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Characterization of Adenovirus DNA in KB Cells Infected with Adenovirus Type 12

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In KB cells infected with adenovirus type 12, the newly synthesized viral DNA contains molecules of different sizes. Sedimentation studies show that fragments of about 0.5, 0.25 and 0.1 of the whole genome are formed, besides molecules of normal length. The fragments have the same average base composition and hybridization characteristics as parental DNA, which suggest that they represent the whole genome.

The degree of degradation of type 12 parental and progeny DNA is not influenced by the multiplicity of infection ranging from 1000 to 5000 physical particles per cell. Coinfection of cells with adenovirus types 5 and 12 reveals that, in contrast to *in vitro* experiments, type 5 DNA is not affected by type 12 endonuclease. These results indicate that the structural viral components do not freely interact during uncoating and fragmentation.

INTRODUCTION

After infection of KB cells with adenovirus type 5 (Ad 5) or type 12 (Ad 12) the fate of the parental DNA of both types is rather different. Ad 12 parental DNA is partially degraded, while Ad 5 DNA remains intact (Sussenbach, 1971). Degradation of Ad 12 DNA may be performed by an endonuclease present in Ad 12 virions (Burlingham and Doerfler, 1971). No endonuclease activity is observed in Ad 5 virions, which may explain the absence of degradation of parental Ad 5 DNA *in vivo* (Sussenbach, 1971).

New viral DNA, synthesized in Ad 5- or Ad 12-infected cells shows the same difference in size distribution as parental DNA.

In Ad 5-infected cells new DNA has the same length as parental DNA. However, in the Ad 12 system, besides molecules of normal size, also new DNA fragments are observed, which are probably synthesized on parental DNA fragments as templates (Sussenbach, 1971).

This paper concerns the characterization of the newly synthesized Ad 12 fragments in the nucleus and the role of compartmentation on the fragmentation of parental Ad 12 DNA.

METHODS

The growth of KB cells, the purification of adenovirus types 5 and 12, the preparation of purified adenovirus DNA from virions, the infection conditions and the preparation of nuclei have been described earlier (Sussenbach, 1971). For the preparation of viruses containing labeled DNA, 100 μCi [6-^{3}H]-thymidine (10 Ci/mmole), 200 μCi NaH_2 [$^{32}PO_4$] (10 Ci/mg) or 20 μCi [2-^{14}C]thymine (60 mCi/mmole) were added to 3×10^7 infected cells in 100 ml culture medium. The newly synthesized viral DNA was isolated following a slightly modified Hirt procedure (Hirt, 1967).

Nuclei were suspended in 0.01 *M* Tris, 0.01 *M* EDTA pH 8.1 and sodium dodecyl sulfate was added to a concentration of 0.06 %. After 30 min NaCl was added to 1 *M*. The suspension was dialyzed for 4 hr against 0.01 *M* Tris, 0.01 *M* EDTA, 1 *M* NaCl pH 8.1 at 4° and subsequently for 16 hr against SSC (0.15 *M* NaCl–0.015 *M* trisodium citrate pH 7.5). The dialyzed suspension was

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centrifuged for 10 min at 8000*g*. Under these conditions 80–100 % of the labeled DNA was extracted.

Viral DNA was analyzed by sucrose gradient centrifugation in isokinetic sucrose gradients containing 0.2 *M* NaCl, 0.01 *M* Tris pH 8.1, 0.001 *M* EDTA and 0.1 % sarkosyl in the Spinco SW 41 rotor at 37,000 rpm for 4.5 hr at 15°.

DNA–DNA hybridization was performed in 2 × SSC containing 50 % formamide (Okanishi and Gregory, 1970). [^{14}C] Ad 12 (0.25 μg) or KB DNA was immobilized on MF 30 membrane filters (Sartorius, Göttingen, Germany).

[^{3}H]DNA was sonicated to fragments of 200,000 ± 30,000 daltons MW and denatured for 10 min at 100° in 0.1 × SSC. Varying concentrations of sonicated denatured [^{3}H]DNA were incubated with the filters for 16 hr at 37°. After hybridization the filters were washed with 200 ml 2 × SSC, dried and counted. Annealing experiments carried out at 66° in the absence of formamide gave identical results (Aloni, Winocour, Sachs and Torten, 1969). Sarkosyl (sodium *N*-lauroylsarcosinate) was obtained from Geigy, Basel, Switzerland.

