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Structure and Function of Adenovirus Type 12 Defective Virions

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Purified human adenovirus type 12 preparations contain defective virions with a lighter density. These defective virions were isolated, and their biological functions and DNA were characterized. They can induce early and late antigens in infected cells and tumors in newborn hamsters with similar efficiency as complete virions. The majority of the DNA molecules from light virions contain deletions mapping near 16% from the left-hand end of the genome. Mechanisms for the generation of these molecules are discussed.

Preparations of animal viruses contain defective or incomplete virions which have deletions, substitutions, or duplications in their genome. Such defective viruses have been found in RNA as well as in DNA viruses and have been well documented (see reference 13). Often they are detected as having lower buoyant densities when banded in CsCl density gradients. Both in SV40 and adenoviruses, these virions retain some biological functions, such as cell killing, tumorigenicity, and cellular transformation (14, 19, 26, 34). Thus, characterization of the DNA structure and biological functions of these defective virions should allow the mapping of biological functions on the viral genome.

Defective virions have been described for many types of adenoviruses including members from groups A, B, and C of the human, bovine, and simian adenoviruses (5, 7, 19, 21, 26, 31, 33). Generally, they have shorter DNA molecules than do the full-length genome. Daniell reported that the DNA from the Ad 3 as well as Ad 2 incomplete virions is heterogeneous and enriched in left-hand end sequences (7). These short DNA molecules may be the results of an aberrant abortive DNA replication. More recently, Tibbetts studied the DNA sequences in human Ad 7 incomplete virions and suggested that these may represent intracellular pools of assembly intermediates in which the incompletely packaged DNA has been fragmented in vivo during preparative procedures (31).

We reported earlier that human adenovirus type 12 (Ad 12) preparations contain functional defective virions (19). In the present report, we have further studied the biological functions and characterized the DNA of these virions. It was found that about 50% of the DNA molecules contain deletions ranging from 4.5 to 8.8% starting at about 8% from the left-hand end. These virions are still tumorigenic in hamsters and can synthesize viral structural antigens.

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MATERIALS AND METHODS

Cells. Human KB cells in suspension culture were used to propagate virus. KB cells grown as monolayer cultures using minimal essential medium (MEM) plus 10% fetal calf serum were also used. A human cell line (HEP-2) grown as monolayer cultures using MEM plus 10% fetal calf serum was used for immunofluorescence assay. Primary human embryonic kidney (HEK) cells were grown in MEM plus 10% fetal calf serum. 293 cells (HEK transformed by Ad 5 DNA fragments) were grown in monolayer using MEM (Joklik modified) plus 5% horse serum.

Preparative separation of light virions from complete virions. Human Ad 12 (Huie strain), purified by CsCl density gradients, was used. We reported earlier that purified preparation of Ad 12 contain defective virions with lighter density (19). To further characterize these virions, relatively large amounts of light virions were needed. To ensure a reasonably clean separation of light and complete virion bands, purified virion preparations were banded in CsCl density gradients by the relaxation method (3, 19). Then the light virion bands were removed from the top of the gradients and pooled. The region between the two virion bands was removed and discarded. The complete virion bands were then collected and pooled. These pooled virion fractions were rebanded in CsCl density gradients as before. If there was visible contamination of one virion by another, the separation procedure was repeated. The apparently homogeneous light and complete virion bands were then used for further physical and biological characterization.

Biological functions of virions. Plaque formation was carried out using KB cells, 293 cells, or HEK cell cultures as described previously (19).

The ability of virions to induce the synthesis of viral structural antigens (V-antigens) and "early" antigens (T-antigens) was assayed by immunofluorescence. An-

tibody against V-antigens was prepared by immunizing rabbits with purified virions. Sera from these animals were used without fractionation. Sera from hamsters bearing Ad 12-induced tumors were used as a source of anti-T antibody. For immunofluorescence, HEP-2 cells were infected with Ad 12 in suspension. The cells were then seeded onto Leighton tubes and incubated with MEM plus 10% fetal calf serum plus 0.3% anti-Ad 12 antiserum (raised in rabbits against whole virions) to prevent reinfection. The cells were fixed at 48 h after infection and stained for T- and V-antigens by using the direct and indirect immunofluorescence techniques, respectively (19, 24).

Tumorigenicity was assayed by injecting purified virions (0.05 ml) into newborn Syrian hamsters. The animals were observed for more than 4 months.

Viral DNA preparation. Viral DNA was extracted from purified virions essentially by the method of Green and Pina (11). The virions were lysed with 0.5% sodium dodecyl sulfate and treated with self-digested pronase. The lysate was extracted with phenol at freezing temperature. The DNA was dialyzed extensively, with either 0.01 M Tris (pH 7.9) or 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Sometimes the viral DNA was precipitated by the addition of ethanol.

Electron microscopy. Methods for the formation and analysis of heteroduplex DNA molecules are essentially those of Davis et al. (8). The DNAs were denatured by NaOH and neutralized, and renaturation was carried out in 50% formamide at 37°C for 3 h. Under these conditions, more than 85% of the DNA was renatured. The DNA was then spread on Parlodion-coated grids by the formamide technique and then shadowed with platinum-palladium.

For contour-length measurements, the image of the DNA molecules was projected onto paper and then traced. This added procedure gives a total magnification of about 4×10^4 to 6×10^4. Length measurements were done by using a Keuffel and Esser map measurer. ϕ29 phage DNA was used as length standard.

