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Density Differences Between Hybrid and Nonhybrid Particles in Two Adenovirus-Simian Virus 40 Hybrid Populations

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The adenovirus 7-simian virus 40 hybrid virus population E46+ was subjected to fixed-angle equilibrium density gradient centrifugation in CsCl. A difference in buoyant density between the hybrid virion and its nonhybrid adenovirus 7 counterpart was noted, the hybrid virion possessing the lower buoyant density. This difference in buoyant density appeared to be accentuated in a population of adenovirus 2^{+t7}, a derivative of E46+ in which the adenovirus 7-simian virus 40 genome had been transferred to an adenovirus 2 capsid.

The E46+ strain of adenovirus 7 has been shown to contain some virions in which segments of deoxyribonucleic acid (DNA) of adenovirus 7 and of simian virus 40 (SV40) are present together in an adenovirus 7 capsid (1, 17). These virions, referred to as ⊕ (15) or PARA (11) particles, appear to contain a true hybrid genome in that the adenovirus 7 and SV40 DNA segments are linked by covalent bonds (1).

The hybrid virion is defective in both its SV40 and adenovirus characteristics, and requires co-infection with nonhybrid adenovirus (designated ⊖ particles) for its replication.

Purification and further characterization of the hybrid virion's functional capabilities have thus far been hampered by the inability to obtain the defective particle free from the nonhybrid. Unsuccessful attempts have been made to separate these particles by virtue of differences in growth (15), and in immunological, biochemical, and physical attributes (2, 16). Although no differences in density between ⊕ and ⊖ particles could be demonstrated by equilibrium density gradient centrifugation performed in a swinging-bucket rotor (2, 16), the increased resolution achieved when centrifugation is performed in a fixed-angle rotor (5) suggested that further attempts at separation were justified.

MATERIALS AND METHODS

Viruses. The identification and characterization of the E46+ strain of adenovirus 7 and its transcapsidant adenovirus 2^{+t7} have been described in detail (6, 13–16). The latter population contains particles consisting of the adenovirus 7-SV40 hybrid genome in adenovirus 2 capsids; it also contains adenovirus 2 ⊖ particles, and it is free from adenovirus 7.

Pools of these viruses were made by passage in African green monkey kidney (AGMK) cells at a high multiplicity of infection. All virus stocks were shown by complement-fixation reaction to be free from all known types of adeno-associated virus.

Virus purification. Virus was extracted from infected AGMK cells and sedimented onto a CsCl cushion (density, 1.45 g/cc) by centrifugation at 57,000 × *g* for 90 min at 4 C by a modification of a method previously described (13).

Separation of hybrid and nonhybrid particles. A CsCl solution (density, 1.35 g/cc) was prepared in 0.01 M tris(hydroxymethyl)aminomethane buffer, *p*H 8.1. Cushioned virus, suspended in the CsCl solution, was subjected to isopycnic banding in a Spinco no. 40 fixed-angle rotor. Centrifugation was done at 4 C for 62 hr at 70,000 × *g*.

Fixed-angle equilibrium density gradient centrifugation and the reasons for its increased resolution over centrifugation in swinging-bucket rotors have been described (4, 5). A sample of the virus suspension was set aside and maintained at 4 C for the duration of each run. This suspension (designated as starting material) served as a control for the possible selective inactivation of hybrid or nonhybrid particles by prolonged exposure to CsCl.

At the end of centrifugation, 0.1-ml fractions of the gradient were collected through a hole in the bottom of the tube.

Starting material and the gradient fractions to be assayed for infectious virus, or for antigen-inducing capacity, were diluted and dialyzed against phosphate-buffered saline (*p*H 7.4), and then were further diluted in Eagle's minimal essential medium contain-

ing 10% fetal bovine serum. The samples were either assayed immediately or stored at −70 C until used.

Infectivity assay. The amount of infectious nonhybrid adenovirus present in each fraction was measured by plaque titration in human embryonic kidney (HEK) cells according to the method of McAllister et al. (8). The number of infectious hybrid particles was measured by plaquing on AGMK cells previously infected with a "lawn" of nonhybrid adenovirus 7. This method yields a one-hit dose response curve for plaques resulting from infection with a hybrid particle (11).