RESULTS

a. Characterization of fragmented DNA. Infection of KB cells with Ad 12 leads to the synthesis of normal and fragmented viral DNA with sedimentation coefficients of 30 S and about 20 S, respectively. In some experiments also smaller fragments have been observed for new as well as for parental DNA. This phenomenon does not depend on the viral dose (see under *b*) but is probably influenced by the physiological state of the cells. From previous experiments it may be concluded that these fragments are synthesized on fragmented parental templates (Sussenbach, 1971).

To characterize new DNA, cells were infected with Ad 12 and 15–18 hr post infection (p.i.) viral DNA was labeled by addition of ^{3}H-labeled thymidine. At 18 hr p.i. DNA was extracted as described under Methods and centrifuged on a linear sucrose gradient 5–20 % w/v (Fig. 1).

The radioactivity distribution reveals that

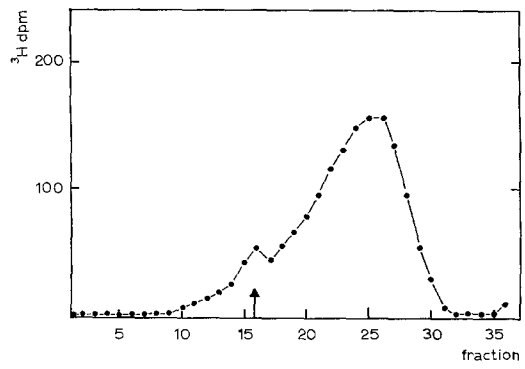

FIG. 1. Sucrose gradient centrifugation in 5–20% sucrose, 0.01 *M* Tris pH 8.1, 0.001 *M* EDTA and 0.1% sodium dodecyl sulfate of new viral DNA synthesized 15–18 hr p.i. in nuclei of infected KB cells. Centrifugation was performed in the Spinco SW25 rotor for 6 hr at 24,000 rpm at 15°. In all figures concerning sucrose gradients the position of ^{14}C-labeled Ad 12 DNA (30 S) is marked by an arrow. The direction of sedimentation is from right to left.

in this particular experiment almost 90 % of the new DNA sedimented slower than the marker as a broad peak of 20 S. Each fraction of this peak (fractions 16–30) was separately recentrifuged in a Spinco SW 41 rotor on a isokinetic sucrose gradient which allows a good resolution. These centrifugations showed that the 20 S peak consisted of molecules of three distinctive size classes with sedimentation coefficients of 24, 18 and 13 S, respectively (Fig. 2). It can be calculated (Studier, 1965) that these three classes represent molecules of 0.5, 0.25 and 0.1 of the whole genome, respectively.

The 24, 18 and 13 S molecules were analyzed by CsCl density centrifugation. All new DNA banded at the same position in the gradient as normal parental DNA, although the width of the peaks increased with decreasing size of the fragments (Fig. 3).

To detect whether the whole viral genome is present in the 20 S peak, 20 S fragments or intact 30 S molecules, both ^{3}H-labeled, were hybridized to parental [^{14}C]Ad 12 DNA under conditions of excess of [^{3}H]DNA in solution. At every concentration tested, the same hybridization percentage was reached for both types of DNA with a maximum of