Restriction endonuclease digestion, gel electrophoresis, and recovery of DNA fragments from gels. Ad 12 DNA was digested with restriction endonuclease *Eco*RI in a buffer containing 90 mM Tris (pH 7.9)-10 mM $MgCl_2$. The DNA fragments were separated by electrophoresis through 0.7 or 1% slab or cylindrical agarose gels for 8 to 12 h at 2 V/cm. The electrophoresis buffer was composed of 40 mM Tris (pH 7.8), 5 mM sodium acetate, and 1 mM EDTA. The DNA bands were stained with 0.5 μg of ethidium bromide per ml and visualized under shortwave UV illumination (27).

[^{3}H]DNA fragments were recovered from appropriate agarose gel slices by freezing and thawing (30). The liquid containing the DNA was withdrawn. More [^{3}H]DNA was recovered by incubating the gel slices with 0.01 M Tris (pH 7.9) for 3 h at room temperature. The buffer containing [^{3}H]DNA solution was made to 4 M sodium perchlorate. After 30 min at 0°C, an equal volume of 0.01 M phosphate (pH 6.8) was added, and the [^{3}H]DNA was adsorbed to a hydroxyapatite column at room temperature. The column was washed with 0.01 M phosphate (pH 6.8), and the [^{3}H]DNA was eluted with 0.4 M phosphate (pH 6.8) and dialyzed against 1 M NaCl and then against 0.01 M Tris (pH 7.9), followed by ethanol precipitation.

Quantitation of radioactivity from gels. [^{3}H]-DNA fragments separated by slab agarose gel electrophoresis were detected and quantitated by fluorography as described by Laskey and Mills (16). The gels were dehydrated by methanol, impregnated with Omnifluor (New England Nuclear) (10% [wt/vol] in methanol), and then soaked in water. They were dried under vacuum and exposed at −70°C to Kodak RP Royal X-Omat film previously exposed to white light. Exposure was sufficient to bring the background density of the film to 0.15 D above that of unexposed film. To quantitate the relative amounts of radioactivity, the film was scanned with a Joyce Loebel microdensitometer, and the areas under the peaks were integrated by using a planimeter.

DNA-DNA hybridization. A 2-μg amount of Ad 12 DNA was digested with restriction endonuclease *Eco*RI. The DNA fragments separated by agarose gel electrophoresis were denatured and transferred to nitrocellulose filter strips as described by Southern (28). The filters were heated for 3 h at 80°C and incubated in 2× SSC, 0.01 M Tris (pH 7.0), and 0.1% SDS at 65% for 24 h with sonicated, denatured [^{3}H]Ad 12 DNA fragments generated by *Eco*RI. The filters were washed with hybridization buffer and incubated further in the same buffer at 65°C overnight. To further remove the unhybridized [^{3}H]DNA, the filters were washed with 0.1× SSC in 0.01 M Tris (pH 9.4) at room temperature. After drying, the filter was dipped in 20% (wt/vol) Omnifluor (New England Nuclear) dissolved in toluene, air-dried, and exposed to X-ray film that previously had been exposed to white light.

RESULTS

Electron microscopic studies of DNA from light and complete virion bands. We suggested earlier that the virions with lighter density were due to a shorter viral DNA molecule. It was estimated that the DNA in the light virions is shorter than that from complete virions by about 3%, based on the difference of virion densities in CsCl (19). To provide another estimate of viral DNA lengths, we extracted DNA molecules from virion bands and examined them under the electron microscope. Figure 1 shows the contour lengths of DNA molecules prepared from light and complete virions, respectively, together with ϕ29 DNA molecules as standard. It was found that the average length of DNA from a complete virion is 11.0 μm compared with 10.3 μm for the light virion DNA. The average length of ϕ29 DNA molecules has been normalized to be 6.2 μm for both DNA preparations. Also, there is considerable overlap in the DNA lengths between those from light virions and those from complete virions. This overlap may be due in part to variation in the determination of DNA lengths under our experimental conditions since the standard deviation of ϕ29 DNA contour lengths was 3.5% of the

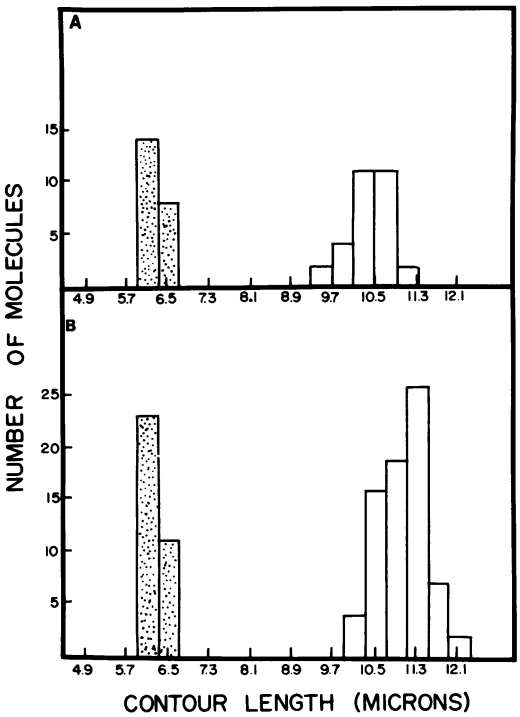


FIG. 1. *Length distribution of DNA molecules isolated from complete and light virions. The DNA molecules were examined under an electron microscope using the Kleinschmidt technique.* □, *Ad 12 DNA molecules.* ▨, *φ29 DNA molecules (6.2 μm) used as length standard. (A) Light virion DNA; (B) complete virion DNA.*

mean. However, the possibility of some contamination of light virions by complete virions cannot be ruled out. Thus, it seems reasonable to conclude that the light virion DNA molecules are shorter than those from complete virions by about 6.5%. This value is larger than that estimated previously (19).

