JOURNAL OF VIROLOGY, Apr. 1971, p. 426-433
Vol. 7, No. 4

Defective Virions in Human Adenovirus Type 12

STANLEY MAK

Department of Biology, McMaster University, Hamilton, Ontario, Canada

Received for publication 16 November 1970

Purified preparations of human adenovirus type 12 showed two bands when subjected to isopycnic centrifugation in a density gradient of cesium chloride. Their density difference was about 0.003 g/ml, suggesting a small difference in their deoxyribonucleic acid to protein ratio. Virions with a lighter density can kill human KB cells and induce T antigen as efficiently as the heavy virions. However, they appeared incapable to form plaques. Two passages of the heavy infectious virions at low multiplicity of infection did not produce significant amounts of light virions; however, when it was passed at high multiplicity of infection, the light band became visible in a cesium chloride density gradient.

It has been reported that purified preparations of simian virus 40 (SV40) obtained by serial undiluted passages of the virus contain virions of lighter density (20). These defective virions can carry out some of the viral functions, such as T-antigen induction and transformation of mouse 3T3 cells in vitro (19), but are unable to form plaques. Preparations of oncogenic human adenovirus type 12 (Ad12) were shown to contain a high proportion of virions which, although capable of cell killing, are unable to form plaques or to induce intranuclear inclusion bodies (15); however, it was not possible to determine whether this resulted from the presence of defective virions of limited functions or from the fact that some of the infected cells are unable to complete the complex process of plaque formation. In this study, we have been able to achieve partial separation of the defective virions from the plaque formers on the basis of buoyant density. The lighter virions have limited functions such as cell killing and induction of T antigen.

MATERIALS AND METHODS

Virus and cells. Ad12 (Huie) and adenovirus type 2 (Ad2) propagated in human KB cells in suspension and purified by CsCl equilibrium centrifugation were used (7). The multiplicity of infection was usually 0.3 and 100 plaque-forming units (PFU)/cell for Ad12 and Ad2, respectively. To obtain radioactively labeled virus, ^{14}C-thymidine (0.04 μCi/ml) or ^{3}H-thymidine (0.3 μCi/ml) was added to the infected cultures during growth. KB cells maintained in monolayer cultures with Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) were used for plaquing of the virus and for assay of cell killing by the virus. A human epithelial cell line (HEp-2) maintained in monolayer cultures by using MEM plus 10% FCS was used for the assay of T-antigen and intranuclear inclusion body induction by the virus.

Immunofluorescence techniques. HEp-2 (10^5) cells were seeded in Leighton tubes containing cover slips. After overnight incubation at 37 C, the cells were washed with MEM and infected with 0.4 ml of Ad12 preparation. After a 2-hr adsorption period with occasional shaking, they were washed with medium and incubated with 1 ml of MEM plus 10% FCS together with dilute anti-Ad12 antiserum to prevent reinfection by the progeny virus. At about 48 hr, the cells were washed with phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}, dried in air, and fixed with CCl_4. They were incubated for 2 hr at 37 C with fluorescein-tagged antiserum from hamsters bearing Ad12-induced tumors, washed exhaustively with PBS without Mg^{2+} and Ca^{2+}, and mounted for fluorescence microscopy. Usually, 1,600 cells were examined for the presence of T antigen.

Virus-induced inclusion bodies. HEp-2 cells were infected and incubated as for fluorescence staining. At about 48 hr after infection, cells were fixed with acetic acid-alcohol mixture (1:3) and stained with orcein. Usually, 1,600 cells were examined for the presence of virus-specific inclusion bodies.

Cloning of cells. The ability to form a clone was used as a criterion for cell survival after virus infection. The method used has been described (15), except that dilute anti-Ad12 antiserum from immunized rabbits was added to the cloning medium to prevent reinfection by the progeny virus.

Infectivity assay. The infectivity was assayed by plaque formation by the method described by Green et al. (8).

UV irradiation of virus. Source of ultraviolet (UV) light, dose rate, and the method of irradiation have been described (15).

