JOURNAL OF VIROLOGY, Apr. 1970, p. 421-426

Vol. 5, No. 4
Printed in U.S.A.

Equilibrium Density Gradient Studies on Simian Virus 40-Yielding Variants of the Adenovirus Type 2-Simian Virus 40 Hybrid Population

WILLIAM H. WIESE, ANDREW M. LEWIS, JR., AND WALLACE P. ROWE
Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014

Received for publication 3 December 1969

The simian virus 40 (SV40)-yielding variants of the adenovirus type 2 (Ad.2)-SV40 hybrid (Ad.2^{++}) population were studied by means of fixed-angle equilibrium density gradient centrifugation in cesium chloride. The hybrid virions of the Ad.2^{++} high-efficiency yielder population banded at densities of 0.004 g/cm^3 lighter than the nonhybrid Ad.2 virions. The degree of separation of the hybrid particles was sufficient to permit greater than 100-fold relative purification by two cycles of centrifugation. Hybrid particles that produce adenovirus plaques in African green monkey kidney cells by two-hit kinetics (one-hit kinetics when assayed on lawns of nonhybrid adenovirus) were not separable from the particles that yield SV40 virus. The hybrid particle in the Ad.2^{++} low-efficiency yielder population was not separable from the nonhybrid Ad.2 virions.

Two genetically stable variants have been isolated from the adenovirus type 2 (Ad.2)-simian virus 40 (SV40) hybrid (Ad.2^{++}) population (6). Designated Ad.2^{++} high-efficiency yielders (HEY) and Ad.2^{++} low-efficiency yielders (LEY), these populations are distinguished by the relative efficiency with which adenovirus-encapsidated particles containing the SV40 genome synthesize SV40 virus. Both populations consist of nonhybrid Ad.2 virions, small quantities of nonhybrid SV40 virions, and adenovirus-encapsidated particles containing infectious SV40 genome. Both populations induce adenovirus plaques by two-hit kinetics in African green monkey kidney (AGMK) cells. Adenovirus plaque formation in monkey cells can be converted to one-hit by co-infecting with lawns of nonhybrid adenovirus virions. Progeny from either one-hit or two-hit adenovirus plaques reproduce the parent population.

In the Ad.2^{++} HEY population, adenovirus-encapsidated particles containing the infectious SV40 genome produce SV40 plaques efficiently in AGMK cells at high titers and with one-hit kinetics. Plaque studies on the Ad.2^{++} LEY population demonstrate that it contains similar quantities of adenovirus-encapsidated particles containing the infectious SV40 genome; however, only 1 of every 10^4 such particles produces SV40 plaques in AGMK cells. The plaquing characteristics of the Ad.2^{++} HEY and LEY variants are described in the accompanying paper (6).

Studies on the adenovirus 7 (Ad.7; E46$^+$) hybrid have demonstrated that partial separation of the hybrid and nonhybrid components of this population can be obtained by fixed-angle equilibrium density gradient centrifugation in cesium chloride (CsCl; 2). The purpose of this study was to attempt to separate hybrid from nonhybrid virions and to characterize the sedimentation properties of the biologically different components in the Ad.2^{++} HEY and LEY populations by using the centrifugation procedures described by Baum et al. (2). This was a particularly relevent approach to the question of whether the hybrid particle which induces SV40 plaques contains markedly less deoxyribonucleic acid (DNA) than the particle inducing adenovirus plaques; this would be anticipated if the former were simply adenovirus-encapsidated SV40 DNA.

MATERIALS AND METHODS

Virus. The isolation and characterization of the HEY and LEY variants of the Ad.2^{++} population have been described (6). Pools of Ad.2^{++} HEY and Ad.2^{++} LEY representing the third AGMK passage level after isolation from the Ad.2^{++} parent pool were prepared in AGMK monolayers infected with an inoculum of 10 plaque-forming units (PFU) or more of nonhybrid Ad.2 per cell. Cultures were harvested

when 75% of the monolayer exhibited adenovirus cytopathic effect. The Ad.2^{++} HEY pool was grown in the presence of SV40 rabbit antiserum. The pools consisted of the cells from two 32-oz flask cultures scraped into a small volume of culture fluid.

