Transformation Potentials of the Noninfectious (Defective) Component in Pools of Adenoviruses Type 12 and Simian Adenovirus 7

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Pools of adenovirus 12 and simian adenovirus 7 were separated into four or five fractions by density gradient centrifugation in cesium chloride. Each fraction was analyzed for total *in vitro* infectivity units, total transformation activity, and for total virus particle (VP) content. Two major subpopulations were separated with mean densities of 1.30 ± 0.02 and 1.34 ± 0.02 g/ml, respectively. Virions in the 1.34 g/ml range were highly infectious (10^2 to 10^3 VP per infectivity unit) in contrast to virions at 1.30 g/ml density (10^4 to 10^5 VP per infectivity units). Transformation capacity was evenly distributed throughout fractions of both viruses, indicating that genetically incomplete or defective virus particles were not deficient in their ability to induce transformation. The average VP per transformation unit for adenovirus 12 (2.85×10^6) and for simian adenovirus 7 (4.00×10^6) did not vary significantly from fraction to fraction. These values were obtained with optimal input multiplicities of 16 to 64 VP per cell. At higher multiplicities the apparent increase in VP per transformation unit was attributable to the viral cytotoxic effect on hamster cells. These studies revealed that quantitation of *in vitro* transformation based on VP multiplicities was more reliable than on the basis of infectious units. These estimates were independent of method of virus production, extraction, and purification.

Thorough understanding of DNA virus oncogenesis will require compilation and assimilation of many separate aspects of cell-virus interaction. A critical area of investigation has involved analysis of the basic physical and biochemical differences between permissive and nonpermissive types of infection (7, 15, 16, 28, 33). Those studies have described differences in the early events of viral infection, i.e., in adsorption, penetration, and uncoating of virions (22, 33), as well as differences in the physical and chemical state of the infecting viral nucleic acid during early phases of infection (6, 37). In general, adenovirus structural proteins are not synthesized during abortive or nonpermissive infections, although transcription followed by translation of early viral proteins often occurs. It is under these conditions that viral-induced malignant cell transformation generally occurs.

A related aspect of cell-virus interaction concerns the range of minor genetic differences found within a given virus population. These differences are probably responsible for the variations in quantitative relationships between infectivity and transforming capacities of different lots of the same oncogenic virus. It has been

proposed by Sauer et al. (27) that all populations of oncogenic DNA viruses contain defective virions that play a major role in determining overall oncogenic potential. This hypothesis was based upon an analysis of the differing capacities of simian virus 40 pools to induce viral structural or nonstructural complement-fixing antigens. This analysis supported the contention that virus particles containing defective genomes may have been responsible for abortive infection of cells *in vitro*. It is therefore conceivable that defective adenovirions may undergo abortive infection of normally permissive cells, creating the necessary conditions for transformation of such cells.

Analyses of the heterogeneity of adenovirus populations (19, 24) have revealed noninfectious particles which are capable of a variety of biological and genetic functions, such as cytolysis, T-antigen production, and inclusion body formation in permissive cells. Using a similar rationale, this study was undertaken to determine the heterogeneity of adenovirus populations with respect to transformation of nonpermissive cells. Our intent was to determine whether genetically incomplete or defective

adenovirions would transform nonpermissive hamster cells in vitro as efficiently as would intact, complete virions.

MATERIALS AND METHODS

Viruses. Simian adenovirus (SA7) was obtained from B. Casto (Biolabs, Northbrook, Ill.) and passaged in the rhesus monkey kidney cell line LLC-MK₂. Human adenovirus type 12(Ad12), strain Huie, was obtained from R. Huebner (National Cancer Institute, Bethesda, Md.) and was passaged in KB cells, in spinner and monolayer cultures.

Cell cultures. Cell cultures of LLC-MK₂ (13) were grown in 32-oz. (0.946-liter) prescription bottles or in roller bottles. Growth medium consisted of Eagle minimal essential medium (MEM) with 10% fetal calf serum (FCS). Cells in roller bottles were used to prepare SA7 virus pools.

