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A unique subgenomic species of adenovirus 2 DNA generated under high multiplicities of infection

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ABSTRACT

We have identified a novel subgenomic viral DNA in KB cells infected with adenovirus 2 (Ad2) under high multiplicities of infection. KB cells were infected with Ad2 at multiplicities of infection greater than 100 PFU/cell. ^{32}P-labeled viral DNA was selectively extracted by a modification of the method of Hirt (8) from the infected cells and analyzed by electrophoresis on agarose gels. In addition to full-length DNA (22 to 23 x 10^6 daltons), a unique subgenomic DNA species of about 12 to 13% (2.6 x 10^6 daltons) of full-length DNA in size was found in the infected cells. This subgenomic DNA was found to be double stranded and was not packaged inside the virus particles. This DNA could be isolated in large amounts (30 to 50% of total viral DNA) from infected cells. When cleaved with restriction endonuclease KpnI, the subgenomic DNA yielded two fragments, each corresponding to about 6% and 7% of the full-length genome in size.

INTRODUCTION

When human adenoviruses are purified from infected human cells by banding in a buoyant CsCl gradient (5) about 5 to 15% of the virus particles band at lower densities than complete virions. These particles have been described as "incomplete" or "immature" virions which may represent intermediates in the assembly of complete virions (3,12,13,14,15,17,24,27). Generation of incomplete particles has been reported to be independent of the multiplicity of infection (3,17). However, it has been shown that specific deletions of Ad2 (26), Ad12 (13,29) and bovine adenovirus type 3 (11) are generated under high multiplicity passage of the virus.

The DNA extracted from incomplete particles is shorter than full-length virion DNA (1,2,3,24). Analysis of the subgenomic DNA extracted form incomplete particles of adenovirus 2 (Ad2), Ad3,and Ad7 after cleavage with restriction endonucleases revealed that the sequences from the left end of the viral genome are preferentially present (3,24). A model has been proposed to account for this observation based on the selectivity of packaging of viral DNA; i.e., the left end of the viral genome gets

packaged preferentially over the right end sequences (24). The subgenomic DNA from incomplete particles has been shown to contain unique structural features such as extended inverted terminal repetitions (3). In some cases it has also been shown that incomplete particles contained DNAs of predominantly cellular origin (23).

Recently, discrete size classes of subgenomic DNA have been isolated from the nuclei of adenovirus infected cells (4). These subgenomic species of DNA contain sequences from both termini and are generated in a manner that is independent of multiplicity of infection (MOI). The presence in the unpackaged subgenomic DNA species of both molecular ends which serve as origins (10,22,28) and as termini of viral DNA replication (19,21,25) suggests that these subgenomic DNA species may contain the cis acting elements of viral DNA replication.

In the present study we describe a novel and unique subgenomic species of viral DNA generated in KB cells infected with Ad2 under high multiplicities of infection. This subgenomic species represents about 12 to 13% of the Ad2 genome in length and could be isolated in large amounts (30 to 50% of total viral DNA) from cells infected under high MOI. This species of DNA is shown to include sequences from both termini of the viral genome.

MATERIALS AND METHODS

Cells and virus

KB cells were cultured in monolayers using Eagle's minimal essential medium (MEM) supplemented with 10% calf serum or in suspension using Joklik's modified MEM supplemented with 5% horse serum. Ad2 (strain, Adenoid 6) was obtained from M. Green. Virus stocks were propagated on KB cells grown in suspension.

Infection of cells, labeling, and isolation of DNA

Monolayers of KB cells were infected with virus at MOI ranging from 10 to 1000 PFU/cell as described in the text. Normally 1 x 10^6 cells contained in a 60 mm dish were infected with 0.25 ml of virus in MEM supplemented with 2% calf serum. One hr after addition of virus, 5.0 ml of the above medium was added to each dish and was replaced with 2 ml of phosphate-free media at 20 hr after infection. The cells were labeled from 20 to 36 hr postinfection with 250 μCi/ml carrier-free $H_3{}^{32}PO_4$. At the end of the labeling period the medium was removed and the viral DNA was selectively extracted by the method of Hirt (8) with modifications. Cells were lysed with 0.6% SDS in 10 mM Tris-Cl (pH 7.4), and 10 mM EDTA. The lysate was

treated with 500 μg/ml Pronase at 37°C for 1 hr. High molecular weight cellular DNA was precipitated with 1 M NaCl at 4° overnight. The viral DNA was collected by centrifugation at 12,000 rpm for 20 min in a Sorvall (SS-34 rotor) centrifuge. The supernatant containing the viral DNA was further purified by extraction with phenol and a mixture of chloroform and isoamyl alcohol (24:1).