Assay for radioactivity. After ^{14}C-Ad2 and ^{3}H-Ad12 were centrifuged in a CsCl density gradient and the fractions were collected, the total content of each fraction was treated with 5% cold trichloroacetic acid together with 100 μg of yeast ribonucleic acid (RNA) as

carrier. The precipitate was collected on nitrocellulose filters by filtration and washed three times with cold trichloroacetic acid. After drying, the radioactivity in the filter was determined by using a Beckman scintillation counter with 5 ml of counting fluid which consists of 4 mg of Omnifluor (New England Nuclear Corp.) dissolved in each milliliter of toluene.

Electron microscopy of virions. The negative staining technique of Watson et al. (21) was employed with slight modifications. Purified virus preparations were dialyzed against 0.01 M tris(hydroxymethyl)-aminomethane (Tris), *p*H 8.1. A sample of 0.1 ml was mixed with 0.01 ml of a suspension of polystyrene latex of known concentration containing 0.25% bovine serum albumin. With a platinum loop, a drop of this mixture was placed on a grid coated with parlodion-carbon and allowed to stand for 1 min. The excess fluid was removed by blotting from the side with a filter paper. The preparation was stained with 0.5% phosphotungstic acid, and the ratio of latex to virus particles was determined by counting several thousands of virus particles on electron micrographs taken with a Zeiss EMU9, at a magnification of 5,600.

Isopycnic gradient centrifugation. For analytical analysis, a purified preparation of Ad12 together with Ad2 virus as marker was suspended in 0.01 M Tris (*p*H 8.1 containing CsCl to give an average density of about 1.34 g/ml. This mixture was centrifuged at 44,770 rev/min at 20 C in a Spinco model E analytical ultracentrifuge. After 22 hr of centrifugation, a UV absorption photograph was taken. Light transmission at various parts of the absorbance photograph was measured by using a microspectrophotometer (Leitz) with a calibrated moving stage and a 10× objective lens. Quantitative light transmission was determined by a photomultiplier and Photovolt power supply and amplifier.

To separate the virions of different densities, Ad12 purified by two equilibrium centrifugations was suspended in CsCl solution (density 1.34 g/ml) prepared with 0.01 M Tris (*p*H 8.1) and centrifuged in a Spinco no. 65 fixed-angle rotor by using the relaxation method (1, 6). Centrifugation was carried out at 5 C for 18 hr at 48,000 rev/min, followed by 24 to 26 hr at 28,000 rev/min. At the end of centrifugation, a hole was punctured at the bottom of the tube and three-drop fractions corresponding to about 0.045 ml were collected for the region of the virus band. These fractions were immediately diluted with 0.45 ml of TBS (22), and 0.16 ml was used for optical density determination at 260 nm. The remainder was either assayed for biological activities immediately or frozen at −45 C in the presence of about 0.08 ml of glycerol until used.

RESULTS

Adenovirus virions with different densities. When purified preparations of Ad12 were centrifuged in CsCl density gradients, with the relaxation method, two very closely spaced opalescent bands could be observed, suggesting that these virus preparations contained virions with different densities. The double band was not due to contamination with Ad2 (the only other adenovirus used in the laboratory) as shown in the following experiment. When purified ^{14}C-labeled Ad2 was centrifuged together with ^{3}H-Ad12 in a CsCl density gradient, the ^{14}C-Ad2 having a higher density (14) sedimented much farther to the bottom of the tube compared to the double bands of ^{3}H-Ad12 (Fig. 1). These results further suggested the presence of Ad12 with different densities.

Density difference between the two Ad12 bands was determined by analytical centrifugation in a Spinco model E ultracentrifuge by using Ad2 as marker. A photograph of UV absorption at 22 hr after centrifugation at 44,770 rev/min is shown in Fig. 2. Light transmission of this absorbance photograph is shown in Fig. 3. Using the equation from Schildkraut et al. (16), it was calculated that the density of Ad2 is greater than the heavy Ad12 band by 0.009 g/ml, assuming that the density of Ad2 is 1.340 g/ml. This corresponds to about an 8% difference in their deoxyribonucleic acid (DNA) content, similar to that reported (14). The density difference between the two Ad12 bands was found to be about 0.003 g/ml, corresponding to a difference of 3 to 4% in their DNA content assuming that the protein contents are the same for the virions in these bands.