To locate the deletion(s), heteroduplex DNA molecules were examined under electron microscopy. Figure 2 shows examples of heteroduplex DNA having a single-stranded loop (molecules A and B). This category of molecules constituted about 30% of the duplexes when equal amounts of complete virion and light virion DNAs were renatured, whereas none was observed in the homoduplex samples. A total of 54 molecules with large single-stranded loops were measured, and the location of the single-stranded regions are shown in Fig. 3. We have placed all the deletions near the left-hand end of the molecules because data presented in a later section justify this orientation. It can be seen that the deletions are confined within the region between 0.08 and 0.23, with deletions ranging from 6 to 10% of the genome. The heterogeneity of the location as well as the extent of the deletion partly may be due to the lack of precision in our determinations of contour lengths of such small stretches of DNA.

Heteroduplex molecules with different aberrations were also observed, but at a much lower frequency. Examples are shown in Fig. 2: molecule C shows two small single-stranded loops; molecule D shows a single-stranded tail.

Restriction analysis of light virion DNA. Restriction endonuclease *Eco*RI cleaves Ad 12 DNA into six fragments, having the order C, D, B, E, F, A, C being the left-hand end (10, 22). The cleavage pattern of ^{3}H-labeled light and complete virion DNAs were examined using fluorography. Microdensitometer tracing of an exposed X-ray film is shown in Fig. 4. The light virion DNA (A) shows five new fragments in addition to fragments A through E. Fragment F was run out of the gel due to its small size. It should be noted that small amounts of these fragments also appear in the complete virion DNA digest (Fig. 4B). These minor bands are probably the results of incomplete separation of complete and light virions.

The size of these fragments was found by comparing their electrophoretic mobilities with those of DNAs of known molecular weight (fragments A through E of Ad 12 DNA digested by *Eco*RI). Figure 5 shows the relationship between the size of the fragments, expressed as the percent of the virion genome, and electrophoretic mobility. The size of the extra fragments from light virion DNA is shown in Table 1. Fragment I (46% of the genome) is larger than any two contiguous fragments; its formation must involve the elimination of at least two restriction sites. Since a significant number of light virion DNA molecules has deletions near one end (Fig. 3), fragments II through V may be the result of a deletion in fragment A or a deletion encompassing the junction of *Eco*RI fragments C and D. The fusion of fragments C and D with a deletion of about 6 to 10% should give new fragments having sizes from 18 to 22% of the genome. Fragments II through IV can be such candidates. Fragment V may result from a deletion totally in fragment C or D.

Origin of the extra restriction fragments from light virion DNA. To determine the origin of fragments II through V, the amounts of radioactivity associated with each DNA fragment generated by *Eco*RI was determined by quantitative fluorography. The results are shown in Table 1, column 4. For the complete virion DNA, the amount of radioactivity associated with each fragment is approximately pro-

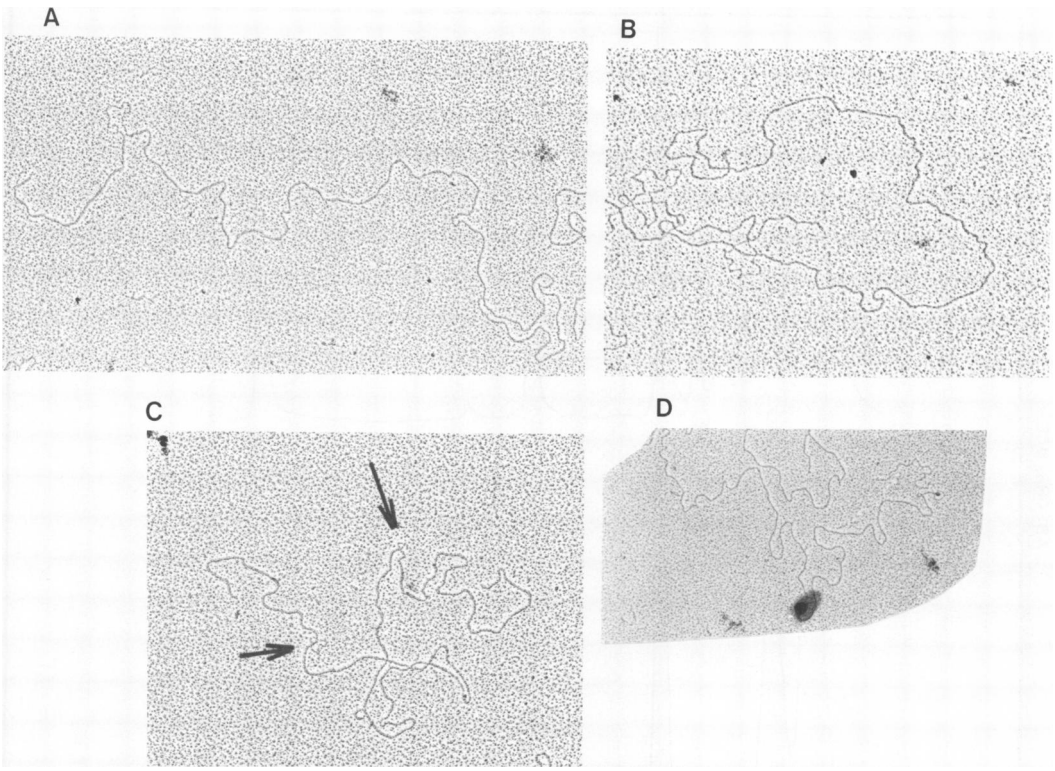
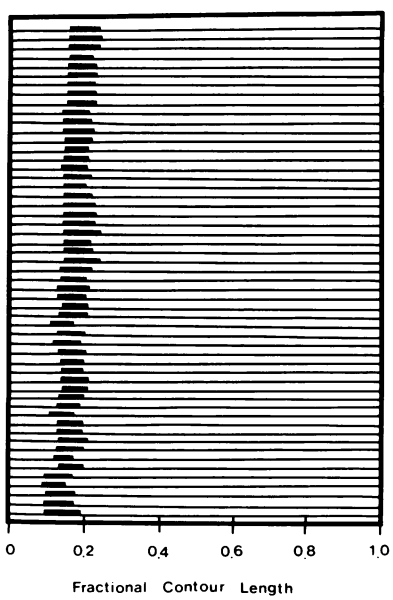