Virus purification. A modification of the enzyme digestion technique of Burnett (3) was used to purify the virus pools. Infected cell packs were frozen and thawed three times and suspended in tris(hydroxymethyl)aminomethane (Tris)-buffered saline (*p*H 7.4), with calcium (0.001 M) and magnesium (0.001 M). The cell suspensions were digested at 37 C with pancreatic ribonuclease and deoxyribonuclease one (Worthington Biochemical Corp., Freehold, N.J.), 100 μg/ml of each for 30 min, followed by a second 30-min digestion with crude snake venom (*Crotalus adamanteus*, K & K Laboratories, Jamaica, N.Y.), 100 μg/ml for 30 min. Finally, the cell packs were treated with alpha-chymotrypsin (Worthington Biochemical) and subtilisin, (Nargase and Co., Ltd.) 100 μg/ml of each for 45 min. The digest was layered on a cushion of 4.5-ml CsCl, density of 1.45 g/cm^3 and centrifuged in a Spinco SW25.1 rotor at 55,000 $\times$ *g* for 90 min at 4 C. The layer of virus below the interface was collected with a finely drawn Pasteur pipette in a volume of approximately 1.0 ml; this cushioned virus constituted the starting material for the separation runs.

Electron microscopy of an Ad.2 cell pack carried through this procedure and banded in CsCl showed the virions to be monodispersed and free of contaminating debris (M. D. Hoggan, *personal communication*). In addition, the cushioned Ad.2^{++} LEY virus was sonically treated for 1 sec by using a Branson sonic oscillator at 6 amp.

Centrifugation. The application of fixed-angle equilibrium centrifugation in CsCl for the separation of adenovirus-SV40 hybrid populations has been described (2). In the present experiments, a Spinco 40 rotor was used at 60,000 $\times$ *g* for 60 to 64 hr at either 4 or 19 C. For each run, the virus sample was suspended in optical grade CsCl dissolved in 0.01 M Tris buffer, *p*H 8.1. The density was adjusted to 1.35 g/cm^3, and 4.5 ml was used in each tube. A sample was set aside at the same temperature as the centrifuge for the duration of the run and subsequently diluted and handled in parallel with the centrifuged material. At the end of the run, 0.1-ml fractions were delivered by a constant-volume displacement device through a hole in the bottom of the tube made by needle puncture. Densities were determined by refractive index on representative fractions on either side of the virus band region; in general, each fraction corresponded to density increments of 0.0015 g/cm^3. The fractions were diluted in Eagle's minimal essential medium containing 10% fetal bovine serum and dialyzed against phosphate-buffered saline (*p*H 7.2 or 7.4), further diluted, and stored at -70 C until used.

Plaque assays. The techniques for determining adenovirus plaque titers in HEK; hybrid-induced, SV40 plaque titers in AGMK; and the defective adenovirus hybrid plaque titers in AGMK with an adenovirus lawn have been described (4, 6, 9). Adenovirus 1 (strain Ad.71) at 0.1 PFU/cell was used as the lawn. This lawn was sufficient to allow a proportionate (one-hit) dose-response curve without itself inducing cytopathic effect. Due to the differences in sensitivity of various lots of primary HEK and AGMK cells, all fractions from each centrifugation run were titrated for any given plaque type on the same lot of cells.