KB cells, a human cell line derived from an oral carcinoma (9), were used for production of Ad12 virus pools. Human epidermoid carcinoma no. 2 (HEp-2) (20) cells were used for the Ad12 fluorescent focus assay and were grown in 32-oz. prescription bottles with Eagle MEM containing 10% FCS. Primary human embryonic kidney cell cultures grown in MEM and 20% FCS were obtained from Flow Laboratories, Inc., Rockville, Md. Primary hamster embryonic cell cultures, used in transformation assays, were prepared from embryos at 12 to 14 days of gestation.

Virus growth and purification procedures. Stock preparations of SA7 were prepared by infecting confluent roller-bottle cultures of LLC-MK₂ cells with virus multiplicities of from 1 to 10 PFU per cell; stock pools of Ad12 were prepared similarly in KB cells. Infected cell pellets were suspended in MTS buffer (0.25 M sucrose, 10^{-3} M MgCl₂, and 0.02 M Tris-chloride) at pH 7.4 or in TE buffer (0.05 M Tris-chloride and 5×10^{-4} M ethylenediaminetetraacetate) (22) at pH 7.4 and portions were frozen at -20 or -70 C.

A modified procedure of Green and Pina (11) was used for virus purification. Infected cell pellets were thawed at 37 C, chilled in ice, and sonically treated for 30-s intervals in the flow cell of a Branson sonifier (model LS75; Branson Instruments, Co., Stamford, Conn.) equipped with a $\frac{3}{8}$ -inch (.9525-cm) diameter step horn. The sonically treated cell preparations were centrifuged at 3,000 rpm ($1,500 \times g$) for 20 min. The supernatants were extracted two to three times with an equal volume of Racon 113 (trichlorotrifluoroethane; Racon Inc., Wichita, Kan.) by homogenization in a Sorval Omnimixer. Aqueous phases were pooled and layered over 7 ml of CsCl (density, 1.5 g/ml) in MTS or TE buffer. After centrifugation for 18 h at $90,000 \times g$, the broad interface band was collected and placed in a discontinuous gradient consisting of 7 ml of CsCl (density, 1.4 g/ml), 10 ml of virus-banded material adjusted to a density of 1.3 g/ml, and 20 ml of CsCl in TE buffer at a density of 1.2 g/ml layered on top. This gradient was centrifuged at $80,000 \times g$ for 24 to 72 h and fractionated with an Isco model D fractionator, equipped with a flow cell for optical density (OD) measurement at 254 or 260 nm (3).

Appropriate density ranges were pooled and dialyzed against two changes of TE buffer and a third

change of MTS buffer. Fractions were frozen at -70 C in portions (0.2 to 0.5 ml) in sealed glass ampules.

Infectivity titrations. Titrations of SA7 and Ad12 infectivity were done by different techniques. A modified method of Green (10) was used for plaque titration of SA7 on LLC-MK₂ cells grown in 60-mm Linbro plates. Virus inocula (0.2 ml) were allowed to adsorb to the indicator cells for 2 to 3 h at 37 C. After removal of inoculum, 8 ml of agar overlay was added, and the plates were incubated in a humidified 5% CO₂ atmosphere at 37 C. An additional 5 ml of overlay containing neutral red was added at day 7. Final plaque counts were made 2 to 3 days after the addition of the second overlay.

The mean tissue culture infective dose end point dilution technique on human embryonic kidney cells was used for titration of some Ad12 pools (29). The mean tissue culture infective dose values were calculated by the method of Reed and Muench (25).

Infectivity of Ad12 on HEp-2 cells was determined by a modification of the fluorescent focus assay method of Thiel and Smith (34). Confluent cell monolayers on circular cover slips were infected with virus dilutions and allowed to adsorb for 2 to 3 h. The inoculum was removed, and 5 ml of Eagle MEM and 10% FCS was added to each dish. After incubation for 48 h in 5% CO₂, the cover slips were fixed in absolute ethanol at -70 C and stored in this manner until tested by indirect immunofluorescence for viral structural antigens. The cover slips were removed from low-temperature ethanol at -70 C and placed immediately into phosphate-buffered saline at pH 7.4. Rabbit anti-Ad12 (neutralizing antibody titer, 1:200) was added to each cover slip and allowed to adsorb for 30 min at room temperature. Cover slips were washed with phosphate-buffered saline (three changes, 15 min each), and the secondary antibody (monkey anti-rabbit globulin conjugated with fluorescein isothiocyanate) (21) was added and allowed to adsorb for 30 min at room temperature. The cover slips were washed with phosphate-buffered saline as before and mounted in 10% buffered glycerin.