Calibration of different viral functions. To facilitate the quantitation of the various viral functions in the various fractions throughout the density gradient, calibration curves for cell

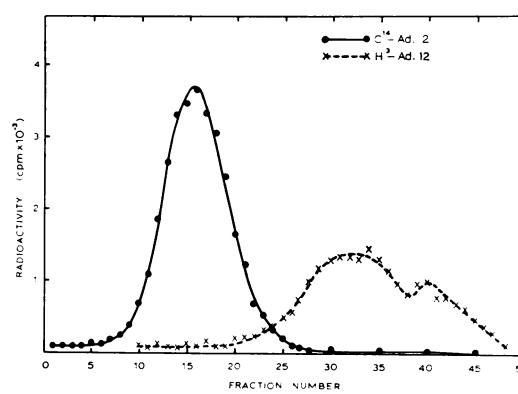

FIG. 1. *Distribution of Ad2 and Ad12 virions in a cesium chloride density gradient. Purified ^{14}C-Ad2 and ^{3}H-Ad12 were mixed with cesium chloride and centrifuged at 48,000 rev/min for 18 hr, followed by 26 hr at 28,000 rev/min in a 65 rotor (Beckman). Three-drop fractions were collected from the bottom of the tube and assayed for radioactivity. Symbols: ●, ^{14}C-Ad2; ×, ^{3}H-Ad12.*

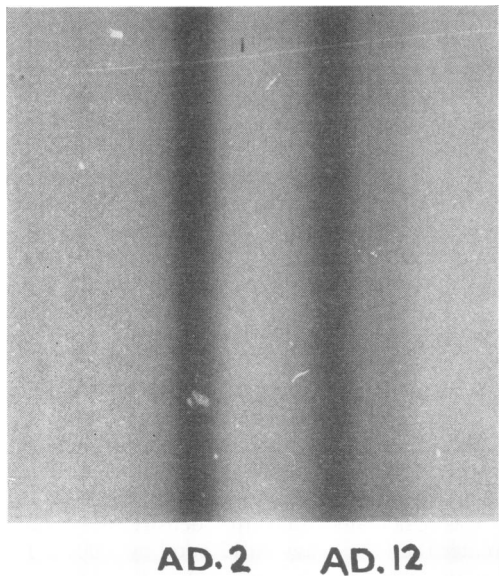

FIG. 2. *Photograph of the UV absorption of the Ad2 and Ad12 virion bands in a cesium chloride density gradient formed by centrifugation at 44,770 rev/min. The band at the far left is the Ad2, whereas the other two bands belong to the Ad12 virions.*

killing and T-antigen and IB induction by the virions were established. The data are shown in Fig. 4, with the fraction of cells surviving that particular viral function plotted against the amount of virus added. For cell killing and T-antigen induction, the per cent of cells surviving declined exponentially as expected for single-hit kinetics (13). From the exponential portion of the dose-response curves, it was calculated that the number of virions added to give 37% survival (on the average one unit per cell) was about 150 to 300 for cell killing and about 3,000 to 10,000 for T-antigen induction. This difference may in part be a reflection of adsorption efficiencies in the two assay systems, since it has been suggested that cell killing ability and T-antigen induction are closely related (17). At moderately higher virus input, IB induction deviated from the one-hit kinetics. Only the initial exponential portions of these curves were used in determining the relative amount of virions capable of that particular function.

For convenience, the optical density (OD) at 260 nm was determined for each fraction to establish the biological efficiency of the virions with different densities. Therefore, it was necessary to convert OD to virion concentration. Figure 5 shows that virion concentration was linear to OD in the range tested, and from the slope of the line it was calculated that 1.0 OD unit with a 1-cm path length corresponds to 3.5 $\times 10^{11}$ adenovirus virions. The amount of DNA per OD of virus was found to be 20 μg with the diphenylamine reaction (3), after the DNA had been extracted with 5% trichloroacetic acid at 90 C for 15 min. Assuming that each virion contains a single DNA molecule with a molecular weight of 23 $\times 10^6$, it was calculated that this corresponds to 5.2 $\times 10^{11}$ virions, about 33% higher than that found by direct particle counts. Our average value of 4.4 $\times 10^{11}$ virions per OD was lower than the reported value of 7.1 $\times 10^{11}$ (12), probably because of a higher light-scattering factor in our OD determination.