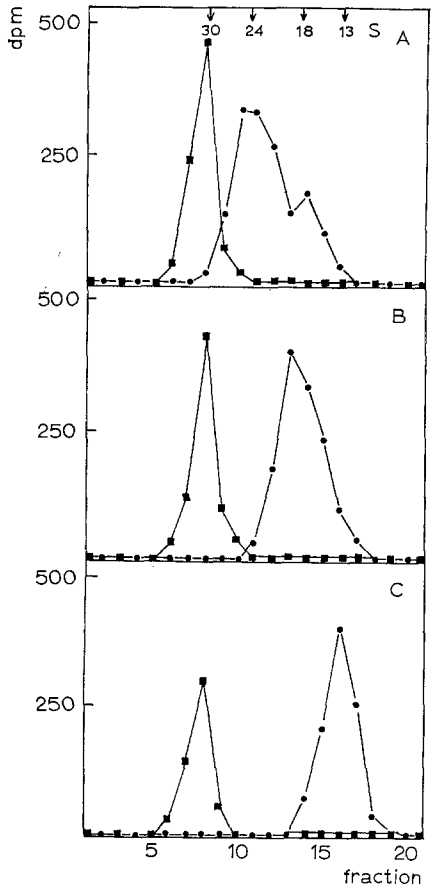

FIG. 2. Sucrose gradient centrifugation in isokinetic sucrose gradients containing 0.2 *M* NaCl, 0.01 *M* Tris pH 8.1, 0.001 *M* EDTA and 0.1% sarkosyl of viral DNA (●) present in fraction 19 (A), 23 (B) and 28 (C) of Fig. 1. Centrifugation of each of fractions 16–30 of Fig. 1 showed that no other size classes were present than those indicated in this figure. 24 S molecules were found in fractions 16–19, 18 S in fractions 19–24 and 13 S in fractions 24–30. [^{14}C]Ad 12 DNA was used as 30 S marker (■). Centrifugation was performed in the Spinco SW41 rotor at 38,000 rpm for 4.5 hr at 15°.

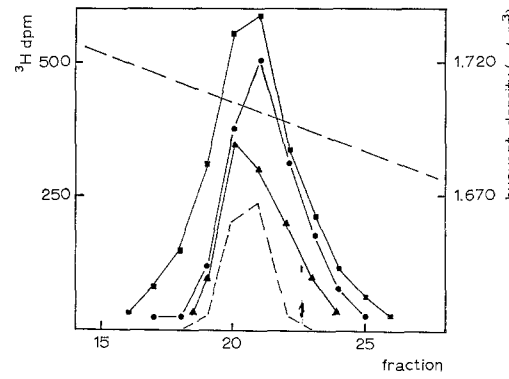

FIG. 3. CsCl equilibrium density centrifugation of newly synthesized viral DNA obtained as described in Fig. 1. The buoyant densities of DNA of fraction 19 (▲), 23 (●) and 28 (■) were compared to that of parental 30 S DNA (---). The density of the DNA-containing solutions was adjusted to 1.700 g/cm^3 prior to centrifugation. Samples were centrifuged at 38,000 rpm for 70 hr at 10° in the Spinco rotor R 50.

30% for a fourfold excess (Table 1). No hybridization was observed with KB DNA.

b. On the process of fragmentation. Burlingham *et al.* have shown that the pentons of Ad 2 and Ad 12 contain endonuclease activity (Burlingham and Doerfler, 1971; Burlingham, Doerfler, Petterson and Philipson, 1971). The enzyme can be released from the virion by degrading the particles by aging. In contrast to Ad 2 and Ad 12, no endonuclease activity has been observed for Ad 5 (Sussenbach, 1971). As has been shown by Burlingham *et al.* (1971), the endonuclease of Ad 2 attacks at GC-rich regions and is able to affect DNA obtained from a variety of sources, e.g., Ad 12 DNA. We were interested whether Ad 5 DNA could be degraded by Ad 12 endonuclease. Ad 12 and Ad 5 virions containing ^{3}H- and ^{32}P-labeled DNA, respectively (3×10^{10} particles each per milliliter) were aged as described earlier for 2 days at 37° (Sussenbach, 1971). Viral DNA was isolated and analysis on isokinetic sucrose gradients showed that *in vitro* Ad 5 DNA and Ad 12 DNA were both degraded by endonuclease of Ad 12 into fragments ranging from 30 to 9 S.

To investigate whether breakdown of Ad 5 DNA can also occur *in vivo*, a coinfection experiment was performed. Cells were infected during 15 hr, either with Ad 5 only or with Ad 5 and Ad 12 together. The parental DNA of Ad 5 contained ^{32}P, while Ad 12 DNA was labeled with [^{3}H] thymidine. Since about 6000 particles of each type penetrated into the cells, coinfection of individual cells was very likely. After infection DNA was extracted from the nuclei and analyzed. In this particular experiment degradation of Ad 12 DNA was very extensive, while paren-

TABLE 1

HYBRIDIZATION OF NEW LOW MOLECULAR WEIGHT DNA[a]

[3H]DNA in solution	Concentration (μg/ml)	DNA on filter	Label in hybrid (dpm)		% Hybridization
			3H	14C	
Ad 12	0.25	Ad 12	1253	97	12.1
Ad 12	0.50	Ad 12	1919	95	19.0
Ad 12	1.00	Ad 12	2938	96	28.8
Ad 12	0.50	KB	5	—	0.1
New	0.25	Ad 12	936	102	8.5
New	0.50	Ad 12	1983	105	17.7
New	1.00	Ad 12	3209	107	27.8
New	0.50	KB	20	—	0.2