FIG. 2. *Electron micrographs showing heteroduplex DNA molecules between complete and light virion DNAs. Heteroduplex formation and electron microscopic analysis were described in Materials and Methods. Molecules A and B show large single-stranded loop near one end. The loop in molecule A spans the EcoRI fragments C and D. The loop in B is contained entirely within EcoRI fragment C. Molecule C shows two small single-stranded loops (arrows). Molecule D shows single-stranded tail at one end.*



portional to the fractional length of the genome (column 3). For the light virion DNA, there is no significant decrease of radioactivity associated with fragment A. On the other hand, amounts of radioactivity associated with fragments C and D are reduced. These data suggest that at least some of the fragments II through V can be the results of a deletion near the junction of *Eco*RI fragments C and D. It should be noted that the radioactivity associated with fragments C and D are reduced to a similar extent. There-

FIG. 3. *Location and the extent of the deletion of 54 heteroduplex molecules between the complete and light virion DNAs. Heteroduplex molecules were formed and examined under electron microscopy as described in the text. The heavy bars represent the single-stranded regions. The horizontal axis represents the total length of complete virion DNA. The regions containing the single-stranded loops have been placed at the left-hand end of the molecule (see text for justification).*

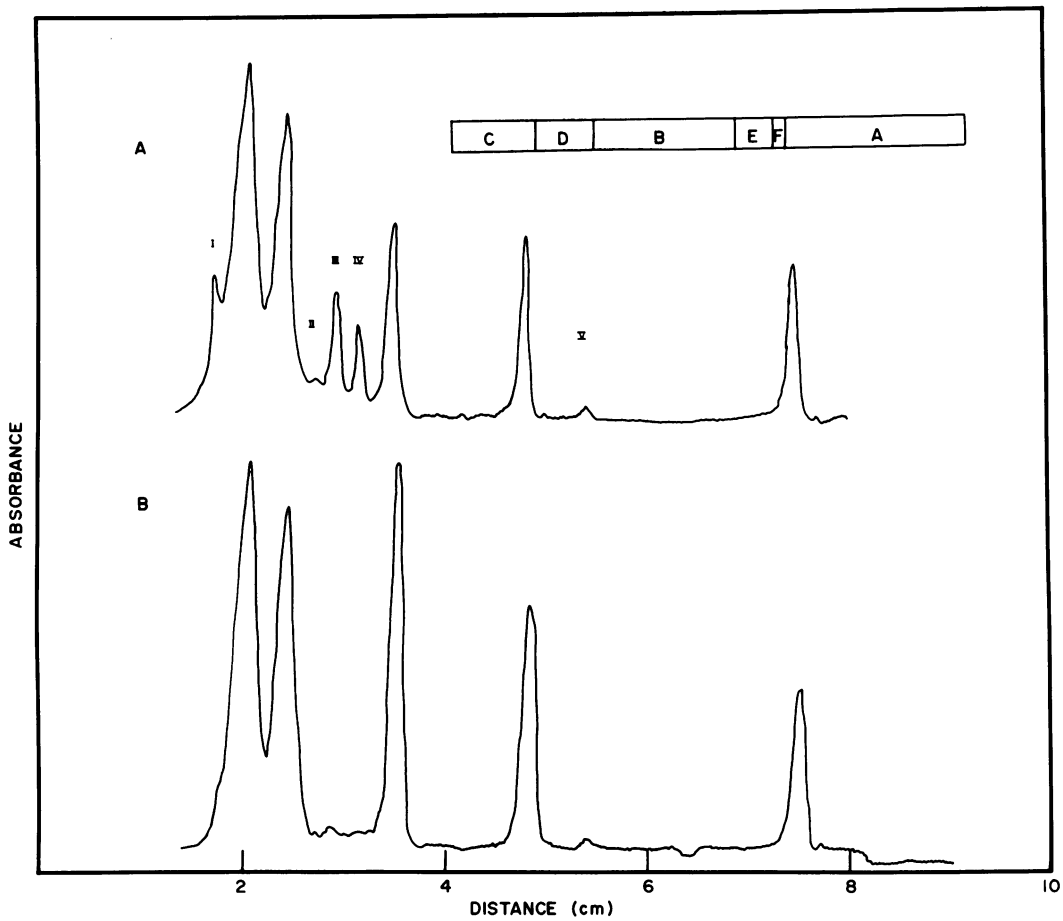


FIG. 4. *Distribution of radioactivity among Ad 12 DNA fragments generated by restriction endonuclease EcoRI. [^{3}H]Ad 12 DNA was digested with EcoRI, and the resulting fragments were separated by slab agarose gel electrophoresis. Fluorographs were made. The graph shows microdensitometer tracings of an exposed X-ray film. (A) DNA isolated from light virions. (B) DNA isolated from complete virions. The extra fragments are labeled from I to V. Also shown is the EcoRI cleavage of Ad 12.*