Functional capacity of virions with different densities. After the Ad12 virus preparation was centrifuged in CsCl solution by using the relaxation method, three-drop fractions were collected and assayed for OD at 260 nm and several viral functions (infectivity by plaquing, killing of human KB cells, the ability to induce T antigen, and induction of IB in HEp-2 cells). For the last three functions, the percentage of cells escaping that particular viral function was determined for an appropriate dilution of the virus. These values were converted into relative amounts of virion capable of that function by using the calibration curves. Representative data are shown in Table 1. To compare the distribution of virions capable of different viral functions in the various fractions, the data have been normalized such that the peak of denser virions has a value of 100 for all viral functions. Figure 6 shows results

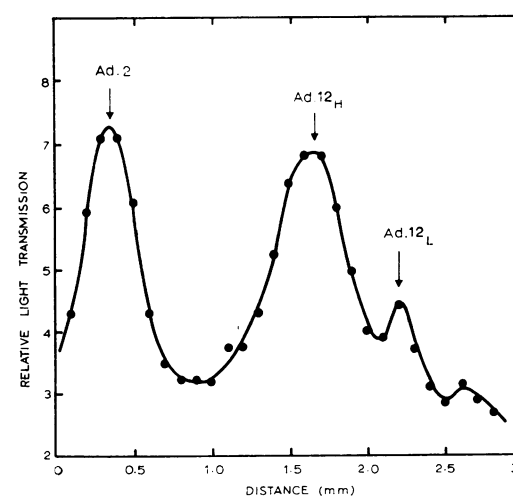

FIG. 3. *Relative light transmission of the photograph shown in Fig. 2.*

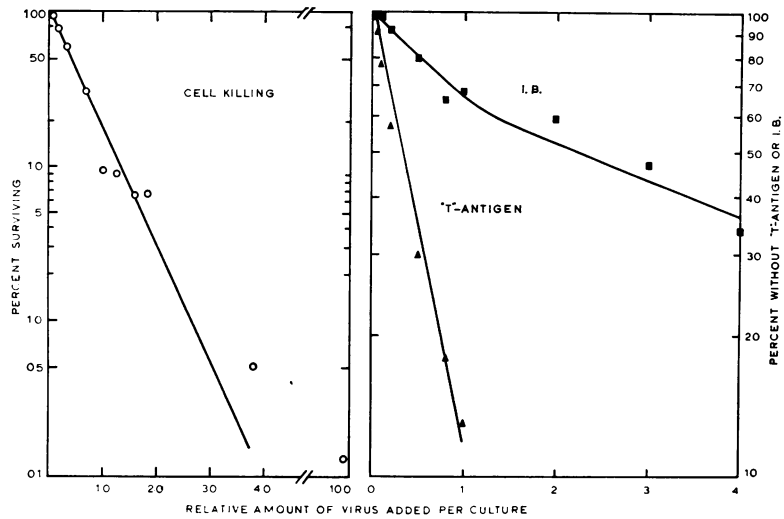

FIG. 4. *Calibration curves for several viral functions. The per cent of cells not showing that particular viral function is plotted against the amount of virus added in a semilogarithmic plot. Symbols:* ○, *cell killing;* ▲, *T-antigen;* ■, *IB induction. On the horizontal scale, 1 unit represents about 300 virions/cell for cell killing and about 7,000 virions/cell for T-antigen and IB induction.*

