline (pH 7.4), and frozen at -70°C . One-liter KB cell suspension cultures containing 2×10^6 cells/ml were infected with 10^6 PFU of virus. At 48 h after infection, the cells were pelleted, suspended in 60 ml of Tris-buffered saline, and stored at -70°C .

After three cycles of freeze-thawing, cell suspensions were treated with 1.0% sodium deoxycholate and 0.1% trypsin and cushioned on cesium chloride (density = 1.45 g/ml) in an SW25.1 rotor at $55,000 \times g$ for 90 min at 4°C . The virus band at the interface was collected, and the virus was rebanded twice in cesium chloride (density = 1.34 g/ml), using an SW39 rotor at $100,000 \times g$. After a 5- to 6-h dialysis against 0.01 M Tris (pH 7.8), purified virus was stored at -70°C .

Methods for the growth and purification of SV40 have been described (19).

Preparation of DNA. For viral DNA extraction (9), sodium dodecyl sulfate (SDS; final concentration, 0.3%) and 1 mg of nuclease-free pronase B per ml (Calbiochem) was added to the virus band. The mixture was incubated at 37°C for 2 h with gentle agitation every 15 min. An equal volume of buffer (0.05 M Tris-hydrochloride, pH 8.1, with 0.1 M NaCl and 0.01 M EDTA)-saturated, redistilled phenol was added, and the mixture was inverted gently 30 to 50 times and allowed to stand for 10 min at room temperature. The mixture was again inverted 30 to 50 times and centrifuged at 1,800 rpm for 10 min at 25°C , and the phenol was removed from the bottom of the tube. The phenol extraction was repeated a second time, and the aqueous phase was then removed with a wide-bore Pasteur pipette. DNA was dialyzed in collodion tubes (Schleicher and Schuell) against $0.01 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M}$ sodium chloride + 0.015 M sodium citrate, pH 6.9) for 24 h at 4°C , and stored at the same temperature.

Micrococcus lysodeikticus DNA and *Escherichia coli* DNA were prepared by the method of Marmur (18). All DNA samples were quantitated by the diphenylamine reaction.

In vitro synthesis of complementary viral RNA (cRNA). Tritium-labeled RNA complementary to Ad2 DNA (^3H -Ad2 cRNA) and to form I SV40 DNA (^3H -SV40 cRNA) was synthesized in vitro by a modification (13) of the method of Chamberlin and

Berg (2). The specific activity of the ^3H -Ad2 cRNA was estimated to be 8.6×10^6 counts per min per μg and that of ^3H -SV40 cRNA, 1.1×10^7 counts per min per μg , assuming equimolar uptake of isotopic precursors.

RNA-DNA hybridization. The procedure for hybridization of RNA with single-stranded DNA immobilized on nitrocellulose membrane filters was that of Gillespie and Spiegelman (6) with slight modifications (12). Hybridization reactions were performed with 13-mm filters in a volume of 0.25 ml of $2 \times \text{SSC}$ plus 0.05% SDS for 16 h at 60°C . All RNA-DNA hybrids were treated with pancreatic ribonuclease and ribonuclease T_1 (to remove noncomplementary RNA) before scintillation counting (12).

Zone sedimentation of viral DNA in alkaline sucrose. Linear 12-ml gradients were prepared in albumin-coated polyallomer tubes from 5 and 20% sucrose solutions in 0.02 M trisodium phosphate (pH 12.5). Unlabeled viral DNA (1–2 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 25,000 rpm for 16 h at 6°C in a Spinco SW40 rotor. Fractions (0.4 ml) were collected with an ISCO model D density gradient fractionator. The fractions were neutralized with 0.02 M citric acid, and the DNA was immobilized on nitrocellulose filters for use in RNA-DNA hybridization studies.

Buoyant density of viral DNA. Viral DNA (1–2 μg) in 0.7 ml of CsCl solution (density = 1.710 g/ml in 0.01 M Tris, pH 8.1) was studied in a Spinco model E analytical ultracentrifuge in a two-cell D rotor. One cell contained Ad2, SV40, and *M. lysodeikticus* DNA, and the second cell contained DNA from a hybrid virus and *M. lysodeikticus* DNA. The cells were centrifuged at 44,700 rpm for 18 h at 25°C , and ultraviolet absorption photographs of the DNA bands were taken on Kodak commercial film. The developed films were analyzed in a Spinco analytical microdensitometer. Densities were calculated using the equation of Schildkraut et al. (20) and taking the density of the *M. lysodeikticus* marker (22) to be 1.731 g/ml (19).