[a] Ad 12 DNA or low molecular weight DNA, synthesized 15–18 hr p.i. in nuclei of infected KB cells was hybridized to viral or cellular DNA. KB DNA (0.25 μg) or [14C]Ad 12 DNA (0.25 μg, 465 dpm/μg) was immobilized on membrane filters and incubated with sonicated denatured viral [3H]DNA (50,000 dpm/μg) isolated from virions or with newly synthesized [3H]DNA (50,000 dpm/μg) with sedimentation characteristics as shown in Fig. 1. More than 90% of total new DNA sedimented in the 20 S peak. Incubation was performed in closed vials for 16 hr at 37° in 1 ml 2 × SSC containing 50% formamide. Each vial contained two DNA filters and one filter without DNA. The radioactivity of the latter, being always less than 0.5% of the input, was subtracted. The hybridization percentage was calculated from the $^3H/^{14}C$ ratio in the hybrid, thus correcting for loss of [14C]DNA during incubation.

tal Ad 5 DNA was not affected in absence or presence of Ad 12 (Fig. 4). This observation provides information on a possible compartmentation of the viral constituents or a tight binding of the endonuclease to the inoculum DNA of Ad 12. These aspects were also studied by comparing the degree of degradation of viral parental and new DNA present in nuclei of cells, which had been infected with different doses of Ad 12. Cells were infected with Ad 12 containing [3H]DNA. The multiplicities of infection were 1000, 2000 and 5000 physical particles/cell, respectively. After infection nuclei were prepared and DNA was extracted and analyzed (Fig. 5). No considerable difference in degradation was observed.

The same results were obtained for new viral DNA synthesized 15–18 hr p.i. under the conditions mentioned above. Apparently the fragmentation is not influenced by the multiplicity of infection (m.o.i.) ranging from 1000 to 5000 physical particles per cell.

DISCUSSION AND GENERAL CONCLUSIONS

Progeny DNA, synthesized in Ad 12-infected cells consists of molecules of normal length and fragments. These fragments can be separated by sucrose gradient centrifugation in three distinctive size classes with sedimentation coefficients of about 0.5, 0.25 and 0.1 of the whole genome size, respectively. Analysis of parental Ad 12 present in the nucleus shows the same species of molecules. In contrast to Burlingham and Doerfler (1971) we observed no viral DNA integrated into cellular DNA and sedimenting > 45 S. These observations indicate that the Ad 12 endonuclease causes double-stranded breaks at distinctive positions on the parental molecule. The same phenomenon has been observed for the Ad 2 endonuclease (Burlingham *et al.*, 1971).

The Ad 2 enzyme attacks Ad 2 DNA at GC-rich regions, which can be visualized by electron microscopy of partially denatured DNA (Doerfler and Kleinschmidt, 1970). This leads to fragmentation of Ad 2 DNA into segments of about 0.25 of the whole genome.

The denaturation map of Ad 12 DNA shows that G-C and A-T base pairs are distributed rather homogeneously and no extended GC-rich regions can be detected. (Doerfler and Kleinschmidt, 1970).

Therefore it is surprising that, in spite of this observation, distinctive breaks are produced, which are located at about the same positions as in Ad 2 DNA. It suggests that the involved GC-regions are rather small or that Ad 12 endonuclease has a different specificity.

CsCl equilibrium density centrifugation of the different species of Ad 12 DNA shows that there is no striking difference in average base composition.

This is in accordance with the rather uniform distribution of the different bases as has been concluded from denaturation maps of Ad 12 DNA. Therefore, separation of the

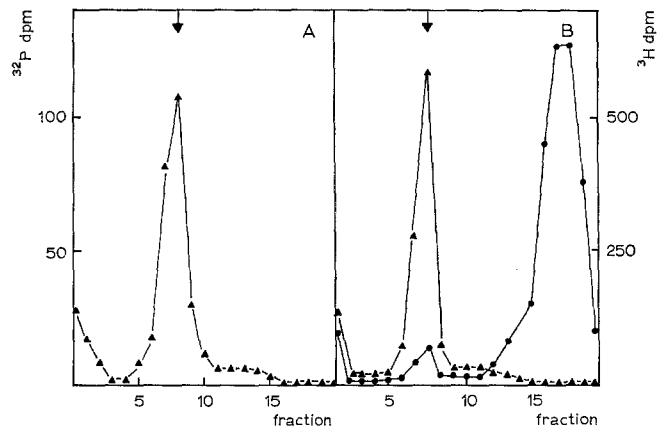

FIG. 4. Sucrose gradient centrifugation in isokinetic sucrose gradients containing 0.2 *M* NaCl, 0.01 *M* Tris pH 8.1, 0.001 *M* EDTA and 0.1% sarkosyl of parental Ad 5 and Ad 12 DNA obtained from cells infected with Ad 5 only (A) or coinfected with Ad 5 (▲) and Ad 12 (●) (B). Centrifugation was performed in the Spinco SW 41 rotor at 38,000 rpm for 4.5 hr at 15°.