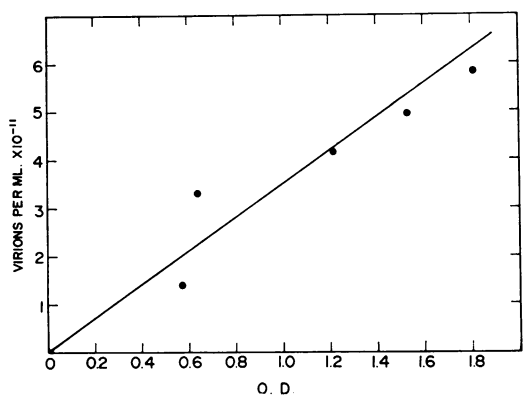

FIG. 5. *Number of Ad12 virions, determined by electron microscopy, is plotted against OD at 260 nm, with a path length of 1 cm. One OD unit is equivalent to 3.5* × 10^{11} *virions per ml.*

from an experiment. The lighter fractions had a much reduced specific infectivity (PFU/virion) compared to the virions of the heavy band. In the virus preparation presented, the lighter fractions contained many more virions capable of inducing IB than plaqueformers. In other experiments, the difference in these functions for the lighter virions was less pronounced. However, in all instances, the distribution of IB-inducing virions in the CsCl density gradient was found between the profiles of PFU and T-antigen induction. Since IB formation in human cells correlates with the production of viral structural antigens, (J. Weber, Ph.D. Thesis, McMaster Univ., Hamilton, Ontario, 1969), these data indicate that some of the virions can carry out late functions but are unable to form plaques. Using radiobiological techniques, the ability to form IB was found to be more resistant to radiation damage than plaque formation (A. J. Rainbow, *in manuscript*), supporting the notion that virions with less genetic information than the plaque former can still induce IB. The efficiency of cell killing and T-antigen induction followed the virion concentration curve more closely. The apparent higher efficiency of T-antigen induction in fractions 14, 16, and 18 may be due to the method of normalization. The normalized values of the T antigen-producing virions at the heavy side of the gradient may be underestimated since the presence of dense IB in some infected cells interfered with the detection of T antigen. These data suggest that the virions with lower density have only limited viral functions. Similar results were obtained for several Ad12 preparations, but the normalized amount of the virions in the lighter density region varied. Ad12 freshly prepared from infected cells gave results similar to those with purified virus that had been stored at −45 C. Virus preparations used in this analysis showed no detectable adeno-associated virus when examined under an electron microscope.

Inactivation of cell killing ability of virions by

TABLE 1. *Amount of Ad12 virions capable of various viral functions in different fractions from a CsCl density gradient*[a]

Fraction no.	Virion concn		Infectivity (PFU per ml)	IB induction		T-antigen induction		Cell killing ability	
	OD at 260 nm	Virion[d] per ml		Per cent without IB[c]	Relative amt of virions	Per cent without T antigen[c]	Relative amt of virions	Per cent of cells surviving[b]	Relative amt of virions
5	0.197	8.7×10^{10}	8.6×10^{6}					5.9	1.6
6	0.213	9.4×10^{10}		77	0.60	38	0.45		
18	0.136	6.0×10^{10}		93.7	0.10	42	0.40		
19	0.124	5.4×10^{10}	0.77×10^{6}					5.9	1.6

[a] An Ad12 preparation was centrifuged in CsCl density gradient and three-drop fractions were collected from the regions of the virus band and assayed for infectivity (PFU), cell killing in KB cells, and the induction of T antigen and IB in HEp-2 cells.

[b] Surviving is defined as the ability to form clones; it is expressed as per cent of the noninfected control cells which has a cloning efficiency of about 70%.

[c] Greater than 1,600 cells were examined.

[d] One $OD_{260} = 4.4 \times 10^{11}$ virions/ml.