Thermal denaturation. The T_m value (temperature corresponding to half the final increase in relative absorbance) for each viral DNA was determined in approximately $0.1 \times \text{SSC}$ at a DNA concentration of 10 to 20 $\mu\text{g}/\text{ml}$. Thermal denaturation profiles were obtained with the Gilford 2400 recording spectrophotometer used in conjunction with a Haake temperature control circulating water bath. The heating rate was $0.5^\circ\text{C}/\text{min}$ between 60 and 90°C . All hyperchromicities were corrected for solvent expansion upon heating. The concentration of sodium in each DNA solution, as determined by flame spectrophotometer (Perkin-Elmer), was 0.022 M.

Electron microscopy of viral DNA. DNA was prepared for electron microscopy by using a modification of the protein film technique described by Davis, Simon, and Davidson (4). The spreading solution contained 1 μg of DNA per ml, 0.1 mg of cytochrome C per ml, 0.5 M ammonium acetate, and 0.003 M Tris (pH 8.5). A 50- μl sample was applied to a microsome slide supported at a 25° angle to the surface of a

0.25 M ammonium acetate solution (hypophase) in a Teflon trough. Parlodian-coated copper grids were touched to the floating film, stained immediately by immersion for 30 s in a 10^{-4} M solution of uranyl acetate in 90% ethanol, rinsed in isopentane, and examined in an RCA EMU3 electron microscope. Photographs were made at a magnification of approximately $\times 4,000$ and enlarged to a magnification of $\times 40,000$ by a projector. Tracings of the projected images were measured with a Keuffel and Esser map measurer, and lengths were determined by calibration with companion micrographs of a grating replica with known spacing.

RESULTS

Linkage of Ad2 and SV40 DNA. DNA from each of the nondefective Ad2-SV40 hybrid viruses was studied by sedimentation through an alkaline sucrose gradient. The results (Fig. 1) demonstrate that the curves corresponding to hybridization of DNA with ^3H -Ad2 cRNA or ^3H -SV40 cRNA coincide exactly, thus indicating that the Ad2 and SV40 components are not separable by alkaline denaturation of the DNA. In addition, the Ad2 and SV40 DNA components from each of these hybrids could not be separated in neutral or alkaline CsCl equilibrium density gradients (experiments not

shown), whereas mixtures of Ad2 and SV40 DNA analyzed under the same conditions were clearly resolved (11).

Buoyant density. Figure 2A shows the results of a study in which Ad2, SV40, and *M. lysodeikticus* DNAs were analyzed in a single cell. The bands obtained were Gaussian and unimodal, indicating a high degree of homogeneity in the DNA preparations. The densities obtained for Ad2 (1.715 g/ml) and SV40 (1.700 g/ml) are consistent with those previously published (3, 7).

The result obtained with Ad2⁺ND₂ DNA (Fig. 2B) is representative of the data obtained with the DNA from each of the Ad2-SV40 hybrid viruses. The densities of Ad2⁺ND₂ DNA (1.714 g/ml) and the other hybrid DNAs are not significantly different from that of non-hybrid Ad2 (Table 1).

Thermal denaturation. The thermal denaturation profiles for Ad2 DNA and for Ad2⁺ND₂ DNA are shown in Fig. 3. Again, the Ad2⁺ND₂ profile is representative of the data obtained with each of the hybrid virus DNAs (Table 1). A smooth absorbance-temperature profile with a single inflection point was observed for each viral DNA. That the DNA preparations were

FIG. 1. Rate zonal sedimentation of Ad2-SV40 hybrid DNA molecules in alkaline sucrose. The counts per minute of ^3H -SV40 and ^3H -Ad2 cRNA bound by DNA filters prepared from 0.4-ml fractions of each gradient are shown. Each hybridization mixture contained a DNA filter from the gradient fraction and 25,000 counts/min of ^3H -Ad2 cRNA or ^3H -SV40 cRNA in a total volume of 0.25 ml of $2 \times \text{SSC}$. In addition, some vials contained *E. coli* DNA filters (0.1 $\mu\text{g}/\text{filter}$) as blanks. The 20 to 50 counts/min bound to the *E. coli* DNA filters have been subtracted. Symbols: $\bullet$, ^3H -Ad2 cRNA; $\circ$, ^3H -SV40 cRNA.