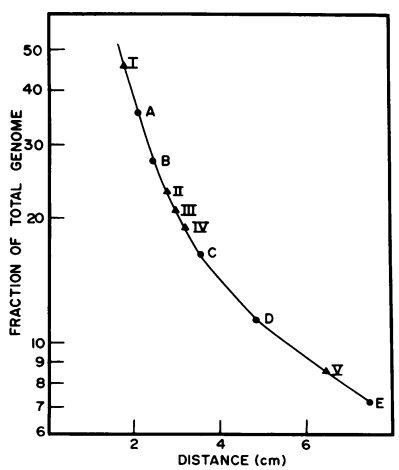


FIG. 5. *Size determination of "extra" DNA fragments generated by EcoRI digestion of light virion DNA. The electrophoretic mobility of EcoRI fragments of Ad 12 DNA was plotted against the size of the fragments expressed as percent of the whole Ad 12 genome. The electrophoretic mobility of the extra fragments I through V are indicated on the graph.*

fore, the C-D junction must be near the middle of most of the deletions.

To provide definitive proof, the DNA-DNA hybridization method was used. A 2-μg portion of light virion DNA was digested with *Eco*RI and the fragments were separated by agarose gel electrophoresis, denatured, and transferred to nitrocellulose filters. Specific restriction fragments of [^{3}H]Ad 12 DNA generated by *Eco*RI were isolated and hybridized to the light virion DNA fragments. The results are shown in Fig. 6. It clearly shows that fragments II, III, and IV hybridize to ^{3}H-labeled *Eco*RI fragments C and D. Fragment V hybridizes with ^{3}H-labeled frag-

TABLE 1. *Distribution of radioactivity in the Ad 12 DNA fragments generated with EcoRI*

Fragment	Size of fragment (% of genome)	Relative amt of radioactivity[a]	
		Complete virion DNA	Light virion DNA
I	46.0		5.5
A	35.6	35.3	33.4
B	27.4	25.8	22.9
II	23.3		1.8
III	20.8		6.4
IV	19.0		3.5
C	16.4	17.7	10.2
D	11.5	11.2	7.4
V	8.5		0.4
E	7.5	7.3	6.6

[a] Determined by microdensitometer tracing of a fluorograph and integration of peak areas.

ment C only. These fragments must originate near the left-hand end of the genome. Fragment I hybridizes to ^{3}H-labeled *Eco*RI fragment A, and probably to fragment B. It does not appear to hybridize with fragment C, D, or E. Thus its origin may be from the right-hand end.

Since radioactivity is a good measure of the amount of DNA present in each fragment (Table 1, column 3), it is feasible to quantitate the proportion of the molecules with a particular deletion. This is summarized in Table 2 for molecules with left-hand deletions. The radioactivity associated with different classes of molecules have been corrected for the amount deleted to calculate the number of molecules in that class. About 48% of the molecules have left-hand end deletions, with the majority having deletions encompassing the junction of fragments C and D.

Biological functions of light virions. We reported earlier that the light virions had a reduced plaquing efficiency on KB cells (19). Host range mutants of Ad 5 can plaque on HEK or human cells transformed by Ad 5 DNA (293 cells), but not on HeLa cells (12). Thus, it is important to test if the light virions behave as host range mutants. Table 3 shows the plaquing efficiency of light and heavy virions on HEK 293 cells as well as KB cells. Data indicate that the plaquing efficiency of the light virions is about 5 to 14% that of the complete virions. These data agree with those reported earlier for plaquing of defective virions on KB cells. The small fraction of plaque formers is probably due to the incomplete separation of the two virion bands.

We have shown earlier that these light virions can induce an early antigen (T-antigen). We examined whether these virions can also synthesize late viral structural polypeptides (V-antigens) by the immunofluorescence technique. Figure 7 shows the relationship between the multiplicity of infection and the fraction of cells that survive that particular viral function. Within the range of multiplicities used, the curves follow single-hit kinetics (20, 25). From these graphs, the number of virions added per cell to give a 63% positive immunofluorescence (on the average one hit per cell) is shown in Table 3 for T- and V-antigen for both complete and light virions. It can be seen that light virions can induce T- and V-antigens as efficiently as the complete virions. The data are consistent with the conclusion that virions capable of inducing T-antigens are also capable of inducing viral structural antigens.

Tumorigenicity of the virions was tested in newborn hamsters and is shown in Table 4. The data indicate that the tumorigenicity of the light virions are retained.

DISCUSSION

Several investigators have studied DNA from incomplete virions having different buoyant densities (7, 32, 33). They found that the DNA is generally heterogeneous in size. Tibbetts suggests that the defectives are the result of damaged assembly intermediates (31). Daniell postulates that the defective DNA arises from mistakes in DNA replication (7).