Purification of the subgenomic species of DNA by sucrose gradient centrifugation

^{32}P-labeled DNA from the Hirt supernatant was phenol extracted and concentrated by ethanol precipitation. The DNA was layered onto a 5 to 22% neutral sucrose gradient containing 0.01 M Tris-Cl (pH 7.4), 0.01 M Na_2-EDTA and 1 M NaCl, and centrifuged at 24,000 rpm in a Beckman SW 41 rotor at 15°C for 15 hr. Fractions were collected from the bottom of the tubes and aliquots of each fraction were spotted on Whatman DE-81 filter discs. The filters were washed four times with a solution of 5% Na_2HPO_4, once with distilled water, twice with ethanol, and once with acetone and were dried and counted using a toluene based scintillation fluid. Fractions containing the subgenomic DNA species (Fig. 3, II) were pooled, dialyzed and concentrated by ethanol precipitation.

Gel electrophoresis, "blotting" and hybridization

Electrophoresis of viral DNA was carried out in 0.6% or 1% agarose slab gels at 40V for 10 to 14 hr. For blotting, the DNA fragments in the agarose gels were denatured and transferred to nitrocellulose sheets by the method of Southern (20). Hybridization of ^{32}P-labeled DNA with DNA fragments immobilized on nitrocellulose sheets was carried out as described by Wold et al. (30).

RESULTS

Effect of multiplicity of infection on the generation of subgenomic species of Ad2 DNA

Ad- DNA labeled with ^{32}P during late stages of infection (20 to 30 hr postinfection) of KB cells could be selectively extracted from infected cells by the method of Hirt (8) with modifications as described under "Materials and Methods." Using this method we analyzed the effect of different MOI on the generation of subgenomic species of Ad2 DNA. As illustrated in Fig. 1, when KB cells were infected at multiplicies of greater than 100 PFU/cell, a unique subgenomic species of DNA about 12 to 13% of the full-length Ad2 DNA could be seen that was absent from DNA

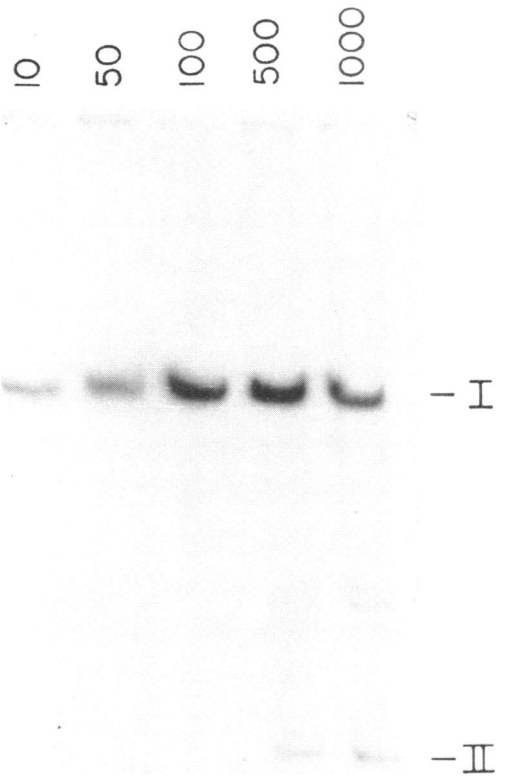

Fig. 1. Gel electrophoresis of Hirt supernatant DNA extracted from KB cells infected with Ad2 at different MOIs. Infected cells were labeled with 250 μCi/ml of carrier-free $H_3{}^{32}PO_4$ from 20 to 36 hr postinfection. Viral DNA was selectively extracted from the infected cells by a modification of the method of Hirt (8) as described under "Materials and Methods." The DNA was electrophoresed in 0.6% agarose gel for 10 hr at 40 V and the gel was autoradiographed. The numbers at the top represent MOI. I. Full-length DNA; II. Subgenomic DNA.

