*BIOPHYSICAL EVIDENCE FOR LINKAGE OF ADENOVIRUS AND SV40 DNA'S IN ADENOVIRUS 7-SV40 HYBRID PARTICLES**

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The existence of virus particles in a strain of adenovirus 7 (E46+) consisting of simian virus 40 (SV40) genetic material inside an adenovirus 7 capsid has been demonstrated in several laboratories.[1-3] These particles have variously been called "⊕," "hybrid,"[4] "transcapsidant," or "PARA."[5] They are able to induce SV40 T and transplantation antigens, but not SV40 V antigen or virus.[1-6]

Understanding of the possible association of the SV40 DNA with the adenovirus DNA in the "hybrid" particles has been hampered by their defectiveness; they are able to replicate only in cells infected with "nonhybrid" adenovirus and thus cannot be obtained as a pure population free of "nonhybrid" adenovirus. A cell infected with a "hybrid" particle alone yields neither adenovirus nor SV40 progeny.

Biologic evidence that the SV40 DNA is linked with Ad. 7 DNA in the "hybrid" was obtained by showing that transfer of the SV40 determinant from one adenovirus type to another regularly resulted in transfer of some genetic material of Ad. 7 into the same particle.[7]

Nucleic acid homology techniques[8] provide a method for specific identification and quantitation of purified DNA's. Ad. 7 complementary RNA (cRNA) prepared *in vitro* reacts with Ad. 7 DNA but not to any measurable extent with SV40 DNA, and the converse is also true.[9] Application of the DNA-cRNA technique to purified E46+ DNA showed that SV40 DNA as well as Ad. 7 DNA was present and that the quantity of each DNA was sufficient to allow biophysical examination of possible DNA-DNA linkage.[9] We have used the DNA-cRNA homology technique and a variation of preparative density gradient ultracentrifugation to attempt to separate the SV40 DNA from the adenovirus DNA in the "hybrid" populations under conditions which do separate these DNA's when present in a mixture.

Materials and Methods.—Viruses: E46+ virus passed in African green monkey kidney (AGMK) cultures was used as seed material. Pool 1 was prepared by Dr. Maurice Green by infecting KB-cell spinner cultures at high multiplicity; pool 2 was grown in AGMK cell monolayers, and pool 3 was grown in KB spinner cultures by Dr. Paul Burnett of the Eli Lilly Co., Indianapolis, Indiana.

Human embryonic kidney (HEK) cells were acutely infected with these pools. The ratio of the percentage of these infected cells showing adenovirus T antigen to those showing SV40 T antigen (as measured by fluorescent antibody assay)[4] was approximately 2:1. This is similar to that of all AGMK-grown pools of E46+, and indicates that the "hybrid" and "nonhybrid" particles were present in comparable numbers.

E46− virus, the "nonhybrid" Ad. 7 recovered from E46+,[2] was grown in KB cells, as was a pool of Gomen strain Ad. 7 virus prepared by Dr. Burnett. This latter pool of Ad. 7 was used as a source of DNA for priming for Ad. 7 cRNA in the second E46+ centrifugation.

SV40 virus (strain 777)[10] was obtained from Dr. Paul H. Black, and was grown in BSC-1 cell monolayers.

All adenovirus preparations were free of adeno-associated viruses (AAV) as judged by electron microscopic examination or complement fixation testing of the preparations against antisera to all four known serotypes.[11]

DNA: All DNA preparations were made from virus banded twice in cesium chloride. DNA

was extracted from pool 1 of E46$^+$ by Dr. Green using his published method.[12] The other preparations were extracted in our laboratories, using a modification of the extraction procedure described previously.[13] In this modified procedure papain treatment was reduced to 45 min after which 1% sodium dodecyl sulfate (SDS) was added and the solution held at 37°C for 15 min. The solution was extracted with phenol, and NaCl (final concentration, 1 *M*) was added to the aqueous phase. After 18 hr at 4°C, the precipitate was removed by centrifugation. The DNA was precipitated from the supernate by the addition of 2 vol of 95% ethanol.

SV40 DNA was extracted by the method of Black and Rowe[14] or by the method described for adenoviruses.