Niiyama (21) showed that DNA from bovine Ad 3 virions with a density of 0.002 g/ml less than that of the complete virions has a specific deletion near one end of the molecule. We have shown in an earlier publication that Ad 12 virions having a buoyant density of 0.003 g/ml less than that of complete virions are defective in some biological functions, such as plaque formation (19). In this study, we have shown that the defective virions contain shorter DNA molecules and that a high proportion (50%) of the defective viral DNA has a deletion near the left-hand end of the viral genome. Garon et al. (9) have observed DNA molecules with a deletion loop mapping at approximately 0.14 molecular lengths from one end, when denatured Ad 12 DNA was renatured. Similar observations were made by Tibbetts (quoted by Garon et al.). However, the orientation of the deletion(s) was not determined.

The heteroduplex DNA molecules with single-stranded loops must be the product of complete virion DNA and defective virion DNA since contour length measurements show that the length of the double-stranded regions equals the contour length of defective virion DNA and the sum of the lengths of single-stranded and double-stranded regions equals that of complete vir-

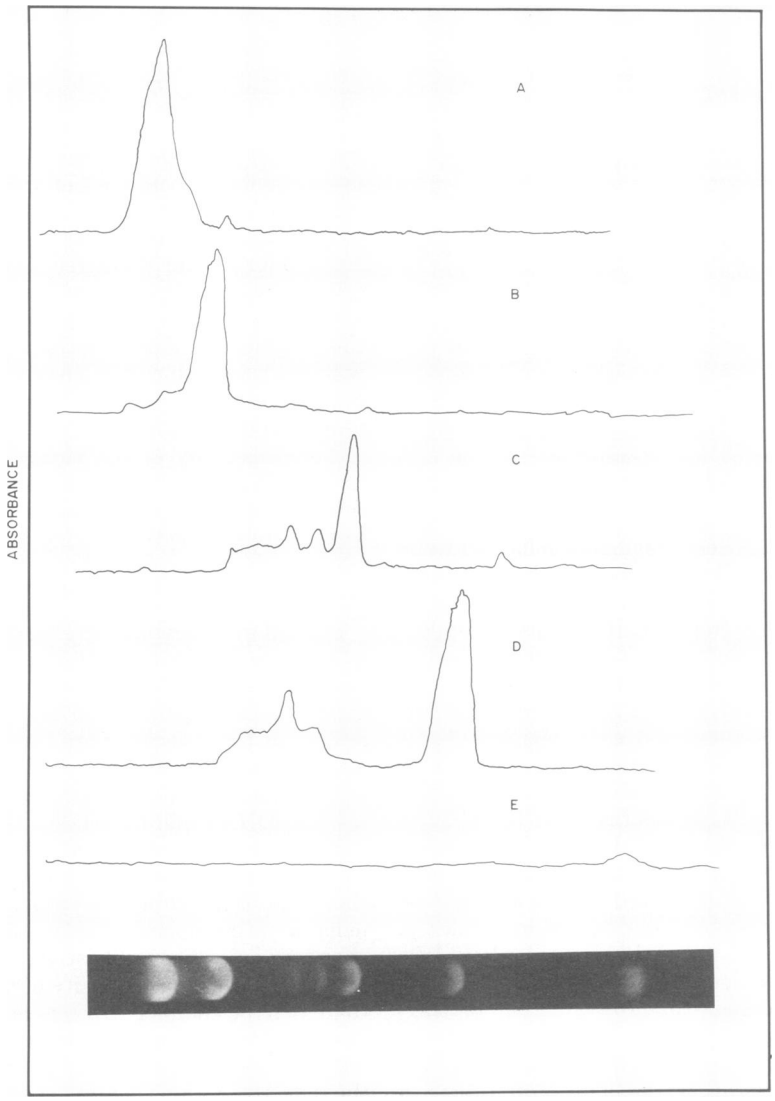


FIG. 6. *Hybridization of ^{3}H-labeled DNA restriction fragments to light virion DNA fragments generated by EcoRI. ^{3}H-labeled Ad 12 DNA was restricted with EcoRI and the fragments were isolated after agarose gel electrophoresis. Each ^{3}H-labeled fragment was hybridized to nitrocellulose strips containing light virion DNA fragments generated by EcoRI. Hybridized radioactivity was detected by fluorography (as described in Materials and Methods). Microdensitometer scans of the resulting exposed film are shown. [^{3}H]Ad 12 DNAs used in the hybridization are fragments A through E starting from the top. Photograph of light virion DNA fragments stained with ethidium bromide is shown at the bottom of the graph.*

ion DNA (data not shown). Furthermore, renaturation of complete and defective virion DNA alone produced no molecules with single-stranded loops. These observations suggest that the deletions must be from one specific end.