Antigen assay. The methods and reagents used in growing HEK cells on cover slips, infecting them, and testing for virus-induced antigens by the direct and indirect fluorescent-antibody (FA) technique have been described (10, 14). These methods allow accurate quantitation of the number of cells in which viral antigens are produced. Since antigen induction is a one-hit phenomenon, the number of cells stained is proportional to the number of ⊕ or ⊖ particles in the inoculum (16).

RESULTS

Density difference of ⊕ and ⊖ particles in adenovirus 7 E46$^+$. Fixed-angle equilibrium density gradient centrifugation of cushioned E46$^+$ virus yielded two visible opalescent bands. The lower, larger band formed at a density of 1.345 to 1.350 g/cc and represented infectious virus. The smaller band of less dense material, presumably empty capsids, formed at a density of 1.335 and induced neither SV40 T antigen nor adenovirus antigen or plaques.

Figures 1 and 2 show the results of assays of the gradient runs of two pools of E46$^+$. The first is an FA assay of antigen induction by pool one; the second, an assay of plaque formation by pool two. In both experiments, as well as in three other tests not shown here, there was a relative enrichment of ⊕ particles in the lower density region of the virus band, and a relative depletion of these particles in the higher density region. This was accompanied in each case by a displacement of the peak of ⊕ particles one gradient fraction to the less dense side of the ⊖ peak. This displacement represented 0.001 to 0.002 g/cc difference in density between the ⊕ and ⊖ particles.

The lower portion of Fig. 1 shows the ratios of SV40 T-inducing particles to adenovirus 7 V-inducing particles (presumably only ⊖). It is seen that this ratio in the starting material was approximately 12:1. At the region of relative ⊕ depletion (fraction 18) this ratio is 1:1, and at the zone of ⊕ particle enrichment (fraction 21) it is 25:1. The ratios of SV40 T to adenovirus T shown in Fig. 1 follow a similar pattern. Similarly, in Fig. 2 the ratio of ⊕ particles to ⊖ particles in the starting material is 1:10 and, after banding, varies from a low of 1:110 to a high of 1:8.

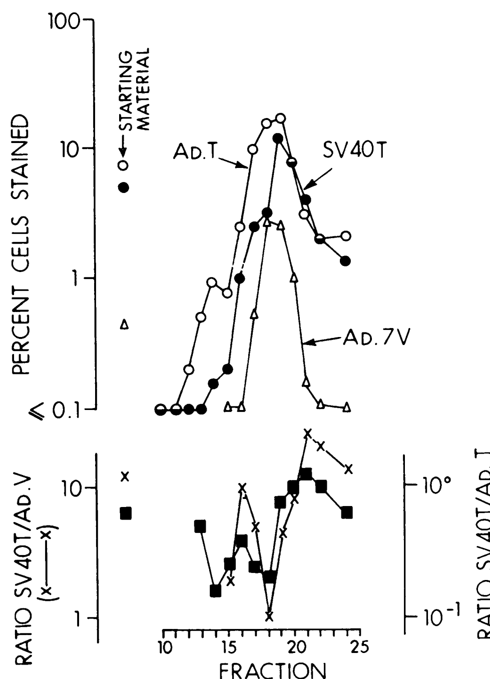

FIG. 1. *Fixed-angle density gradient centrifugation of E46$^+$. Fluorescent-antibody assay of adenovirus 7 V, SV40 T, and adenovirus T antigen-inducing particles in each gradient fraction. The ratios of SV40 T to adenovirus V and of SV40 T to adenovirus T antigen-inducing particles in the gradient fractions are shown at the bottom of the figure. Gradient fractions were all assayed at the same dilution, and the percentage of cells stained by the starting material was normalized to this dilution. Lower fraction numbers correspond to higher density.*

Density difference of ⊕ and ⊖ particles in adenovirus 2^{+t7}. The data presented above show that the ⊕ particles in E46$^+$ are detectably less dense than the ⊖ particles. This density difference was not of sufficient magnitude to permit a preparatively useful degree of physical separation.