FIG. 2. Buoyant density of viral DNA species. Microdensitometer tracings of the UV absorption photographs of DNA samples at equilibrium in a CsCl density gradient are shown. Centrifugation was performed at 44,700 rpm for 18 h at 25 C. *M. lysodeikticus* DNA is represented by the band at the far left of each tracing. A, Ad2, SV40, and *M. lysodeikticus* DNA (approximately 1 μ g each). B, Ad2+ND₂ and *M. lysodeikticus* DNA (approximately 1 μ g each).

not partially denatured is indicated by the absorbance increase of 30 to 40% at the highest temperature.

The guanine plus cytosine (G + C) content of each viral DNA was calculated from both the buoyant density and T_m determinations (Table 1). The four hybrids do not differ significantly from each other or from nonhybrid Ad2. In addition, the results of the two methods agree well for each DNA, indicating the absence of unusual bases.

Electron microscopy of the DNA from Ad2 and the Ad2-SV40 hybrid viruses. Nonhybrid Ad2 DNA was used as an external standard for comparison with the DNA from each of the Ad2-SV40 hybrid viruses. This Ad2 DNA had an $s_{20,w}$ value of 31.4 in 1.0 M NaCl, corresponding to a molecular weight of 23.9×10^6 (21).

Each hybrid virus DNA preparation contained only linear duplex molecules, and these were indistinguishable from those of Ad2 DNA. There was minimal fragmentation of the DNA molecules from these preparations; only molecules between 7 and 14 μ m were plotted in the

histograms. No circular SV40 DNA was noted, there were no looped-out regions, and there was no evidence of circularity for Ad2 or the hybrid virus DNAs.

The mean molecular length of the Ad2 standard was 10.4 μ m (Fig. 4) which corresponds to a molecular weight of 20×10^6 , assuming a linear density of 1.92×10^8 daltons/ μ m for the hydrated form B of DNA (1). The mean molecular lengths for the hybrid virus DNAs did not,

TABLE 1. Composition of Ad2, SV40, and the nondefective Ad2-SV40 hybrid virus DNAs

Viral DNA	Buoyant density ^a	G + C % ^b	T_m (C) ^c	G + C % ^d
Ad2	1.715	56	77.5	57
Ad2+ND ₂	1.714	55	76.9	56
Ad2+ND ₃	1.716	57	77.8	58
Ad2+ND ₄	1.714	55	76.9	56
Ad2+ND ₅	1.713	54	77.5	57
SV40	1.700	40		

^a Buoyant density (ρ) measurements were performed in CsCl with *micrococcus lysodeikticus* DNA ($\rho = 1.731$) as a standard.

^b Calculated from buoyant density value (20): G + C % = $(\rho - 1.660)/0.098$.

^c T_m determinations were performed in approximately $0.1 \times$ SSC (Na^+ concentration = 0.022 M).

^d Calculated from T_m value (16): G + C % = 2.44 ($T_m - 81.5 - 16.6 \log M$), where M = molar concentration of Na^+ .

FIG. 3. Thermal denaturation profiles of Ad2 and Ad2+ND₂ DNA. The ordinate represents the ratio of absorbance at 260 nm at the elevated temperature (A_t) to the absorbance at 25 C (A_{25}) in $0.1 \times$ SSC. The T_m value is indicated by an arrow.

FIG. 4. Length distribution of Ad2 and Ad2-SV40 hybrid DNA molecules measured in electron micrographs. Only molecules between 7 and 14 μm were plotted.

differ significantly from that of the nonhybrid Ad2 standard (Table 2).

Percentage of SV40 DNA in the genome of the nondefective Ad2-SV40 hybrid viruses. The percentage of SV40 DNA contained in each hybrid virus DNA was estimated by hybridization with ^3H -SV40 cRNA (11). The preparations of viral DNA used were determined to be free of contaminating cellular DNA by hybridization with ^3H -RNA from human (HEK) and monkey (Vero) cells. In addition, there was no hybridization between human (KB) or Vero cell DNA and the Ad2 or SV40 ^3H -cRNAs employed in these experiments.