Immunofluorescence tests. The techniques for growing HEK and AGMK cells on cover slips, infecting them, and testing for viral antigens by the indirect fluorescent-antibody procedure have been described (5, 7, 8). Infecting cover slips with low dilutions of fractions taken from a centrifugation run required modification of the standard technique to conserve materials. A cover slip (11 by 22 mm), bearing a confluent HEK monolayer, was transferred onto a small piece of blotting paper in a dry petri dish so that the edges of the cover slip were free from contact with the dish or blotting paper. Four drops of infecting inoculum were placed onto the cover slip and allowed to incubate with occasional manual rotation at 37 C in an atmosphere of humidified 5% CO_2. After 1 hr of adsorption, the fluid was shaken from the cover slip, which was then washed twice and fed with medium containing 2% dialized agammaglobulinic calf serum and 10^{-5} M 5-fluorodeoxyuridine to prevent completion of the infectious cycle and secondary infection. After 24 hr, the cover slips were harvested and fixed. To test for Ad.2 T antigen, a broadly reactive antiserum pool from hamsters bearing adenovirus 12-induced tumors was used (10). Antiserum used to test for SV40 T antigen was pooled from hamsters bearing transplanted SV40-induced tumors.

RESULTS

A purified pool of Ad.2^{++} HEY was centrifuged in a CsCl equilibrium gradient in an attempt to separate the hybrid and nonhybrid components. At the end of the run, two bands were visible: a dense lower band in fractions 17 to 20 and a faint upper one at approximately fraction 24. Plaque assays were performed on the 0.1-ml fractions collected in the area of the bands (Fig. 1A). The highest virus titers were all in the vicinity of the lower band, at a density of 1.345 to 1.348 g/cm^3. The fraction having the highest titer of nonhybrid Ad.2 was more dense by two fractions than the fraction having the highest titers of both SV40-yielding adenovirus-encapsidated particles and the defective hybrid. The titration curves of the latter two were remarkably similar; the titers of SV40-yielding particles ranged from 0.8 to 1.2 $\log_{10}$ higher than the titers of hybrid particles which form adenovirus plaques on lawns.

To reduce the titer of nonhybrid Ad.2 in the fractions rich in hybrid particles, a second cycle of centrifugation was done. Samples of 0.4 ml of 10^{-1} dilutions of fractions 22, 23, and 24 were pooled and centrifuged again under similar conditions. A single, barely visible band