Adenovirus 2 virions have a greater density than do adenovirus 7 virions (9). This difference is thought to be due to the greater ratio of DNA to protein in the adenovirus 2 particle. We postulated, therefore, that the density difference between ⊕ and ⊖ particles would be enhanced in the adenovirus 2^{+t7} population. That is, if a difference in the DNA component of a virus is reflected in the density of the virion, then the density of a particle containing the relatively light adenovirus 7$^+$ genome should differ more from the density of an adenovirus 2 ⊖ particle than from that of an adenovirus 7 ⊖ particle.

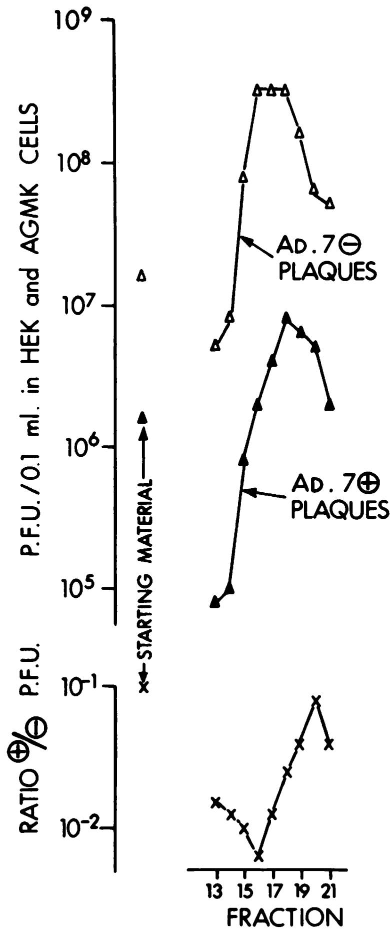

FIG. 2. *Fixed-angle density gradient centrifugation of E46+. Infectivity assay of ⊕ and ⊖ particles in each gradient fraction. The ⊖ particles are titrated in HEK cells; ⊕ particles, in AGMK cells preinfected with a "lawn" of adenovirus 7−. The ratio of ⊕ to ⊖ particles in the gradient fractions is shown at the bottom of the figure. Lower fraction numbers correspond to higher density.*

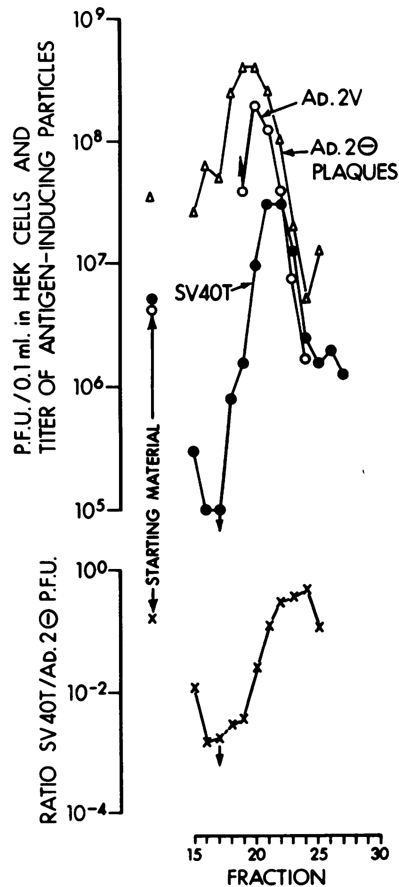

FIG. 3. *Fixed-angle density gradient centrifugation of adenovirus 2^{+t7}. Fluorescent-antibody assay of SV40 T and adenovirus 2 V antigen-inducing particles and infectivity assay of adenovirus 2− particles present in each gradient fraction. The ratios of SV40 T antigen-inducing particles to adenovirus 2− plaque-forming units are shown at the bottom of the figure. Lower fraction numbers correspond to higher densities.*