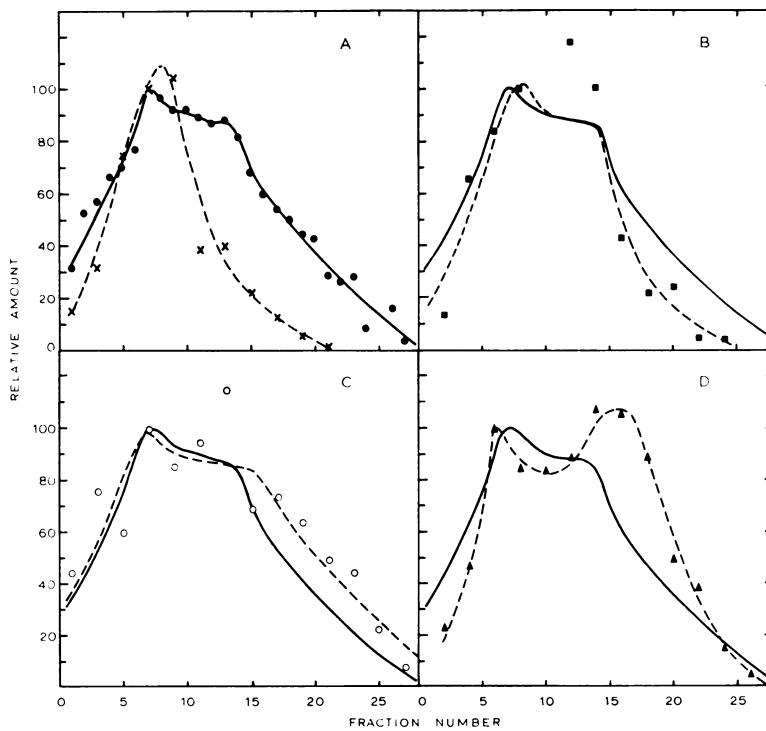

FIG. 6. *Distribution of Ad12 virions capable for various viral functions in a cesium chloride density gradient. Conditions of centrifugation are the same as described in Fig. 1. Symbols: ●, virion concentration; ×, plaque formation; ■, IB induction; ○, cell killing; ▲, T-antigen induction. These data have been normalized (see text). Solid line in panels B, C, and D represent virion concentration.*

UV. As shown in Fig. 6, the efficiency of cell killing was the same for virions of different densities. It is possible that the cell killing is not an expression of viral function, since it has been shown that adenovirus capsid subunits can inhibit mammalian cell metabolism (11). As shown in Table 2, UV can abolish the cell killing ability for virus from both the light and heavy fractions with similar efficiency. On the other hand, UV does not significantly inhibit the

adsorption of the virus to the host (24). Thus, it seems reasonable to assume that, with purified preparations of adenovirus, cell killing requires some functional activity of the viral genome.

Separation of heavy and light virion bands. To confirm the presence of defective virions in the lighter density band, most of the top band of an Ad12 preparation, having been centrifuged in a CsCl density gradient, was usually removed from the top by careful suction. The remainder of the top band together with part of the bottom band was also removed by suction. The remainder of the virus was collected from a hole at the bottom of the tube. The efficiency of plaque formation and T-antigen induction for these fractions is shown in Table 3 for two virus preparations. The heavy band had a 7- to 10-fold higher specific infectivity (PFU/virion) than the light band, whereas the T-antigen induction efficiency was similar within each virus preparation. Thus, these data support the earlier conclusion that the lighter virus band contained more defective virions. It should be pointed out that the efficiency of plaquing was very low compared with that reported with the same cell line (8). It is possible that the characteristics of the virus population or those of KB cells or both have changed during the intervening years to produce such low efficiency in plaquing. However, the difference in the specific infectivity between light and heavy fractions is still valid since they were assayed under identical conditions. When examined under an electron microscope by using negative staining, virions from the light and heavy bands appeared to have the same morphology.

TABLE 2. *UV inactivation of cell killing ability of virions from light and heavy fractions*[a]

Fraction no.	Fraction of KB cells surviving[b]	
	Nonirradiated virus	UV-irradiated virus (200 sec)
11 (Heavy)	0.05	0.98
23 (Light)	0.10	0.96

[a] Three-drop fractions were collected from a CsCl density gradient containing Ad12.

[b] Surviving is defined as the ability to form clones; it is expressed as the per cent of the noninfected control cells. The cells were infected with approximately 100 virions per cell for both fractions.