Transformation assay. Modified procedures of Casto (4) and Duff and Rapp (8) were used for the transformation assays, differing only in the method of virus adsorption. Primary hamster embryo monolayer cultures were prepared in 32-oz. prescription bottles from 12- to 14-day-old embryos, as described elsewhere (4).

The transformation method modified from Casto (4) consisted of adding 0.2 ml of virus suspension to 2×10^5 to 3×10^5 primary hamster embryo cells which had been planted in petri dishes 5 h prior to the addition of the virus suspension. Virus adsorption was permitted for 2 to 3 h after which 5 ml of MEM plus 10% FCS was added to each culture dish. After 24 to 48 h the cultures were fed with 5 ml of Dulbeccos MEM supplemented with 0.1 mM CaCl₂ and 10% FCS. Cultures were fed every 3 to 4 days for 16 to 18 days. Plates were air-dried, fixed in methanol, stained with Giemsa, and scanned for transformed foci at $\times 25$ magnification.

The adsorption method of Duff and Rapp (8) allowed virus to adsorb to primary hamster embryo cells in suspension. Virus dilution and cell suspension

(1 ml each) at a concentration of 8×10^5 cells per ml were placed on a rotary shaker in 15-ml culture tubes at 37 C for 3 h. After virus adsorption the cell suspension was added to each of three 60-mm petri dish wells (Linbro tissue culture tray). Each well contained 5 ml of Eagle MEM plus 10% FCS. Fresh medium was added to the culture tube, and incubation was continued for an additional 24 h. Remaining cells in the culture tube were trypsinized after 24 h and seeded to the fourth well of the Linbro tray. After another 24 h the medium was changed to Dulbeccos MEM containing 0.1 mM CaCl_2 and dialyzed FCS. The remainder of the procedure was as outlined above.

Estimation of VP concentrations. Determination of virus particle (VP) concentration by electron microscopy was by a modification of the technique of Kellenberger and Arber (14). Equal volumes (0.1 ml) of virus suspension, latex particles, and 4% phosphotungstate, containing 0.4% sucrose and 0.5% bovine serum albumin at pH 6.8, were mixed on a Vortex mixer for 30 to 40 s. Formvar- and carbon-coated grids were touched to a drop of the virus mixture, and all but a small amount was removed by blotting. The grids were allowed to dry for 1 to 2 min and were touched to a drop of 2% phosphotungstic acid at pH 6.8. The grid was immediately blotted and allowed to dry. Grids were examined with a Philips 200 electron microscope at a base magnification of 12,500. The number of VP per 100 or 200 latex particles was determined, and the virus concentration was calculated.

Oncogenicity assay in hamsters. PD-4 Syrian hamsters, (Whitney Laboratories, Aurora, N.Y.) were used for determining the *in vivo* oncogenicity of virus fractions. Newborn hamsters were injected subcutaneously with 0.1 ml of virus suspension on the dorsal surface within 24 h after birth. Inoculated animals were weaned at 3 to 4 weeks of age and segregated according to sex. Animals were palpated regularly for tumor formation, and surviving hamsters were sacrificed at 4 months and examined for internal tumors. Representative tumors were fixed in 5% buffered formalin and processed for histological examination.

RESULTS

Fractionation of virus pools by cesium chloride density gradient centrifugation. Examples of the distribution of bands of virions after density gradient centrifugation are shown in Fig. 1 to 3. Variations in banding patterns were attributable to prebanding purification procedures. Figures 1 and 2 were derived from results with an Ad12 pool prepared by sonic treatment of infected cells that were subsequently layered over a cushion of CsCl prior to treatment with fluorocarbon. Extracted aqueous phases were used for the final centrifugation. Large amounts of contaminating host protein were not observed when sonically treated cell supernatants were extracted with fluorocarbon prior to centrifugation. The latter

effect is illustrated by comparing the profile of a SA7 pool (Fig. 3) with that of Ad12 (Fig. 2). The larger virus yield in the SA7 pool represents the yield from eight 2-gallon (7.56-liter) roller bottles.