extracted from cells infected at multiplicities of infection 10 to 100 (Fig. 1, band II). In initial experiments, the DNA-nature of this subgenomic species was determined by DNase sensitivity and by alkali-resistance (results not shown). Apart from this major species of subgenomic DNA (Fig. 1, band II) which in different experiments represented 30 to 50% of the total radioactivity recovered in the Hirt supernatant,

another minor species migrating between the full-length DNA (Fig. 1, band I) and subgenomic DNA species II was also seen in most instances. In the present study, we have not studied this minor subgenomic DNA species.

Comparison of the size of the subgenomic DNA with the DNA fragments of Ad2 generated by restriction endonucleases BamHI and EcoRI indicated that the size of the subgenomic DNA was about 12 to 13% of full-length genomic DNA or 2.6 x 10^6 daltons. Fig. 2 illustrates the size of the subgenomic DNA in comparison with restriction fragments of full-length DNA generated

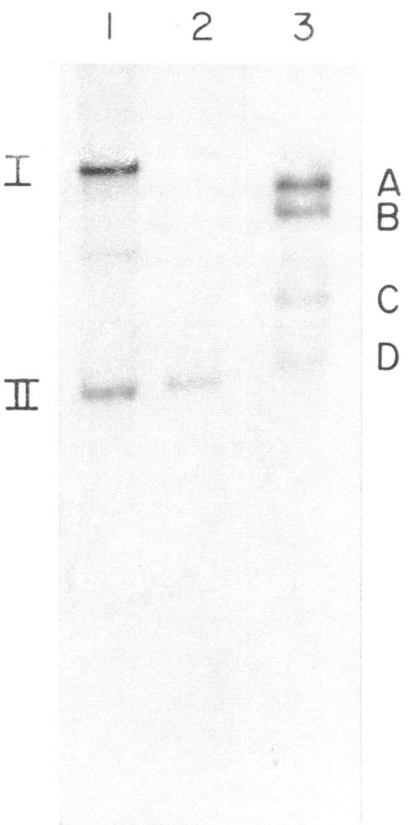

Fig. 2. Autoradiogram of ^{32}P-labeled restriction fragments of Ad2 full-length DNA and subgenomic DNA. DNA was electrophoresed in 0.6% agarose gel and the the gel was autoradiographed. 1, Hirt supernatant DNA from KB cells infected with Ad2 at an MOI of 500 PFU/cell; 2, subgenomic DNA purified through sucrose gradient centrifugation; 3, fragments of Ad2 full-length DNA generated by digestion with restriction endonuclease BamHI. I. Full-length DNA; II. Subgenomic DNA. The MW estimates were obtained from the published BamHI cleavage map (18).

by restriction endonuclease BamHI. In neutral sucrose gradients, the subgenomic DNA sedimented as a molecule corresponding to about 14S compared to the 31S full-length DNA (6) (Fig. 3). This subgenomic species of Ad2 DNA was seen under high MOI (>100 PFU/cell) using four different stocks of Ad2 prepared after plaque purification.

Structure of the subgenomic DNA

The subgenomic DNA species could be separated from the full-length DNA in a 5 to 22% sucrose density gradient (Fig. 3). The ^{32}P-labeled subgenomic DNA species was purified by banding in two consecutive sucrose gradient centrifugations and its homology to the Ad2 genome was determined by hybridization to the unlabeled DNA fragments generated by restriction endonuclease KpnI that had been immobilized on nitrocellulose membrane sheets (20). As shown in Fig. 4, the subgenomic DNA species hybridized to fragments A, B, F, and G. B and G fragments are derived from the left 23% of Ad2 genome whereas A and F fragments are derived from the right 29% of the genome (Fig. 4). The two terminal fragments F and G represent 6.5% and 6.1% of the genome from the right and left ends, respectively. Considering