Development of light virions. Although the virions from the heavy fractions have a relatively homogeneous density, it is possible that, upon passages, "light" virions can develop as in the case of SV40 (20). Virions from four fractions with high density were used to infect KB cells at about 0.02 PFU/cell. The resulting crude extract (sonically treated and clarified by light centrifugation) was passed once more in KB cells at low multiplicity. The purified virus preparations from this passage were subjected to isopynic centrifugation in CsCl; three-drop fractions were assayed for virus concentration and PFU. Figure 7 shows that the distribution of PFU followed that of virus concentration, as contrasted by that shown in Fig. 6a. Thus, it appears that two passages at relatively low multiplicity of infection were not sufficient to generate significant amounts of light virions. It is not known whether further passages at low multiplicity of infection can give rise to "light" virions. However, with further passage of this virus preparation at higher multiplicity (0.3 PFU/cell), the resulting purified virus preparation showed a definite visible light band of virus.

TABLE 3. *Relative amount of Ad12 capable of T-antigen induction and plaque formation in light and heavy virus bands*[a]

Virus band	Virus prepn A		Virus prepn B	
	T-antigen induction (TAU/10^{11} virions)[b]	Infectivity (PFU/10^{11} virions)	T-antigen induction (TAU/10^{11} virions)	Infectivity (PFU/10^{11} virions)
Light	3.7×10^7	9.7×10^5	1.5×10^7	2.8×10^6
Intermediate region	3.5×10^7	1.1×10^6	1.1×10^7	1.3×10^7
Heavy	3.4×10^7	6.7×10^6	1.2×10^7	2.8×10^7

[a] After the virus was centrifuged in a CsCl density gradient, the light virus band was removed from the top of the gradient by suction followed by the removal of the intermediate region also by suction. The heavy band was removed by dripping through a hole at the bottom of the tube.

[b] One TAU is defined as the amount of virus added to induce T antigen in 63% of the cells in a culture, divided by the total number of cells infected. T antigen was detected by immunofluorescence. At least 1,600 cells were examined for each assay.

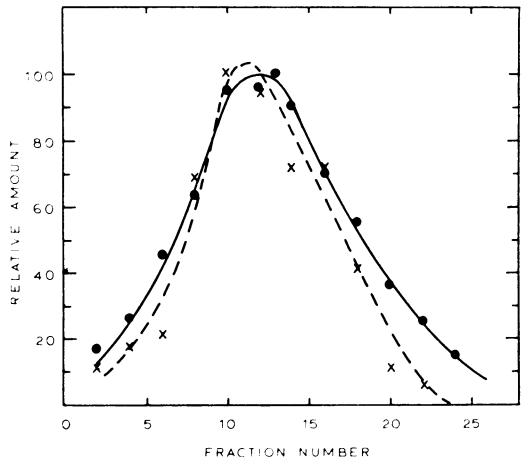

FIG. 7. *Ad12 virions obtained by passages at low multiplicity of infection. Several fractions of heavy Ad12 virions were passed twice at about 0.02 PFU per cell; this purified virus preparation was centrifuged in a cesium chloride density gradient as described in Fig. 1. Symbols: ●, virion concentration; ×, plaque formation.*

DISCUSSION

Defective virions with limited biological functions have been reported in both DNA- and RNA-containing animal viruses (2, 9, 20). These virions contain smaller nucleic acid molecules than the plaque formers (10, 23). Previous reports indicated that human Ad12 contains virions capable of some viral functions such as cell killing (15) and acting as helper virus (4) but is unable to form plaques. This study confirmed the presence of defective virions in purified Ad12 preparations and showed that they are of lower density. It is possible that the virus preparations studied in this work contained a whole spectrum of virions with different degrees of defectiveness. Some of these can carry out the early functions such as T-antigen induction, whereas others may be capable of viral DNA and protein synthesis and yet be unable to produce plaques. "Light" virions of adenoviruses have been reported, but their biological functions were not described (B. T. Burlingham and W. Doerfler, Bacteriol Proc., p. 181, 1969).

It has been reported for several animal viruses that the noninfectious virions induced by radiation and the naturally occurring defective SV40 can induce tumors in newborn animals (5, 18). The oncogenicity of the defective Ad12 described in this report is not known at present but is under investigation.

ACKNOWLEDGMENTS

This investigation was supported by research grants from the National Cancer Institute of Canada.

The author thanks I. Takahashi for his helpfulness in the analytical ultracentrifugation work.