Among the light virion DNA molecules, three to four discrete classes with specific deletions can be identified (Table 2; Fig. 6). These deletions centered around 16% of the left-hand end of the genome, one of the *Eco*RI restriction endonuclease cleavage sites. The heteroduplex data shown in Fig. 2 are consistent with this conclusion. These four classes of molecules account for approximately 49% of the DNA molecules. The remaining 51% of the DNA molecules may be the result of contamination of the light virion band by complete virions, or these molecules have deletions at locations other than that near the *Eco*RI C-D junction, or a combination of both. We feel that not all 51% of the molecules

TABLE 2. *Summary of Ad 12 DNA molecules with deletions*

Fragment	Location of deletion in fragments[a]	Length of deletion (% of genome)	Relative amt of fragment[b]	Corrected relative amt of fragment[c]	Molecules in that class (% of total)
II	C and D	4.6	1.8	2.2	6
III	C and D	7.1	6.4	8.6	25
IV	C and D	8.9	3.5	5.1	15
V	C	7.8	0.4	0.8	2
C		0	10.2	10.2	51
D		0	7.4	7.4	

[a] Letters denote fragments of Ad 12 DNA generated by *Eco*RI.

[b] Quantitated by microdensitometer tracing of a fluorograph and integration of the peak areas.

[c] Column 4 was multiplied by a correction factor (length of fragments C plus D/length of that particular fragment) to give values in column 5.

TABLE 3. *Plaque formation and V-antigen and T-antigen induction by complete and light virions*

Virion band	Plaque formation (virions/PFU)[a]			V-antigen induction (VAU)[b]	T-antigen induction (TAU)[b]
	KB	HEK	293		
Complete	3×10^3	2×10^3	2×10^3	560	140
Light	3×10^4	1.4×10^4	1×10^4	760	210

[a] The number of virions was determined by optical density measurements at 260 nm (OD_{260}). One $OD_{260} = 4.4 \times 10^{11}$ virions per ml. PFU, Plaque-forming units.

[b] More than 1,000 cells in infected cultures were examined for immunofluorescence. One VAU and TAU are defined as the number of virions per cell added to induce V-antigen or T-antigen, respectively, in 63% of the cells.

are the contaminating full-length molecules for the following reasons. (i) When light virion preparations were centrifuged in a CsCl density gradient, no virion band was visible at the position of the gradient where the complete virion would be banded. (ii) In seven separate experiments the number of PFU per optical density of virion from the light band was always less than 15% of that from the complete virion band. (iii) Viral DNA molecules lacking restriction sites were detected by restriction enzyme cleavage and gel electrophoresis (Fig. 4). Heteroduplex molecules showing single-stranded regions not located near the left-hand end of the molecule were observed (Fig. 2C and D) when light virion DNA and complete virion DNA were renatured. (iv) Statistical analysis of the data shown in Fig. 1 suggests that the light virion DNA preparation contains less than 25% full-length DNA molecules, if the true average deletion is 7.2%. The latter value was based on data shown in Table 2.

From these considerations, it can be concluded that the light virions were not grossly contaminated with complete virions and that deletions in these defective DNA molecules are not homogeneous. The presence of fragments C and D after *Eco*RI digestion can be accounted for partly by contamination with complete DNA molecules and partly by molecules having deletions in locations other than the *Eco*RI C-D junction.

The class or classes of defective viral DNA having deletions near the left-hand end reported here are probably the results of mistakes made during replication. A possible mechanism is depicted in Fig. 8. During adenovirus DNA replication, single-stranded molecules have been found (23) and probably are created by strand displacement (17, 29). The segments of DNA represented by heavy bars in Fig. 8 can be

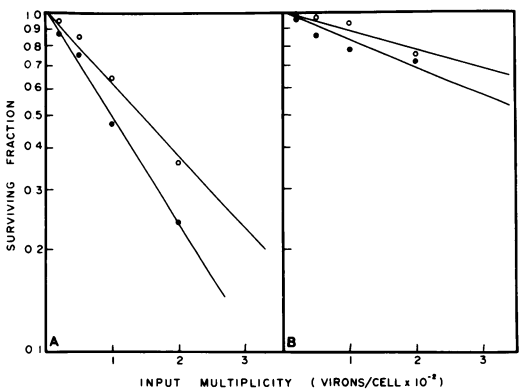


FIG. 7. *Induction of T- and V-antigens by complete and light virions. Fraction of cells without T- or V-antigen is plotted against the multiplicity of infection. This plot allows a quantitative comparison of the efficiency of T- and V-antigen induction by different virus preparations (see text). (A) T-antigen; (B) V-antigen. ●, Complete virions; ○, light virions.*

TABLE 4. *Tumorigenicity of complete and light virions*[a]

Virus band	Virus dose (virions/animal)	Tumor incidence[b]
Heavy	1.5×10^9	6/18 (75%)
	1.5×10^8	14/19 (74%)
	1.5×10^7	1/9 (11%)
Light	1.5×10^9	14/18 (78%)
	1.5×10^8	3/6 (50%)
	1.5×10^7	4/8 (50%)

[a] Newborn Syrian hamsters were injected subcutaneously with purified virions and observed for 4 months.

[b] Ratio of animals with tumors to total number of animals injected.