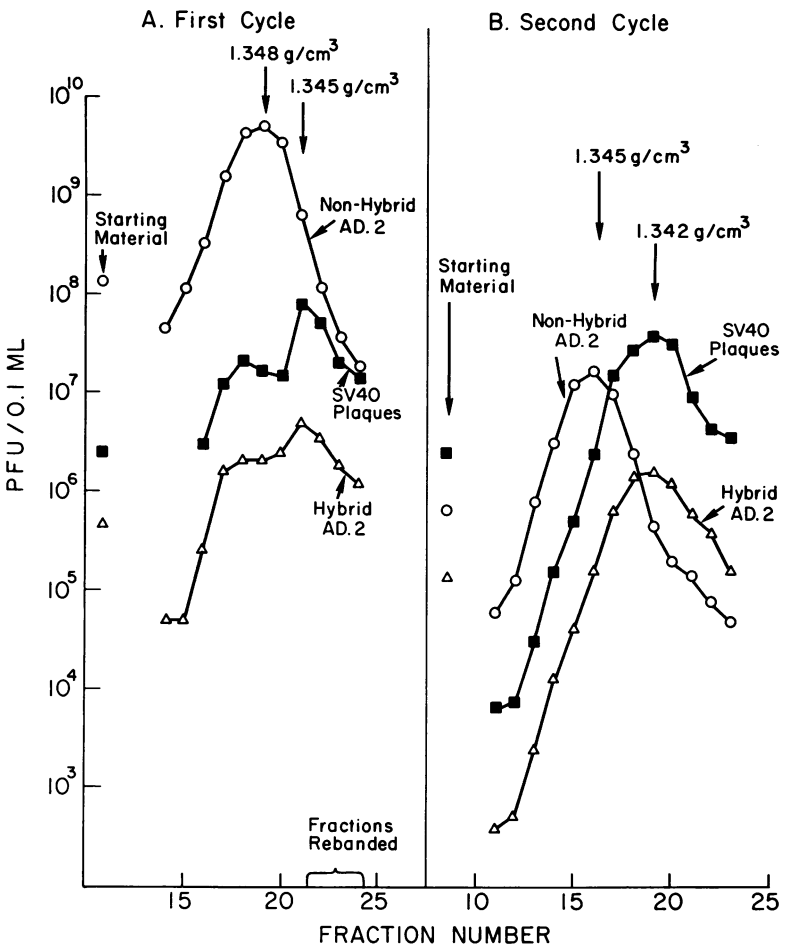

FIG. 1. *Titrations of fractions of consecutive centrifugations of Ad.2++ high-efficiency yielders. A, First centrifugation of purified virus harvested after infection of two AGMK bottles; B, 0.4 ml of 1 to 10 dilutions of fractions 22, 23, and 24 from A. Both runs were at 60,000 × g in a no. 40 rotor at 19 C for 64 hr in CsCl. (A repeat of this entire experiment at 8 C in the first run and 4 C in the recycle effected a similar separation.) Symbols:* O——O, *nonhybrid Ad.2;* ■——■, *SV40 yielded from Ad.2-encapsidated particles;* △——△, *Ad.2++ HEY hybrid assayed on Ad.1 lawn. Discrepancies noted in a comparison of the titers of particles producing SV40 plaques and nonhybrid Ad.2 virions in the starting material in Fig. 1B with the titers obtained for the same material before pooling in Fig. 1A are probably due to differences in the cell lots used for the assays.*

was apparent. Fractions were collected and assayed for infectivity (Fig. 1B). It is apparent from these data that the nonhybrid Ad.2 was again more dense than either the SV40-yielding hybrid or the defective hybrid particles. The curves for these hybrid particles were again parallel. After this second centrifugation, their respective plaque-forming capacities exceeded that of nonhybrid Ad.2 in the upper fractions. In fraction 20, there was purification of the hybrid from the nonhybrid by nearly 10^4-fold, relative to the proportions in the original starting material. The particles producing SV40 plaques and the hybrid plaques were approximately 0.004 g/cm^3 less dense than the nonhybrid Ad.2.

Both of the curves in Fig. 1A, which represent assays of hybrid particles which produce either SV40 or adenovirus plaques, are biphasic with minor peaks occurring in the fraction 17 to 19 region. This minor peak was not present after rebanding (Fig. 1B). A similar, but less clear-cut, biphasic pattern was seen in another banding of HEY, whose results are not shown here. This suggests the presence of another class of hybrid virion in the HEY population, which is slightly more dense than the nonhybrid Ad.2 virions. In

any case, the ratio of SV40-yielders to hybrid particles forming adenovirus plaques on lawns was the same in this region as in the peak at the lower-density region. Clarification of this question must wait further studies.

Having effected some purification of the Ad.2^{++} HEY hybrid virion and the SV40-yielding particle from nonhybrid Ad.2, it was possible to test the hybrid particles for their capacity to induce Ad.2 T antigen. HEK monolayers on cover slips were infected with 10^{-2} dilutions of fractions from the second centrifugation. The resulting cover slip antigen preparation was divided and quantitatively assayed for SV40 and adenovirus T antigens. The results are plotted in Fig. 2 and compared with the plaque titration assays from the same fractions (from Fig. 1B). It is seen that SV40 T antigen induction paralleled plaque induction by hybrid particles, whereas adenovirus T antigen induction was very broad, paralleling the combined nonhybrid and hybrid plaque-induction curves.