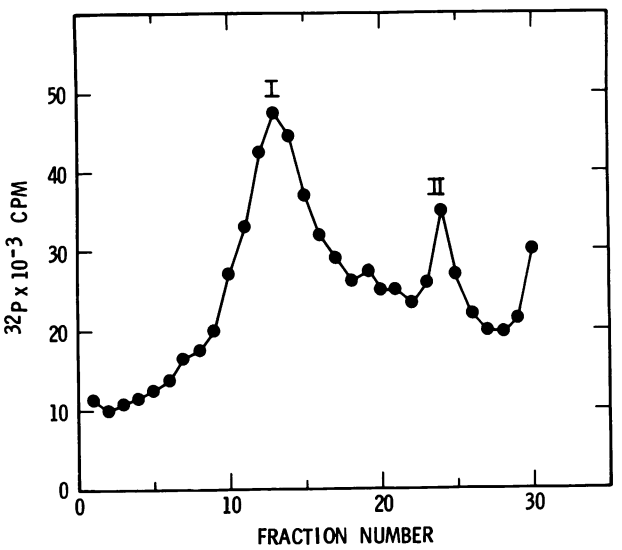

Fig. 3. Velocity sedimentation of viral DNA in sucrose density gradient. ^{32}P-labeled DNA from the Hirt supernatant was layered onto 5 to 22% neutral sucrose gradient [in 0.01 M Tris-Cl (pH 7.4), 0.01 M Na_2-EDTA, and 1 M NaCl] and centrifuged at 24,000 rpm in Beckmann SW 41 rotor at 15°C for 15 hr. Fractions were collected and aliquots of the fractions were counted for radioactivity after adsorbing onto DE-81 filters (see Materials and Methods). I. Full-length DNA; II. Subgenomic DNA.

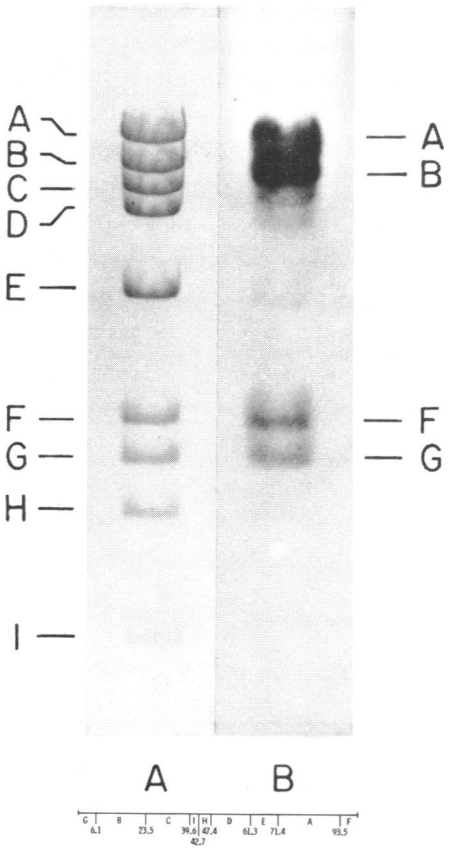

Fig. 4. Hybridization of the ^{32}P-labeled subgenomic DNA to the restriction endonuclease KpnI-generated unlabeled fragments of Ad2 DNA. Two μg of Ad2 DNA was digested with restriction endonuclease KpnI and was electrophoresed in 1.0% agarose gel. The gel was stained with EtBr and photographed. The DNA was denatured, transferred to nitrocellulose sheet by the method of Southern (20) hybridized with ^{32}P-labeled subgenomic DNA and autoradiographed using Kodak x-ray film (XR-5). A. EtBr staining of Ad2 DNA digested with KpnI. B. Autoradiogram of the nitrocellulose transfer hybridized to the ^{32}P-labeled subgenomic DNA.