To quantitate the SV40 component of each of the hybrid virus DNAs, a calibration curve was constructed relating ^3H -SV40 cRNA bound in a hybridization reaction to the amount of SV40 DNA on the filter. Increasing amounts of ^{14}C -labeled SV40 DNA (0.005–0.03 μg), and that quantity of nonhybrid Ad2 DNA needed to yield a total of 0.5 μg of DNA, were immobilized on nitrocellulose filters. These filters were then reacted with a saturating amount (previously determined with identical DNA filters) of ^3H -SV40 cRNA. The ^3H counts per minute bound were observed to be proportional to the amount of SV40 DNA on the filter (Fig. 5). The same saturating amount of ^3H -SV40 cRNA was reacted with approximately 0.5 μg of ^{14}C -labeled DNA from each of the nondefective hybrid viruses. The exact amount of hybrid DNA present was determined from the ^{14}C

TABLE 2. Mean length and standard deviation of the Ad2 and Ad2-SV40 hybrid DNA molecules^a

Source of DNA	No. of molecules	Molecular length (μm): mean and standard deviation
Ad2	34	10.4 ± 0.5
Ad2+ND ₂	110	10.2 ± 1.2
Ad2+ND ₃	295	10.4 ± 1.0
Ad2+ND ₄	134	10.2 ± 1.0
Ad2+ND ₅	156	10.0 ± 0.7

^a As plotted in Fig. 4.

counts per minute on the filter at the end of the hybridization procedure. At least four determinations were performed for each DNA sample, and the counts per minute of ^3H -SV40 cRNA bound were plotted on the calibration curve. The results estimating the percent of SV40 DNA in the various hybrid DNAs are shown in Fig. 5.

DISCUSSION

The Ad2 and SV40-specific components of the DNA from each of the hybrid viruses could not be separated by rate-zonal sedimentation in alkaline sucrose (Fig. 1). This observation, plus evidence from CsCl equilibrium sedimentation, indicates that no separate SV40 polynucleotides exist, and that each of these hybrid viruses contains Ad2 and SV40 DNA linked together by alkali-resistant, presumably covalent, bonds.

FIG. 5. Calibration curve for counts per minute of ^3H -SV40 cRNA bound to increasing amounts of SV40 DNA immobilized on nitrocellulose filters. Ten reaction vials were employed, each holding a filter containing a specified amount of ^{14}C -SV40 DNA (13.3×10^3 counts per min per μg) and that amount of unlabeled Ad2 DNA which would yield a total of 0.5 μg , a filter containing 0.5 μg of *E. coli* DNA (the blank), and a saturating amount of ^3H -SV40 cRNA (5.6×10^6 counts/min). The average counts per minute bound to the blank (536) have been subtracted; net ^3H counts per minute and ^{14}C counts per minute have been corrected for spillover of the other isotope. The counts per minute (mean) of ^3H -SV40 cRNA bound to 0.5 μg of each of the ^{14}C -Ad2-SV40 hybrid virus DNAs are indicated on the figure and extrapolated to the equivalent amount of SV40 DNA. Specific activities of the hybrid virus DNAs, counts per minute $\times 10^3/\mu\text{g}$: Ad2+ND₁, 5.1; Ad2+ND₂, 7.2; Ad2+ND₃, 4.7; Ad2+ND₄, 4.5; Ad2+ND₅, 4.5. Four to six filters were used for each hybrid virus DNA. The net, corrected ^3H counts per minute bound to each virus DNA were normalized to 0.5 μg on the basis of ^{14}C counts per minute present on the filter. Normalized ^3H counts per minute, mean $\pm$ S.D.: Ad2+ND₁, $12,978 \pm 1178$; Ad2+ND₂, $33,390 \pm 2176$; Ad2+ND₃, 2682 ± 327 ; Ad2+ND₄, $44,486 \pm 3186$; Ad2+ND₅, $28,153 \pm 1605$. All filters were counted to yield a counting accuracy of $\pm 1\%$ for ^3H and ^{14}C .