Density gradient centrifugation of native DNA: Optical grade CsCl was dissolved in 0.01 *M* Tris buffer. The pH was adjusted to 8.2 with 50% KOH, and the density of the solution measured by its refractive index.

The centrifugations were performed according to the method of Flamm *et al.*[15] DNA preparations were mixed with the cesium solution (final density 1.700 or 1.705 gm/cc) and immediately centrifuged in a fixed-angle #40 rotor in a model L Spinco ultracentrifuge at 33,000 rpm (approx. 70,000 × *g*). The duration of centrifugation was 62 hr at 25° C. The use of the fixed-angle head yields improved resolution over that of swinging-bucket centrifugation because zones of fixed volume (the bands and interband areas) have a smaller diameter when the tube is held vertical for fractionation, than when the bands were forming at a 26° angle to the side of the tube in the rotor. This tends to widen the bands but, more important, to widen the vertical distance between the center of bands of different density. The contents of the tube were collected as 0.115-ml fractions through a puncture in the bottom of the tube using a constant volume displacement device.

Density gradient centrifugation of alkali-denatured DNA: Vinograd *et al.* reported[16] that adenovirus DNA is completely denatured at pH 12.4 with a resultant increase in density of the DNA of 0.06 gm/cc. For the preparation of alkaline density gradients, CsCl was dissolved in 0.08 *M* phosphate buffer, pH 12.55 (instrument reading; glass electrode; Metrohm E300 pH meter). The density of the alkaline cesium solution as measured gravimetrically was approximately 1.760 (0.06 gm/cc greater than that used for native DNA). To ensure denaturation of the DNA's, they were dissolved in 0.01 ml of 0.1 *M* KOH at room temperature prior to their addition to the CsCl solution. Before centrifugation the pH was readjusted to 12.55, and the density to approximately 1.760. Centrifugation was carried out exactly as described for native DNA, except that the fractions were collected in vials containing 0.1 ml of 0.1 *M* citric acid.

DNA:RNA hybridization technique: Tritium-labeled RNA complementary to adenovirus 7 or SV40 DNA (cRNA) was synthesized *in vitro* as previously described.[13] Aliquots (0.025-ml) of the fractions from the preparative gradients were thermally denatured, incubated at 65°C with each RNA, and were assayed in a liquid scintillation counter for the number of counts retained by a cellulose filter after RNase treatment. This procedure has been described in detail.[13, 17]

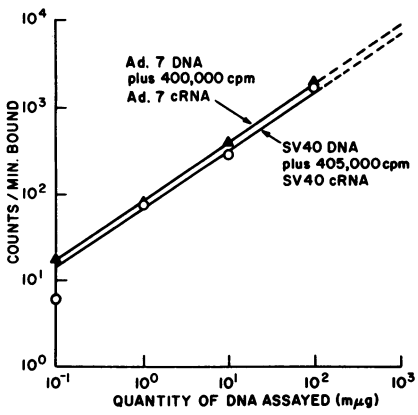

FIG. 1.—Calibration curves plotting cpm of tritiated cRNA bound vs. weight of SV40 and adenovirus 7 DNA added.

Calibration curves (for calculation of the quantity of DNA present from the amount of DNA-RNA hybrid formed) were determined simultaneously with the first experiment (Fig. 1). The specificity of the hybridization reaction using these DNA's and RNA's has been demonstrated previously.[9]

Preparation of labeled RNA in tissue culture: HEK monolayers (~5 × 10^6 cells per flask) were infected with E46$^-$ virus at a multiplicity of approximately 10 $TCID_{50}$ per cell. Twenty-four hr after infection 100 μc of tritiated uridine (sp. act. = 11.9 mc per μmole) were added to the maintenance medium in each culture. Two hr later cells were harvested and RNA was extracted from the cell pellet by the hot phenol method of Scherrer and Darnell.[18] The RNA yield from $10^{7.5}$ cells was approximately 1.5 × 10^6 cpm of radioactive RNA.