Generally the gradients were separated into four or five density regions. Each fraction was assayed for infectivity, VP content, and trans-forming capacity (see below). The relative purity of each fraction was analyzed by OD measurements at 260 and 280 nm (Table 1). The OD ratios at 260/280 nm provided an index of virion integrity, as well as relative purity. The higher the index, the greater the proportion of nucleic acid in the virus particles. Based on these indices, virions in the most dense fraction contained the most DNA, whereas fractions with lower ratios contained virions

FIG. 1. CsCl density gradient demonstrating separation of Ad12 and host cell material. The opaque bands correspond to highly infectious virus (1) at 1.337 g/ml, a second virus band (2) at 1.298 g/ml, and a cell debris containing area (3) at 1.28 g/ml. Centrifugation was for 48 h at $80,000 \times g$.

FIG. 2. OD profile at 280 nm for an Ad12 pool after centrifugation for 48 h at $80,000 \times g$. The virus pool, representing the infected cell-yield from three 2-gallon roller bottles, was sonically treated, and the virus containing suspension was layered over a CsCl cushion (density, 1.50 g/ml). After centrifugation for 6 h at $80,000 \times g$ the virus band was extracted with fluorocarbon. Aqueous supernatants from these extractions were then processed by two equilibrium density gradient centrifugations (48 h at 25,000 rpm in a SW27 rotor of the Beckman 2-65B ultracentrifuge).

FIG. 3. OD profile at 254 nm for a gradient of a SA7 pool after centrifugation for 72 h at $80,000 \times g$. The virus pool was purified by sonic treatment and extraction with fluorocarbon. Aqueous supernatants were layered over CsCl (density, 1.50 g/ml) and centrifuged at 25,000 rpm ($90,000 \times g$) for 18 h. The crude virus band was then subjected to two successive density gradient centrifugations, the second being for 72 h at $80,000 \times g$.

with less nucleic acid. These observations were confirmed morphologically (Fig. 4). In the pool of Ad12 defined by Fig. 1 and 2, the most dense fraction (1.34 g/ml) contained 8.7×10^{10} VP per OD unit (260 nm). Others (23) have reported 4×10^{11} and 7×10^{11} particles per OD unit in preparations of Ad12. This fraction gave the highest 260/280 OD ratio, 1.31.

Biological activity and structural integrity of VP at different buoyant densities. Preliminary experiments with several virus pools indicated that lighter fractions contained a high proportion of virions with diminished infectivity for human cells. For example, fraction 3 of the

pool described in Fig. 2 contained 57.5% of the VP and only 0.1% of the total infectivity. By contrast, the most dense fraction contained greater than 90% of the total infectivity. In this preliminary study, transformation activity, although not identical in each fraction, did not follow the distribution of infectivity in the gradient. Significant amounts of transformation activity were observed at lighter densities. Subsequent experiments (*vide infra*) were performed to explore these differences.

Virus structural differences observed in the various fractions (Fig. 4) were similar to those reported by Smith (29). Virions from the lighter densities (Fig. 4A–D) appeared to have undergone various degrees of capsid rupture. Virions of greater density were more stable. The most abundant structurally intact virions (Fig. 4H) were found in the most dense fraction, although an occasional disrupted virion could be found (Figure 4G).

Effect of multiplicity on in vitro transformation assays. Previous reports have demonstrated significant cytotoxic effects of Ad12 on hamster cells (31, 32). The depression in the frequency of in vitro transformation that occurs with high virus concentrations (4) appears to be due to this cytotoxic effect. At low virus concentrations, the number of transformed foci is linear with dilution, indicating the single-hit nature of viral transformation. Linearity of transformation with dilution does not occur with high virus concentrations. The actual number of transformation foci diminishes greatly at high multiplicities (4). In these studies, the reduction in the number of transformed foci at high multiplicities occurred more readily with Ad12 than with SA7.

Thus, at high input multiplicities (e.g., 2,500 and 1,000 VP per cell) of Ad12, significant cytotoxic activity occurred. With SA7, linearity of focus formation with dilution occurred at input multiplicities of 1,000, 400, and 160 VP per cell. These observations necessitated the use of optimum concentrations (noncytotoxic) of input virus to determine the total transformation capacity of a given virus preparation. Multiplicities routinely used were 160 and 64 VP per cell. All subsequent determinations were based on these conditions.