The cleavage map of KpnI on Ad2 DNA is shown at the bottom of the figure. This cleavage map was constructed by workers at Cold Spring Harbor Laboratories and published at EMBO Ad-Workshop, 1978.

the size of the subgenomic DNA species (12 to 13% of the full-length genome), the hybridization to the A and B fragments should be partial, i.e., to the sequences neighboring the F and G fragments. In addition to the above four fragments small amounts of hybridization were also observed

to C and D fragments. This may be due to contamination of the full-length DNA or the minor subgenomic DNA.

Further attempts were made to elucidate the structure of the subgenomic DNA by cleavage with restriction endonucleases KpnI, HindIII, and XbaI. Cleavage with HindIII always yielded incomplete digestion where only small amounts of radioactive DNA migrated to positions corresponding to fragments F and/or G (Fig. 5, lanes 2 and 3). The G fragment is derived from map position 0 to 7.5 (left end of the genome) whereas F fragment is derived from map position 89.5 to 97.3 (near the right end of the genome) (17) and both fragments migrate together in agarose gels. It is not known at present whether the partial digestion fragment corresponds to the F or to the G fragments (or both). It was also observed that no significant radioactivity was found at the position corresponding to HindIII-K fragment

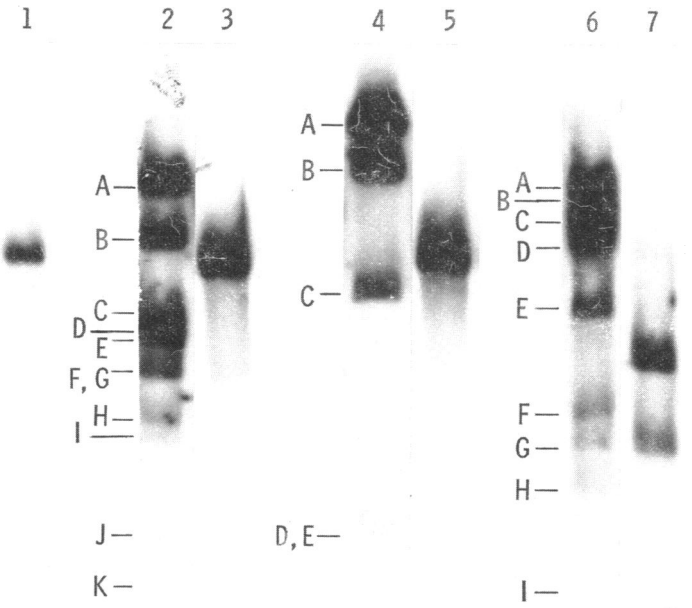

Fig. 5. Cleavage of subgenomic DNA with restriction endonucleases. ^{32}P-labeled full-length DNA and subgenomic DNA were extracted from KB cells infected with Ad2 and were purified through neutral sucrose gradients. The DNA was digested with restriction endonucleases for 2 hr at 37°C, electrophoresed in 1% agarose gel for 10 hr at 40 V, and the gel was autoradiographed. 1, Undigested subgenomic DNA; 2, full-length DNA digested with HindIII; 3, subgenomic DNA digested with HindIII; 4, full-length DNA digested with XbaI; 5, subgenomic DNA digested with XbaI; 6, full-length DNA digested with KpnI; 7, subgenomic DNA digested with KpnI.

which is derived from map position 97.3 to 100 (right end of the genome). Digestion with XbaI, which has a cleavage site located within the left 4.0% of the Ad2-genome (based on a map constructed by workers at Cold Spring Harbor Laboratories published at the EMBO Ad-workshop, 1978), did not yield any cleavage product (Fig. 5, lanes 4 and 5). The reason for this is not known. Possibly, the subgenomic DNA has lost the HindIII and XbaI cleavage sites, or else has some other unique structure that prevents these two enzymes from recognizing these cleavage sites. It is clear that the DNA is susceptible to restriction endonucleases because cleavage with KpnI resulted in two fragments, one of which co-migrated with G fragment (left 6.1% of the genome) and another corresponding to 7.0% of the genome (Fig. 5, lanes 6 and 7). Considering the resistance of the subgenomic DNA to cleavage with restriction endonucleases HindIII and XbaI, we tested whether it is completely double stranded DNA by incubation of purified subgenomic DNA with single-strand specific nuclease S-1. The native subgenomic DNA was resistant to S-1 treatment, whereas the denatured subgenomic DNA was completely degraded indicating that the subgenomic DNA is indeed double stranded (data not shown).