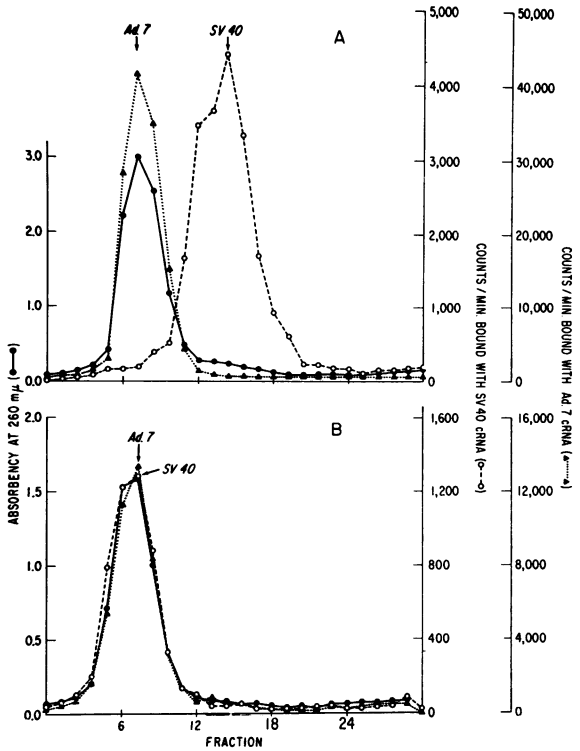

FIG. 2.—Fixed-angle cesium chloride density gradient centrifugation. A plot of the absorbency at 260 mμ and cpm of tritiated SV40 and Ad. 7 cRNA's bound by 0.025-ml aliquots of each gradient fraction. (*A*) Mixture of 40 μg E46⁻ DNA and approximately 4 μg SV40 DNA; starting density = 1.700 gm/cc, pH 8.2. (*B*) Preparation 1 of E46⁺ DNA (28.8 μg); starting density = 1.700 gm/cc, pH 8.2.

Results.—The data from density gradient centrifugation of undenatured DNA are shown in Figures 2 and 3. Figure 2*A* illustrates the UV absorbency and radioactive cRNA bound by adenovirus and SV40 DNA in fractions from the centrifugation of a mixture of E46⁻ and SV40 DNA's. Since only a small amount of SV40 DNA was used, there was only one absorbency peak. This absorbency peak corresponded to the adenovirus DNA and was, in turn, almost completely separate from the SV40 DNA. On the other hand, results of simultaneous centrifugation of the DNA from E46⁺ (Fig. 2*B*) showed that the peaks of absorbency, SV40 DNA, and adenovirus DNA were entirely coincident.

Not only were the peaks at the same place in the E46⁺ gradient, but the widths of the bands were identical, the width of the SV40 band in the E46⁺ DNA being less than that in the mixed DNA run. In density gradients, band width is inversely proportional to the square root of the molecular weight of the banded substance.[19]

Figure 3 shows the results of centrifugation with preparation 2 of E46⁺ DNA, extracted from AGMK-grown virus. These results were similar to those obtained with a preparation 1 from virus grown in human cells. The extraction procedures for the two preparations were substantially different, minimizing the possibility

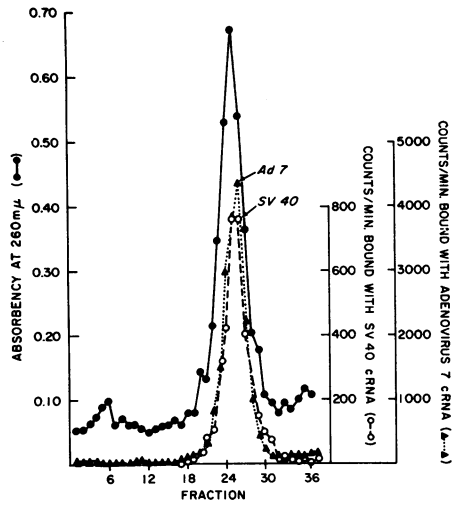

FIG. 3.—Density gradient centrifugation of 20.0 μg of preparation 2 of E46+ DNA. Starting density = 1.705. Rotor, temperature, pH, and duration of centrifugation were identical to those employed in centrifugation of preparation 1 of E46+ DNA.

that the observed association between DNA's in the "hybrid" was a preparative artifact.

Comparison of the calibration curves (Fig. 1) with the E46+ centrifugation results (Fig. 2*B*) indicated the presence of about 20 times as much Ad. 7 DNA as SV40 DNA in the E46+ preparation.