LITERATURE CITED

1. Anet, R., and D. R. Strayer. 1969. Density gradient relaxation: a method for preparative buoyant density separation of DNA. Biochem. Biophys. Res. Commun. 34:328–334.
2. Blackstein, M. E., C. P. Stanners, and A. J. Farmilo. 1969. Heterogenicity of polyoma virus DNA: isolation and characterization of non-infectious small supercoiled molecules. J. Mol. Biol. 42:301–314.
3. Burton, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315–323.
4. Butel, J. S., J. L. Melnick, and F. Rapp. 1966. Detection of biologically active adenovirions unable to plaque in human cells. J. Bacteriol. 92:433–438.
5. Defendi, V., and F. Jensen. 1967. Oncogenicity by DNA tumour viruses: enhancement after ultraviolet and cobalt-60 radiations. Science 156:703–705.
6. Flamm, W. G., H. E. Bond, and H. E. Burr. 1966. Density-gradient centrifugation of DNA in a fixed-angle rotor. A higher order of resolution. Biochim. Biophys. Acta 129: 310–317.
7. Green, M., and M. Pina. 1963. Biochemical studies on adenovirus multiplication. IV. Isolation, purification, and chemical analysis of adenovirus. Virology 20:199–207.
8. Green, M., M. Pina, and R. Kimes. 1967. Biochemical studies on adenovirus multiplications. XII. Plaquing efficiencies of purified human adenoviruses. Virology 31: 562–565.
9. Hackett, A. J., F. L. Schaffer, and S. H. Madin. 1967. The separation of infectious autointerfering particles in vesicular stomatitis virus preparation. Virology 31:114–119.
10. Huang, A. S., and R. R. Wagner. 1966. Comparative sedimentation coefficients of RNA extracted from plaque forming and defective particles of vesicular stomatitis virus. J. Mol. Biol. 22:381–384.
11. Levine, A. J., and H. S. Ginsberg. 1968. Role of adenovirus structural proteins in the cessation of host-cell biosynthetic functions. J. Virol. 2:430–439.
12. Lonberg-Holm, K., and L. Philipson. 1969. Early events of virus-cell interaction in an adenovirus system. J. Virol. 4: 323–338.
13. Marcus, P. I., and T. T. Puck. 1958. Host-cell interaction of animal viruses. I. Titration of cell killing by viruses. Virology 6:405–423.
14. Pina, M., and M. Green. Biochemical studies on adenovirus multiplication. IX. Chemical and base composition analysis of 28 human adenoviruses. Proc. Nat. Acad. Sci. U.S.A. 54:547–551.
15. Rainbow, A. J., and S. Mak. 1970. Functional heterogeneity of virions in human adenovirus types 2 and 12. J. Virol. 5:188–193.
16. Schildkraut, C. L., J. Marmur, and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its buoyant density in CsCl. J. Mol. Biol. 4:430–443.
17. Strohl, W. A., 1969. The response of BHK21 cells to infection with type 12 adenovirus. I. Cell killing and T-antigen synthesis as correlated viral genome functions. Virology 39: 642–652.
18. Uchida, S., and S. Watanabe. 1968. Tumourigenicity of the antigen forming defective virion of simian virus 40. Virology 35:166–169.
19. Uchida, S., and S. Watanabe. 1969. Transformation of mouse

3T3 cells by T-antigen forming defective SV40 virions (T particles). Virology 39:721–728.
20. Uchida, S., K. Yoshike, S. Watanabe, and A. Furuno. 1968. Antigen-forming defective viruses of simian virus 40. Virology 34:1–8.
21. Watson, D. H., W. C. Russell, and P. Wildy. 1963. Electron microscopic particle counts on herpes virus using the phosphotungstate negative staining technique. Virology 19: 250–260.
22. Winocour, E. 1963. Purification of polyoma virus. Virology 19:158–168.
23. Yoshike, K. 1968. Studies on DNA from low-density particles of SV40. I. Heterogeneous defective virions produced by successive undiluted passages. Virology 34:391–401.
24. zur Hausen, H. Association of adenovirus type 12 deoxyribonucleic acid with host cell chromosomes. J, Virol. 2:218–223.