Accordingly, a pool of adenovirus 2^{+t7} was purified and banded as described above. The gradient fractions were assayed in HEK cells for adenovirus infectivity, and for their ability to induce adenovirus 2 V and SV40 T antigens. The results of this experiment are shown in Fig. 3. The curves for adenovirus plaques in HEK and adenovirus 2 V antigen appear to be similar over the range tested. The peak of SV40 T antigen-inducing particles occurred two fractions to the light side of the peak of adenovirus infectivity. In addition, the ratio of SV40 T-inducing particles to adenovirus 2− plaque-forming units, which had been about 1:6 in the starting material, was decreased to approximately 1:600 in the region of greatest ⊕ particle depletion (fraction 16), and increased to 1:2 in the region of maximal ⊕ particle enrichment (fraction 23).

Thus, in this preparation, as in E46+, the hybrid particles appeared to be less dense than the nonhybrid adenovirus in the mixed population.

An attempt was made to enhance the separation of ⊕ and ⊖ particles in adenovirus 2^{+t7} by pooling fractions at either end of the virus band area and subjecting them to a second cycle of fixed-angle density gradient centrifugation. Although it appeared that the initial separation was maintained, it was not enhanced. Also, the loss of about $\log_{10}$ of virus during this rebanding made the procedure impractical for preparative purification of ⊕ particles.

DISCUSSION

Physical and chemical procedures have been used in numerous unsuccessful attempts to separate hybrid and nonhybrid particles in mixed populations so that the hybrid virion could be purified and studied (2, 16).

Biological purification has been hampered by the defective infectivity of the hybrid particle. Because of this, it has been impossible to grow up pools of this virus which were uncontaminated with nonhybrid helper virus, though pure nonhybrid adenovirus could easily be obtained by limiting dilution titration or plaquing in HEK cells (15).

It appeared, from previous studies, that the capsids of ⊕ and ⊖ particles were identical. DNA-messenger ribonucleic acid homology studies demonstrated that SV40 DNA was present in the hybrid population (13), and transcapsidation experiments in which the SV40 genome was transferred from one adenovirus type to another indicated that the DNA of SV40 and that of adenovirus were present within the same virion (17). Fixed-angle density gradient centrifugation of the DNA extracted from the hybrid virus population showed that the SV40 DNA was associated with adenovirus DNA, and alkaline denaturation of the DNA failed to displace the SV40 from adenovirus DNA, indicating that they were linked by covalent bonds (1).

The results of the present experiments show that the ⊕ and ⊖ particles of two mixed populations can be distinguished from one another on the basis of buoyant density differences. Somewhat unexpectedly, in the case of both the E46$^+$ and adenovirus 2^{+t7} populations, the hybrid particle containing part of the SV40 genome appears to have a lower buoyant density than the nonhybrid particle which lacks SV40 genome. It is possible that it is this differing DNA composition of the hybrid and nonhybrid virions which yields particles of different buoyant density, as the capsids of both virions are apparently identical.

By analogy with some of the deletion mutants of λ bacteriophage, this *decrease* in density of a virion which has gained an extra DNA fragment may be explained by a more than compensatory loss of adenovirus DNA in the hybrid particle. Other explanations, such as internal configurational changes in DNA or core proteins, seem less likely to account for a change in the density of a closed structure. The proposed deletion of a piece of adenovirus 7 DNA in the hybrid virion is entirely consistent with its proven defectiveness as an adenovirus.

Pilot experiments with the adenovirus 2^{+t7} population do seem to have provided an additional degree of separation of ⊕ and ⊖ particles, in that the peaks representing these two virions are two fractions apart, but rebanding has resulted in an unacceptable degree of virus loss.

Some adenovirus-SV40 hybrids produce infectious SV40 virus and, therefore, presumably contain the entire SV40 genome (3, 7). The methods described have been used to study these SV40-yielding hybrids (Wiese et al., *in preparation*), and may prove useful in selective enrichment of the plaque progeny of E46$^+$ having different oncogenic potential which have been recently described (12).

It is probable that the separation of peaks of hybrid and nonhybrid virions achieved in these studies approaches the limits of resolution of this method. Preparative separation of these particles, though now theoretically possible, awaits methods with a higher power of resolution.

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