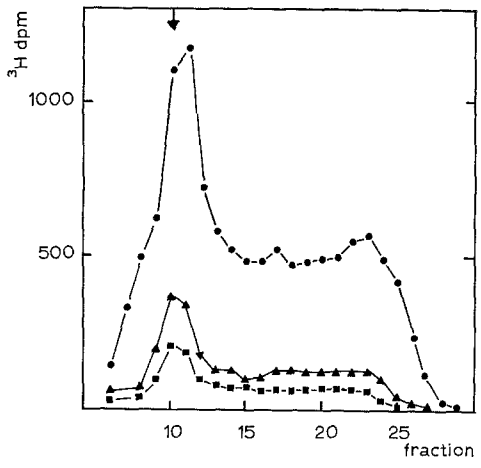

FIG. 5. Sucrose gradient centrifugation in isokinetic sucrose gradients containing 0.2 *M* NaCl, 0.01 *M* Tris pH 8.1, 0.001 *M* EDTA and 0.1% sarkosyl of parental Ad 12 DNA isolated from cells infected with a m.o.i. of 1000 (■), 2000 (▲) and 5000 (●) physical particles/cell, respectively. Centrifugation was performed at 38,000 rpm for 4.5 hr at 15° in the Spinco SW 41 rotor.

fragments based on difference in buoyant density is impossible in contrast to Ad 2 fragments, which have been separated by CsCl density centrifugation (Kimes and Green, 1970).

The hybridization of new Ad 12 fragments with parental Ad 12 DNA confirms the viral origin of this DNA. Although we did not reach the saturation level the similarities in hybridization kinetics and the similar buoyant densities of parental DNA and "20 S" fragments suggest that all genes are represented in the fragments. This makes it unlikely that only fragments containing a specific region are able to replicate and indicates that no specific origin might be needed for replication of Ad 12 DNA. It is interesting that, although low molecular weight molecules are formed in Ad 12-infected cells, no fragment-containing virions have been found. However, recently Mak (1971) observed the existence of defective Ad 12 virions, which perhaps may contain low molecular weight DNA.

We have already pointed out the difference in fate of parental Ad 5 and Ad 12 DNA. Ad 12 DNA is partially degraded, while Ad 5 is not fragmented. This difference can be correlated with the presence or absence of a viral endonuclease. Since Ad 5 DNA can be attacked by the Ad 12 endonuclease, we investigated the fate of Ad 5 DNA in cells which were coinfected with Ad 12 at high m.o.i., making coinfection of individual cells very likely. Our experiments reveal that even in the coinfected cells Ad 5 DNA is not degraded. Since, in the same experiment Ad 12 DNA is extensively fragmented, this indicates that Ad 12 endonuclease does not freely move through the cell, but is restricted in its action. This could be due to a difference in

location in the cell between Ad 5 and Ad 12 during uncoating (compartmentation). This view is in agreement with the observation that the degree of fragmentation of parental Ad 12 DNA and of progeny DNA is not influenced by the multiplicity of infection over the range from 1000 to 5000 physical particles/cell. Although it is hard to predict the effect of varying enzyme and viral DNA concentrations in the cell, it should be possible if free diffusion of enzyme and substrate should occur that increasing concentrations might lead to increased fragmentation. However if some kind of compartmentation is involved no influence on the degree of fragmentation is expected. Our results may be explained by assuming that after penetration the Ad 12 endonuclease has a chance to degrade only Ad 12 DNA molecules, which are located in the same phagocytic vacuole. This compartmentation may also endure in some way in the nucleus. In cells coinfected with Ad 5 and Ad 12, Ad 5 DNA is not attacked probably since they are located in different phagocytic vacuoles. Another explanation may be that Ad 5 DNA is protected in some way by cellular or viral components.

It is not yet clear, whether the difference in fate of Ad 5 and Ad 12 DNA must be correlated with the different ways of penetration, which have been described by Chardonnet and Dales (1970). Anyhow it seems probable that the aspect of fragmentation of viral DNA may shed new light on compartmentation during uncoating of adenovirus.

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