Attempts to separate the hybrid virions in the Ad.2^{++} LEY population were carried out in a similar centrifugation study (Fig. 3A). A single centrifugation failed to separate fractions bearing maximal titers of nonhybrid Ad.2 and the hybrid particles which produced adenovirus plaques on AGMK cells infected with lawns of nonhybrid adenovirus virions. The maximal titer of each was in fraction 23. However, comparison of the ratios of the respective titers in each fraction suggested that the denser fractions were relatively enriched with the hybrid. Accordingly, 0.35 ml of a 10^{-1} dilution of fraction 21 was centrifuged a second time. Evidence for separation could not be confirmed (Fig. 3B). It would have been very desirable to determine the distribution of SV40-yielding particles in the LEY gradients; however, this is a technically difficult, semi-quantitative procedure, and the attempted assay was not successful.

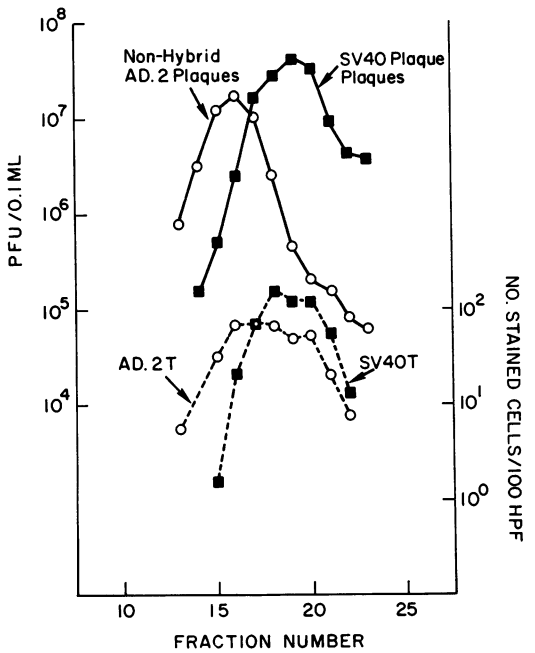

FIG. 2. *Immunofluorescent titration of second centrifugation of Ad.2^{++} high-efficiency yielders for adenovirus T and SV40 T antigens. Lower curves are results of plaque assays from Fig. 1B. Upper curves are T antigen assays. Adenovirus T antigen was found in fractions rich in SV40-yielding particles as well as in those rich in nonhybrid Ad.2 particles. Symbols:* ○——○, *nonhybrid Ad.2 plaques;* ■——■, *SV40 plaques;* ○-----○, *adenovirus T antigen;* ■-----■, *SV40 T antigen.*

DISCUSSION

The results reported here are relevant to two of the chief problems on the nature of the SV40-yielding hybrids, that is, whether a single hybrid genome can alternatively give rise to free SV40 or participate in adenovirus plaque production and whether the genome of the SV40-yielding particle is a true hybrid DNA.

The results with the Ad.2^{++} HEY population are completely in accord with the hypothesis that there is a single hybrid particle. Under conditions which sharply separated the majority of the hybrid particles from the nonhybrid adenovirus particles, there was no indication that the SV40-yielding particles were dissociated from the hybrid particles capable of complementing and potentiating nonhybrid adenovirus.

The alternative model, having two hybrid particles, one yielding only SV40 and the other yielding both types of hybrid particle, cannot be ruled out. It seems highly unlikely that the two particles would have identical buoyant densities, unless the SV40-yielder is derived from the parent hybrid by a small genetic deletion. However, the data essentially rule out the possibility that the SV40-yielding particle is a simple transcapsidant, containing nonhybrid SV40 DNA in an adenovirus capsid. Such a particle would have a buoyant density significantly less than observed here; also, other studies (Crumpacker et al., *unpublished data*) have failed to detect any free SV40 DNA in the adenovirus particles of Ad.2^{++} HEY, the SV40 DNA detected being linked to Ad.2 DNA.