Distribution of VP, infectivity, and transformation activity in density gradients of Ad12 and SA7. The results of assays for VP content, infectivity, and transformation activity for fractions of additional SA7 and Ad12 pools are presented in Tables 2 and 3. Between 80 and 90% of the total virions in the gradient was located primarily at two densities corre-

FIG. 4. Electron micrographs of Ad12 particles from gradient fractions with different densities showing the variation in capsid structural integrity. (A, B) Fractions at 1.267 g/ml; (C, D) fractions at 1.298 g/ml; (E, F) fractions at 1.315 g/ml; (G, H) fractions at 1.337 g/ml.

TABLE 1. *Estimation of the relative purity of fractions from a pool of Ad12 based on virus particles per optical density unit at 260 nm*

Fraction	Density in CsCl (g/ml)	OD (260/280 nm)	OD units ^a (total per fraction)	Total number of virus particles	Virus particles per OD at 260 nm
1	1.267 ± 0.005	0.88	0.54	1.86 × 10 ¹⁰	3.44 × 10 ¹⁰
2	1.281 ± 0.009	0.86	0.46	2.28 × 10 ¹⁰	4.96 × 10 ¹⁰
3	1.298 ± 0.008	1.13	3.69	7.90 × 10 ¹¹	2.14 × 10 ¹¹
4	1.315 ± 0.010	1.16	4.98	3.48 × 10 ¹⁰	6.99 × 10 ⁹
5	1.337 ± 0.013	1.31	3.82	3.33 × 10 ¹¹	8.72 × 10 ¹⁰

^a OD at 260 nm was corrected by subtracting the OD at 300 nm.

TABLE 2. *Biological properties of Ad12 virions separated on the basis of their buoyant density in CsCl*

Approx mean density of fraction (g/ml)	Total in the gradient (%) ^a		
	VP	IU ^b	TU ^c
1.274 ± 0.012	1.2	3.7	0.4
1.294 ± 0.009	66.3	3.7	39.2
1.311 ± 0.008	1.8	5.6	2.4
1.336 ± 0.018	30.7	87.0	58.0

^a Percentage of VP, IU, and TU in the entire virus pool contained in each fraction. This pool was sonically treated and subsequently treated with fluorocarbon. The aqueous phase was then subjected to an equilibrium density gradient centrifugation over a cushion of CsCl (density, 1.5 g/ml).

^b Infectivity determined by fluorescent focus assay on HEp-2 cells.

^c Determined at input multiplicities of 64 or 160 VP per cell.

sponding to a density of 1.30 and 1.34 g/ml. Transformation activity was generally proportional to the VP content of each fraction. In contrast, the majority of infectivity was confined to the heaviest fraction.

Ad12 virions with a density of 1.294 ± 0.009 g/ml contained a significant amount (39.2%) of transformation activity (Table 2). This fraction contained 66.3% of the total VP but only 3.7% of the infectivity. The most dense fraction contained 30.7% of the VP; this represented 87% of the total infectivity in the gradient and 58% of the transformation activity. These results clearly indicate the presence of virions that are unable to replicate in human cells but yet are able to induce transformation in hamster cells.

Analyses of both pools of SA7 revealed an even greater correlation between VP and transformation activity. The fraction of SA7 pool 1 with a density of 1.31 ± 0.01 g/ml contained only 0.8% of the infectivity; in contrast, 49% of the VP and 45.7% of the transformation activity were in this fraction. The most dense fraction (density, 1.347 ± 0.008 g/ml) contained 91% of

TABLE 3. *Distribution of virions and biological properties of SA7 separated on the basis of their buoyant density in CsCl*

SA7 virus pools	Approx mean density per fraction (g/ml)	Percent per gradient fraction ^a		
		VP	IU ^b	TU ^c
1	1.289 ± 0.011	0.1	1.0	3.7
	1.310 ± 0.011	49.0	0.8	45.7
	1.330 ± 0.009	20.3	7.1	31.3
	1.347 ± 0.008	29.6	91.1	19.3
2	1.267 ± 0.005	0.9	0.8	1.6
	1.281 ± 0.009	5.8	1.8	5.4
	1.298 ± 0.008	17.1	2.2	18.5
	1.315 ± 0.009	10.2	5.2	16.3
	1.337 ± 0.013	66.0	90.0	58.2