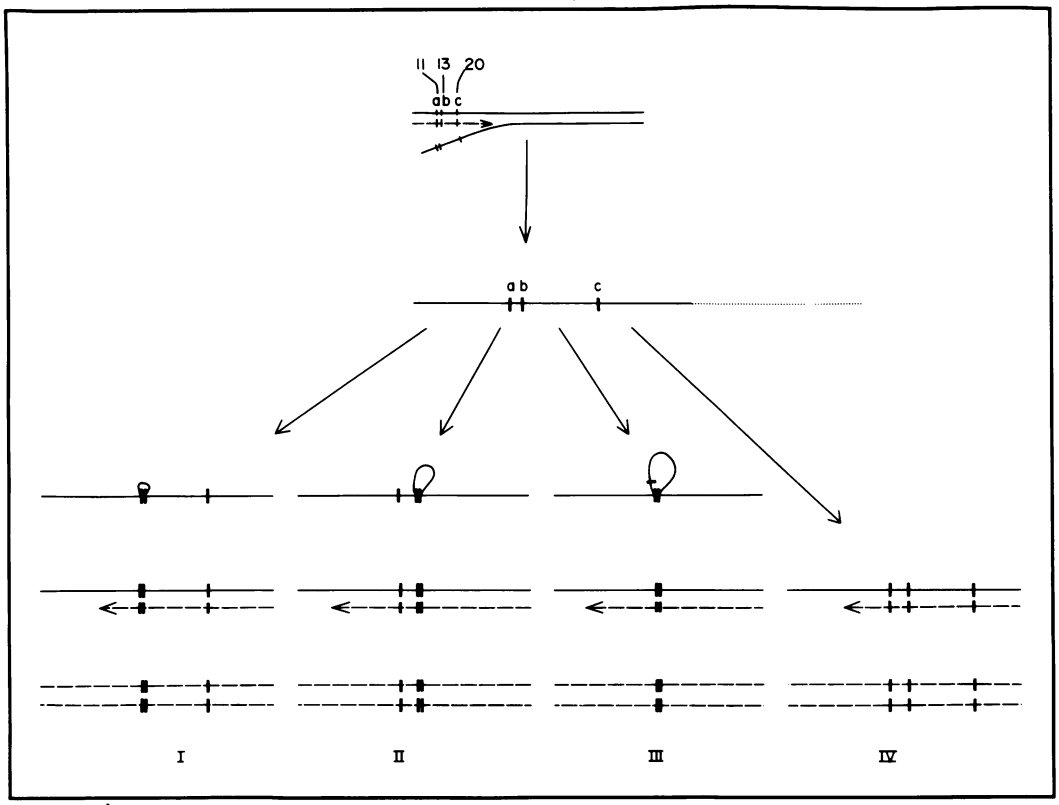


FIG. 8. *Model for the generation of light virion DNA with deletions near the left hand end of the molecule. Regions a, b, and c can be brought together to allow a looping out of a segment of Ad 12 DNA. Subsequent replication of this "looped out" molecule can give rise to DNA molecules having specific deletions. Molecules I, II, III, and IV should result in defective DNA molecules with deletions of 2, 7, 9, and 0%, respectively. The molecule depicted on the top is a full Ad 12 DNA. Only the left hand end is shown in the other molecules.*

brought together by looping out a segment of the DNA. The newly replicated DNA molecule would thus contain a deletion. The length and position of the deletion depend on the specific DNA segments brought together. By using three blocks, as illustrated in Fig. 8, we can account for two of the major classes of defective DNA molecules observed. The deletion (2%) in the class I molecules shown in Fig. 8 may be too small to be detected. The specific DNA segments have been placed at approximate positions 11, 13, and 20 for the following reasons. The spacing of the blocks can account for molecules with 7 and 9% deletions. The deletions must contain about equal lengths of fragments C and D since these fragments are reduced by similar amounts in the light virion DNA (Table 2). The placement of these DNA sequences at approximate positions 13, 20, 22 is also compatible with the observed data.

Recently, it has been reported that Ad 2 mRNA's have leader sequences derived from different regions of the genome. These sequences map approximately at positions 17, 20, and 29 from the left-hand end of the Ad 2 genome (4, 6). It has been postulated that segments of precursor RNA loop out to allow the joining of separated RNA regions to form the leader sequences (15). This mechanism implies that the DNA should possess sequences in the same map positions such that looping out of the DNA is possible. Intrastrand base pairing would facilitate the bringing together of different DNA segments to create loops. Tibbetts et al. reported that several complementary sites may exist in the single-stranded Ad 2 DNA since they observed that fragmented single-stranded DNA was retained on hydroxyapatite in 0.12 M phosphate at 65°C (32). It is reasonable to assume that such DNA sequences also exist in the Ad 12 genome to allow looping out of sequences. It should be noted that the segments of DNA postulated to be brought together to generate the defective DNA molecules map at positions 11, 13, and 20. If these are also involved in the biogenesis of Ad 12 mRNA, the leader sequences

should map at these positions, which are slightly different from those in Ad 2.

Another possible explanation is that there exist homologous or semihomologous regions at the positions discussed above. The pairing of these regions can give rise to unequal crossing over, resulting in one DNA molecule with a deletion and the other with an addition. Although we have not observed that virions have higher buoyant density than complete virions, others have reported such virions in adenovirus (5).

We have determined that these defective virions can synthesize early and late antigens with efficiency similar to that of the complete virions. These results suggest that the deleted regions in the viral genome are not essential for late gene expression. This is reasonable for the 50% of the defective genomes with identifiable deletions since the deleted regions code for late mRNA (27). In Ad 2, the corresponding region (7.5 to 17% from the left-hand end) codes for polypeptides IX and IVa_2, two minor structural polypeptides (1). In Ad 12 genes coding for these polypeptides are deleted, it is expected that the defective virions are incapable of plaque formation. It has been found that less than 16% of the left-hand end of Ad 2 is sufficient to cause malignant transformation of rat cells (35; Mak et al., Virology, in press). Thus, it is not surprising that these defective virions can cause tumors in newborn rodents, since the left 16% of the genome is included in the majority of the virions.

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