The efficiency with which the hybrid-rich fractions of the HEY gradient induced Ad.2 T antigen

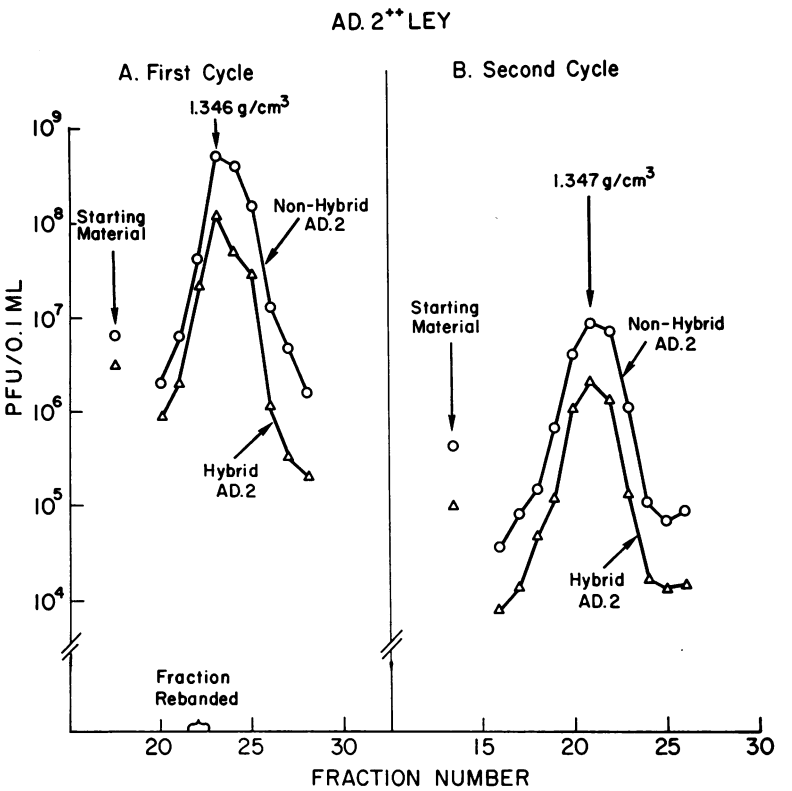

FIG. 3. *Titrations of fractions of consecutive centrifugations of Ad.2++ low-efficiency yielders. (A) First centrifugation of purified virus harvested from two AGMK bottles. (B) Recentrifugation of 0.35 ml of a 1-to-10 dilution of fraction 22 from A. Runs A and B were each at 60,000 × g in a no. 40 rotor at 4 C in CsCl for 61 hr and 60 hr, respectively. Symbols:* ○——○, *nonhybrid Ad.2;* △——△, *hybrid assayed on Ad.1 lawn.*

suggests linkage of the SV40 DNA with the portion of Ad.2 DNA coding for certain T antigens, as with E46+ virus (1, 10). However, this finding could also be due to defective Ad.2 particles, analagous to the finding of defective, T-antigen-producing particles in the upper portion of SV40 bands (11).

The difference in buoyant density between the LEY hybrid particles and the major component of the HEY hybrid particles was striking and suggests that the LEY genome contains a much larger piece of adenovirus DNA than the HEY genome. It is possible that this difference is related to the markedly less efficient production of SV40 by the LEY virus. It should be noted that the relationship of the HEY and LEY viruses is not clear; both are plaque isolates from the same uncloned population, but they may have originated by separate recombination events.

The present data along with those of Baum et al. (2) indicate that the various adenovirus-SV40 hybrid viruses have distinctive buoyant densities relative to their nonhybrid helpers. Ad.2++ LEY bands at the same density as the nonhybrid component, Ad.7 (E46+) is one fraction lighter, and Ad.2++ HEY is two to three fractions lighter. The relative buoyant density should provide a useful marker, particularly in studies of genetic derivatives of the hybrids.

The degree of physical separation achieved with the HEY virus should facilitate study of the biological and chemical properties of the defective hybrid particles by providing preparations in which they are in excess of the nonhybrid Ad.2 virions.

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