Further studies demonstrated that the G + C content of the DNA of each of the hybrid viruses, as determined by buoyant density in neutral CsCl and by thermal denaturation, was not significantly different from that of nonhybrid Ad2 DNA (Table 1).

The approximate molecular weight of the DNA of each of the hybrids was determined by an electron microscope comparison with the

DNA of nonhybrid Ad2. The length of the nonhybrid Ad2 was 10.4 μm , which corresponds to a molecular weight of 20×10^6 , a value approximately 10% less than our previous electron microscope determination for this DNA (3). This discrepancy is probably due to changes in the procedure employed for the preparation of the DNA for electron microscopy. Previously, the DNA was spread onto a water hypophase, whereas in the present studies the hypophase contained 0.25 M ammonium acetate, a change known to produce lengths as much as 20% shorter when studied by electron microscopy (10). This premise is supported by the fact that the $s_{20,w}$ of the nonhybrid Ad2 was 31.4 in the present study, a value quite comparable to our previous value of 31.9 (3), and to $s_{20,w}$ values of 30.5 to 32.0 demonstrated in other laboratories (5, 7, 8). The lengths of the hybrid virus DNAs varied from 10.0 (Ad2+ND₅) to 10.4 μm (Ad2+ND₄) (Fig. 4). We have concluded from this comparison that the molecular weight of each hybrid virus DNA is approximately that of nonhybrid Ad2, i.e., 24×10^6 , but that small deletions of Ad2 DNA are not precluded by the present data.

The reconstruction experiments, utilizing hybridization with ^3H -SV40 cRNA, demonstrate that the relative percentage of SV40 nucleotide sequences is smallest in Ad2+ND₅ and greatest in Ad2+ND₄ (Fig. 5). The data on the approximate size of the SV40 DNA component in each of the molecules, as calculated from the percentage of SV40 per molecule, and assuming a molecular weight of 24×10^6 for Ad2, indicate that Ad2+ND₁ contains 240,000 daltons, Ad2+ND₂—624,000 daltons, Ad2+ND₃—48,000 daltons, Ad2+ND₄—840,000 daltons, and Ad2+ND₅—528,000 daltons of SV40 DNA. The hybrid with the largest SV40 component, Ad2+ND₄, thus contains one-fourth to one-third of the SV40 genome, assuming a molecular weight for SV40 of 3×10^6 . The accuracy of these data depends, in part, on the fidelity of in vitro transcription of form I SV40 DNA by *E. coli* RNA polymerase. If the enzyme failed to transcribe a small area(s) of the SV40 genome, and if this area(s) comprised a relatively large portion of the SV40 sequences in the hybrid genomes, our reconstruction data would underestimate the SV40 component in the hybrids. Electron microscope studies have been completed utilizing the technique of heteroduplex formation between Ad2 DNA and DNA from each of the hybrid viruses (T. J. Kelly, Jr., and A. M. Lewis, Jr., manuscript in preparation). Results of these studies visualizing the lengths of the SV40 segments and their sites of insertion indicate that most SV40 DNA has been ac-

counted for in the reconstruction data presented herein. However, somewhat longer SV40 DNA segments have been demonstrated within each hybrid genome by the microscope technique. For example, the latter method has shown that Ad2+ND₄ contains approximately 43% of the wild-type SV40 genome as compared with 28% by cRNA reconstruction. Thus, although a discrepancy exists in the data obtained by the two techniques, it is clear from either procedure that the SV40 component of each of the hybrid virus DNAs differs in quantity, and that Ad2+ND₄ does contain approximately 30 to 40% of the SV40 genome. Moreover, a correspondence exists between the number of detectable SV40-specific antigens induced in lytically infected cells by three of these viruses and the size of their SV40 moiety. For example, only the hybrid with the largest SV40 segment, Ad2+ND₄, induces three SV40 antigens (U, tumor-specific transplantation antigen, and tumor antigen). It is of considerable interest that Ad2+ND₅, with twice as much SV40 DNA as Ad2+ND₁, does not induce a detectable SV40 antigen, whereas Ad2+ND₁ does induce the U antigen. Qualitative studies on the SV40-specific RNA induced by Ad2+ND₅, as well as by each of the other nondefective Ad2-SV40 hybrids, are described in an accompanying report (A. S. Levine et al.).

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