^a Percentage of VP, IU, and TU in the entire virus pool contained in each gradient fraction. Pool 1 was produced in 16-oz. prescription bottles, extracted from cells by sonic treatment, and placed over a cushion of CsCl (density, 1.5 g/ml). After centrifugation the concentrated virus was subjected to density gradient centrifugation. Pool 2 was produced in 2-gallon roller bottles and extracted by sonic and fluorocarbon treatment. Density gradient centrifugation was similar to that used for pool 1 except that the virus was subjected to two successive density gradient runs and the fractions top collected.

^b Infectivity determined by plaque assay on LLC-MK₂ cells.

^c Determined at input multiplicity of 64 VP per cell.

the infectivity, 29.6% of the VP, and only 19.3% of the transformation activity. SA7 pool 2 yielded similar results.

Comparison of the ratios of VP per unit of biological activity in density gradient fractions of Ad12 and SA7. These analyses were designed to provide estimates of the number of VP per infectivity unit (IU), estimates of the number of VP per transforming unit (TU), and the ratio of IU/TU for each of the fractions.

As noted previously, the major portion of Ad12 and SA7 infectivity resided in fractions of mean density 1.330 g/ml or greater (Tables 2

and 3). The most dense fraction also contained the fewest incomplete particles. In view of these observations, it was not unexpected that virus fractions of greatest density provided the best estimate of the minimum number of VP required to induce infection.

The efficiency of virus infection as measured by VP/IU ratio was found to vary extensively between fractions of the gradients (Table 4). This was also true of the IU/TU ratio when it was used to estimate the efficiency of virus transformation. In contrast, VP/TU ratios were strikingly similar from fraction to fraction for both Ad12 and SA7.

In all virus pools (Table 4), the most efficient infectivity was achieved by virions of the most dense fraction, and the least efficient infectivity was obtained with virions in the 1.30 ± 0.01 g/ml fraction. By contrast, with the IU/TU ratio, transformation was most efficient with virions from the 1.30 ± 0.01 g/ml fraction. However, this ratio is misleading since it does not take into account the number of VP required per IU or TU. Thus, estimates of transformation efficiency based on VP/TU rather than IU/TU ratios indicated that transforming capacity was relatively evenly distributed in the gradients. Differences between SA7 and Ad12 were not significant since the mean number of

VP/TU for SA7 was $4.42 \pm 2.55 \times 10^6$ as compared to $2.87 \pm 2.07 \times 10^6$ for Ad12 (Table 4).

Oncogenicity of Ad12 and SA7 virions of different buoyant densities. Ad12 and SA7 virions of different buoyant densities induced tumors in inbred PD-4 Syrian hamsters with varying efficiencies (Table 5). At concentrations of 10^9 and $10^{9.8}$, light-density (1.30 g/ml) Ad12 virions induced tumors in two out of three female and zero out of nine male hamsters. Heavy-density virions (1.34 g/ml) induced tumors in three out of seven female and three out of nine male hamsters. Collectively, the apparent efficiency of tumor induction was higher for the group receiving heavy virions. On the other hand, females appeared to be equally sensitive to tumor induction by virions of both densities. SA7 virions of light-density induced tumors in two out of four female and one out of two male hamsters. Heavy-density virions induced tumors in one out of five male hamsters.

DISCUSSION

Subpopulations of Ad12 and SA7 separated on the basis of their buoyant density in CsCl differed significantly in infectivity capacities. In contrast, no significant difference was detected in transformation capacities between

TABLE 4. Ratios of VP/IU, VP/TU, and IU/TU for density gradient fractions of Ad12 and SA7