Subgenomic DNA is not packaged into viral particles

To see whether the subgenomic DNA species is packaged into viral particles, KB cells were infected with Ad2 at an MOI of 500 PFU/cell and ^{32}P-labeled virus was prepared essentially as described (5). ^{32}P-labeled virus was centrifuged in CsCl equilibrium density gradients and fractions were collected from the bottom of the centrifuge tube. All the fractions were dialyzed against 10 mM Tris-Cl (pH 7.4) containing 1 mM EDTA, treated with 1 mg/ml Pronase, electrophoresed on a 1% agarose gel, and autoradiographed. ^{32}P-labeled DNA was observed from virus particles banding at a density of 1.34 g/cc which corresponded to full-length DNA in addition to some very minor subgenomic DNA from particles banding at lower densities; no subgenomic DNA species corresponding to band II in Fig. 1 were observed indicating that it is not packaged within the viral particles (results not shown).

DISCUSSION

We have shown that a unique subgenomic viral DNA species is generated in KB cells infected with Ad2 under high MOI (>100 PFU/cell). This novel viral subgenomic DNA species is about 12 to 13% of the full-length genome in size, is produced in large amounts (30 to 50% of the production of

full-length DNA), and contains sequences homologous to both molecular ends of the genome. The subgenomic DNA species is not packaged into virus particles, is double stranded and has a single cleavage site for restriction endonuclease KpnI. This unique subgenomic DNA species was observed with a number of different stocks of Ad2 prepared after plaque purification. This DNA cannot be artifacts of the isolation method such as specific breakage of full-length DNA, because such an artifact would not contain both terminal sequences and a unique cleavage pattern with restriction endonuclease KpnI.

Daniell and Mullenbach (4) have described a number of discrete size classes (separated on agarose gels) of subgenomic DNA species that contain both the molecular ends of the Ad3 genome, and that were isolated from the nuclei of HeLa cells infected at MOIs of 0.2 to 20 PFU/cell. It is not certain whether any of their subgenomic DNA species correspond to the species that we have observed, but this seems unlikely because they used MOIs of less 20 PFU/cell, and our subgenomic species was not detectable unless MOIs of greater than 100 were used.

How this unique subgenomic DNA species is generated is not clear. Presumably, it results from errors in adenovirus DNA replication. It is believed that both molecular ends of the viral genome serve as origins and as termini for viral DNA replication (10,19,21,22,25,28). The subgenomic DNA reported here contains both ends and therefore once having been formed might have the cis acting signals for replication. We have also observed that a subgenomic DNA of almost identical molecular weight (on agarose gels) is as produced in KB cells infected with Ad7 which belongs to a different subgroup [group B (7)]. The Ad7-subgenomic DNA hybridized to the terminal fragments of Ad7 DNA and did not cross hybridize to Ad2 (unpublished observation). If the subgenomic DNA is generated by an error in replication (3), this error may be sequence specific considering the unique size of the subgenomic DNA. Much more work is needed before we attempt to propose a model for the generation of the subgenomic DNA.

It is rather surprising that this subgenomic DNA is not cleaved by HindIII and XbaI, because the blotting analysis indicated that the subgenomic DNA includes the left and right 6% of the viral genome, and therefore should contain cleavage sites for HindIII and XbaI. It is possible that novel nucleotide sequences are created during the generation of this molecule or some other unique structure prevents these enzymes from recognizing their respective cleavage sites. It is interesting to note

that the subgenomic DNA has a single cleavage site for restriction endonuclease KpnI and may be useful as a cloning vector for KpnI-generated fragments of foreign DNA.

A complete nucleotide sequence analysis of this subgenomic DNA and comparison with the termini of full-length DNA may provide several insights into adenovirus DNA replication. The subgenomic DNA will possibly offer the opportunity to isolate the *cis* acting elements of DNA replication and to study these components in detail. We are currently investigating whether the subgenomic DNA species has protein(s) covalently attached to the 5' termini (16), and can replicate autonomously.

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