In the gradients run at pH 12.6 (Fig. 4), the adenovirus DNA was resolved into two components as detected by reaction with *in vitro* cRNA. The denser fraction (1.765 gm/cc) corresponded to the density expected for denatured Ad. 7 DNA and, though less reactive with *in vitro* cRNA than the less dense fraction, constituted the bulk of the total Ad. 7 DNA as indicated by the UV absorbency. This denser fraction was the primary DNA detected by reaction with RNA from Ad. 7-infected HEK cells (Fig. 5); neither fraction showed significant reaction with labeled RNA from uninfected human tissue culture cells. The lower density fraction may represent partially denatured Ad. 7 DNA, but the reason for its more efficient reaction with *in vitro* cRNA is not known; this may represent an effect of selective transcription *in vitro* by *E. coli* RNA polymerase.

In the gradient separation of the mixture of Ad. 7 and SV40 DNA's (Fig. 4*A*), the peak of SV40 DNA was 0.012 gm/cc lighter than the dense Ad. 7 peak, while the SV40 DNA of E46+ was only 0.002 gm/cc lighter. The 0.012 gm/cc density difference corresponds to the known difference in density of Ad. 7 and SV40 DNA's, and the separation obtained is the maximum to be expected. The 0.002 gm/cc difference observed with E46+ DNA (this difference was confirmed in a second experiment) may be attributable to the fact that the Ad. 7 DNA is derived from both the "nonhybrid" and "hybrid" particles in E46+; the presence of an SV40 segment in the DNA from the latter would slightly reduce the buoyant density of the hybrid DNA strand.

The width of the SV40 peak was greater in the mixed run than in the E46+ run, as was seen with the undenatured DNA's.

Discussion.—Utilization of fixed-angle rotors for preparative ultracentrifugation[15] has enabled us to separate SV40 DNA physically from Ad. 7 DNA in either the native or denatured state when present in an artificial mixture, although the densities of these DNA's differ by only 0.010–0.015 gm/cc. These DNA's could not be separated under identical conditions when they were derived from the "hybrid" particle.

The possibility that the molecular combination represents a preparative artifact is unlikely since the same result was obtained with two batches of E46+ DNA differing in host cell origin and method of extraction. In neither extraction pro-

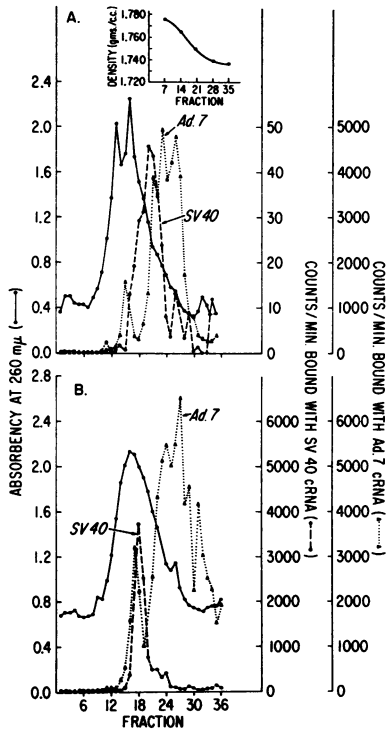

FIG. 4.—Fixed-angle cesium chloride density gradient centrifugation of alkali-denatured DNA. A plot of the absorbency at 260 mμ, and cpm of tritiated SV40 and Ad. 7 cRNA's bound by 0.025-ml aliquots of each gradient fraction. (*A*) Mixture of 50 μg E46⁻ and 5 μg SV40 DNA; starting density = 1.759 gm/cc, pH 12.55. (*B*) Preparation 3 of E46⁺ DNA (100 μg); starting density = 1.761 gm/cc, pH 12.55. *Insert:* A plot of the density measured gravimetrically vs. gradient fraction number.

cedure was the E46⁺ DNA exposed to conditions which would cause denaturation and reannealing. Another possible explanation of the results is that the segment of SV40 DNA present in the "hybrid" has a density identical to that of the adenovirus DNA. This atypical segment could occur if the SV40 genome consists of regions differing greatly from one another in their G-C content. However, if the SV40 DNA segment (mol wt $\leq 2 \times 10^6$) was separate from adenovirus DNA (mol wt = 22×10^6) in E46⁺, the band width of the lower-molecular-weight SV40 would have been greater than that of the adenovirus DNA band. The data in Figures 2 and 4 show that SV40 DNA in an artificial mixture gave a wider band than either the adenovirus DNA from E46⁺ or E46⁻ or the SV40-hybridizable segment in E46⁺ DNA.