	Approx mean density (g/ml)	VP/IU $\times 10^{-20}$	VP/TU $\times 10^{-60}$	IU/TU $\times 10^{-10}$
SA7 pool 1	1.289 $\pm$ 0.011	20.0 $\pm$ 15.0	0.71 $\pm$ 0.54	35.7 $\pm$ 8.9
	1.310 $\pm$ 0.011	1,230 $\pm$ 270.0	2.64 $\pm$ 0.92	2.2 $\pm$ 0.5
	1.330 $\pm$ 0.009	52.5 $\pm$ 15.8	1.62 $\pm$ 0.57	30.8 $\pm$ 7.7
	1.347 $\pm$ 0.008	6.1 $\pm$ 1.8	3.82 $\pm$ 1.34	623.0 $\pm$ 156.0
$\bar{X}$			2.20 $\pm$ 1.34	
SA7 pool 2	1.267 $\pm$ 0.005	27.2 $\pm$ 05.4	4.46 $\pm$ 1.34	164.0 $\pm$ 41.0
	1.281 $\pm$ 0.009	77.6 $\pm$ 11.6	8.53 $\pm$ 2.13	110.0 $\pm$ 28.0
	1.298 $\pm$ 0.008	185.0 $\pm$ 37	7.27 $\pm$ 1.85	39.2 $\pm$ 9.8
	1.315 $\pm$ 0.009	45.9 $\pm$ 09.2	4.97 $\pm$ 1.27	108.0 $\pm$ 27.0
	1.337 $\pm$ 0.013	11.3 $\pm$ 02.3	5.79 $\pm$ 1.80	512.0 $\pm$ 128.0
$\bar{X}$			6.20 $\pm$ 1.68	
AD12 pool	1.274 $\pm$ 0.012	3.2 $\pm$ 1.6	5.57 $\pm$ 2.88	1,760 $\pm$ 530
	1.294 $\pm$ 0.009	176.0 $\pm$ 53.0	3.34 $\pm$ 1.00	19 $\pm$ 6
	1.311 $\pm$ 0.008	3.1 $\pm$ 1.2	1.45 $\pm$ 0.73	467 $\pm$ 117
	1.336 $\pm$ 0.018	3.5 $\pm$ 0.7	1.04 $\pm$ 0.26	299 $\pm$ 60
$\bar{X}$			2.85 $\pm$ 2.07	

^a Infectivity of SA7 was determined by plaque formation on LLC-MK₂, whereas that of Ad12 was determined by fluorescent focus formation on HEp-2 cells.

^b TU determined at an input of 64 VP per cell.

TABLE 5. Comparison of the oncogenicity of light and heavy adenovirions in PD-4 Syrian hamsters

Buoyant density (g/ml)	Virus particles injected	Tumor incidence					
		Ad12			SA7		
		Male	Female	Total	Male	Female	Total
1.30	10 ⁹	0/4	1/1	1/5	1/2	2/4	3/6
	10 ^{9.8}	0/5	1/2	1/7			
1.34	10 ⁹	0/3	1/3	1/6	1/5		1/5
	10 ^{9.8}	3/6	2/4	5/10			

fractions. Defective virions, as measured by infectivity in human cells, banded at densities lower than that of the most highly infectious virus band at 1.34 g/ml, thus reflecting the reduced DNA content of the defective virions. Others (19, 24) have utilized a similar approach in the search for defective subpopulations of adenovirus while investigating the possibility, as suggested by Sauer (27), that defective virions may be more oncogenic. Mak (19) analyzed a subpopulation of Ad12 that contained 3 to 4% less nucleic acid than fully infectious virions. This population, although unable to replicate fully, did induce cell killing and inclusion body formation in susceptible cells. In the present study, even lighter, noninfectious adenovirions that contained up to an estimated 30% less DNA than the most dense fully infectious virions still retained transforming capacity.

The possibility of complementation between defective VP giving rise to transformation must be given consideration, especially if the genome deletions are random. Were this a significant factor required for transformation by defective virions, one would expect at least a twofold increase in the VP/TU ratio of the less-dense (defective) virions over the virions of greatest density (complete). Data presented here (Table 4), although not totally consistent with this expectation, are suggestive of some complementation in Ad12 but not with SA7.

The defective virions also retained cell killing potential but required high input multiplicities of greater than 160 VP per cell. Severe cell killing, observed at high input multiplicities, became negligible at lower input virus-to-cell ratios. This cytolysis may be due to some virus-derived factor, possibly an endonuclease (7); it is probably the cause of marked depression of transformation capacity at high virus input. The studies by Cooper et al. (5) clearly demonstrated a quantitative relationship between virus dose and the degree of chromosomal damage induced by adenoviruses. Extensive damage of this nature is lethal.