The present data indicate that the SV40 DNA in E46⁺ is in a molecule having both a density and molecular weight similar to the extracted adenovirus DNA. This finding, and the genetic evidence of linkage from transfer experiments,[7] strongly indicate that the SV40 and adenovirus DNA's are physically linked within the "hybrid" particle. The results obtained with denatured DNA are consistent with the hypothesis that the linkage is covalent rather than by hydrogen bonding.

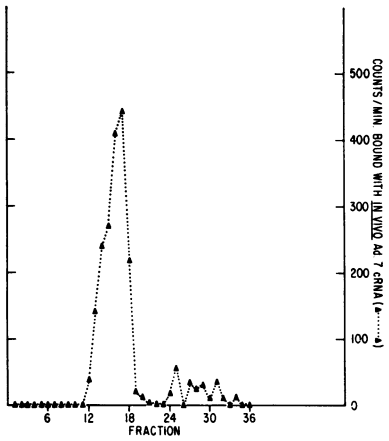

FIG. 5.—Hybridization of aliquots of alkali-denatured E46+ DNA (preparation 3) gradient fractions with Ad. 7 cRNA extracted from E46−-infected tissue culture cells; 37,000 cpm of radioactive RNA were incubated with 0.025-ml aliquots of each gradient fraction. The results are expressed as cpm of tritiated cRNA bound by Ad. 7 DNA in each gradient fraction.

The formation of linkage between SV40 and Ad. 7 DNA's is remarkable in the absence of demonstrable homology as measured by cross-reactivity with their cRNA's.[9]

The demonstration of linkage indicates that the defectiveness of the hybrid particle does not necessarily imply absence of a portion of the SV40 genome. Defectiveness could be due instead to configurational or functional properties of the hybrid molecule.

It is not feasible to estimate the molecular weight of the SV40 DNA segment in the hybrid from the 1:20 ratio of SV40 to Ad. 7 DNA in E46+ since the adenovirus DNA is derived from both "hybrid" and "nonhybrid" particles. The fluorescent antibody-staining ratio, the best available method of estimating the relative proportion of the two particles, can only be taken as a rough estimate. Also, quantitation of the virus DNA may well be biased by selective copying *in vitro* of DNA by *E. coli* polymerase.

The failure to detect SV40 DNA in association with the less dense Ad. 7 DNA fraction (Fig. 4*B*) would occur if the lower density fraction consisted of fragments derived either from a region of the Ad. 7 DNA strand remote from the site of combination with SV40, or from the "nonhybrid" Ad. 7 DNA present in E46+.

Other adenovirus-SV40 "hybrids," notably types 2 and 4,[20] differ from E46+ in yielding SV40 virus with high efficiency when infection is at low multiplicity. Studies are in progress to determine if this class of "hybrid" is also a hybrid at the molecular level.

Summary.—The native and alkali-denatured DNA's of purified hybrid virus E46+ and its antecedents adenovirus 7 and SV40 were subjected to fixed-angle density gradient centrifugation. The resulting gradient fractions were analyzed for the presence of DNA components hybridizable with adenovirus and SV40 cRNA. The SV40 and adenovirus DNA's of the hybrid virus were nondissociable while those in a mixture of adenovirus and SV40 were completely dissociable.

These findings indicate that the DNA's of E46+ are linked by an alkali-stable bond as a single molecule within the hybrid particle and that this particle may be aptly termed a hybrid on biophysical grounds.

Centrifugation of alkali-denatured Ad. 7 DNA yielded two peaks of DNA of different density. The lower density species hybridized preferentially with *in vitro* cRNA while the denser species, composing the bulk of the DNA, and consisting of denatured strands, hybridized preferentially with RNA extracted from Ad. 7-infected tissue culture cells.

The SV40 DNA of E46+ was associated with the denser of these two species.

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