To avoid cell killing and to provide standardization, the critical transformation assays were performed at inputs of 64 VP per cell. Under these conditions, the VP/TU ratios obtained for each virus population independent of density were nearly equal, with no significant differences in variation. These results supported the conclusion that lighter, defective virions were able to induce transformation as efficiently as virions with a density of 1.34 g/ml.

Different methods of virus purification were analyzed to determine the effect of physical manipulation on the distribution of VP in gradients, on their infectivity, and on their transforming potential. Regardless of whether the pools were prepared by sonic and fluorocarbon treatment, by sonic treatment alone, or with fluorocarbon treatment followed by layering in a gradient for centrifugation, the greatest proportion of infectivity resided in the fraction with a density of 1.34 ± 0.02 g/ml. Likewise, no significant alteration of VP/TU, VP/IU, or IU/TU was attributable to the purification methods used.

In several instances (Table 4) the VP/IU ratios of the least dense fraction in each gradient approximated that of the most dense fraction. The most likely explanation is that fully infectious virions remained attached to a cell membrane material that banded at these lighter densities.

Infectivity and transformation efficiencies are known to depend upon the particular virus-cell systems utilized (1, 18, 30, 35). Variations in adsorption, uncoating, and the molecular events that follow may be peculiar for each system. This makes comparisons of transformation efficiencies difficult, particularly when input multiplicities are based on IU. Comparisons are more accurate when the number of VP/TU is used as the basis of comparison. McAllister and Macpherson (17) reported ratios of 1×10^6 to 4×10^6 PFU/TU and 2×10^7 VP/TU for transformation of NIL-2 cells by Ad12 at a multiplicity of 80 PFU per cell. Rat embryo cells seemed to be slightly more sensi-

tive to transformation and yielded ratios of 5×10^6 VP/TU and 1.1×10^6 PFU/TU. Duff and Rapp (8) concluded that the PARA (defective simian virus 40-adenovirus 7) hybrid transforms hamster embryo fibroblast more efficiently than any of the other oncogenic DNA viruses, (PFU/TU = 2.5×10^2). Other investigations (2, 12) have indicated that PARA is the only virus known to contain a defective particle which is responsible for the transforming capacity of the virus stock. We detected subpopulations of Ad12 and SA7 which are as efficient as PARA at inducing transformation (1.90×10^2 PFU/TU for Ad12 and 2.0×10^2 PFU/TU for SA7) when the comparison is made on the basis of infectivity. Further comparisons with the PARA data cannot be made without knowing the number of VP in an IU or TU of PARA.

Although the infectivity-transformation ratios for Ad12 and SA7 virus pools were in the range observed for Polyoma (18, 30) and SV40 (1, 35), the similarity is apparently due to the lower sensitivity of the infectivity assays used for the former viruses. If true similarity in *in vitro* transforming potential existed between adenovirus populations and that of the Papova viruses, similar VP/TU ratios would be expected. The VP/TU ratios obtained here of 10^6 to 5×10^6 are close to those of Polyoma but much higher than those of simian virus 40. Transformation efficiencies of Polyoma and simian virus 40 (1 to 5% [18, 30] and 0.5 to 1.0% [1, 35], respectively) exceed that of adenoviruses. Here the maximum efficiency of transformation of hamster embryo cells by Ad12 and SA7 were 0.006 and 0.01%, respectively. These values are 50- to 1,000-fold lower than for Papova viruses. It would appear that actual differences in transformation efficiency of the Papova and adenoviruses hinge on differences of the assay systems used, as well as on the inherent cytotoxic effect on the cells.

The oncogenicity of "heavy" and "light" adenoviruses in female PD-4 Syrian hamsters was similar. Males appeared less sensitive to the lighter virions, an observation consistent with a well-documented sex difference in susceptibility to adenovirus oncogenesis (36). Although a far greater number of animals would be required for greater precision, it is apparent that the light-density fraction contains significant oncogenic capacity, at least for female hamsters. This observation suggests the speculation that adenovirus virions lacking infectivity but with no appreciable loss of transforming capacity might be capable of transforming systems which normally are not transformable because of productive infection. This would

include human cells. Thus, the sensitive cell infected with defective virions may be permitted to fulfill the important criterion for transformation (28), that being survival of the infected cell.

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