Discrete Subgenomic DNA Fragments in Incomplete Particles of Adenovirus Type 2

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SUMMARY

Discrete subgenomic DNA fragments were found in three out of thirty-two preparations of adenovirus type 2 incomplete particles grown in human Hep-2 cells and examined over the course of 1 year. One preparation contained three fragments corresponding to 5, 14 and 19% of the genome, another contained a 37% fragment and the third a 40% fragment. Each fragment hybridized exclusively to the left end of the genome. Digestion of the nick-translated 37% fragment with HindIII confirmed that it contained the left 37.3% of the genome. Synthesis of these fragments was not dependent on high input multiplicity of infection. Comparable fragments were not found in unpackaged DNA from the corresponding infected cells. This is consistent with the hypothesis that such fragments are generated during virus assembly or, alternatively, may reflect the very small proportion of these fragments relative to the pool of unpackaged DNA within the cells. The possibility that they are generated by errors in DNA replication is discussed.

INTRODUCTION

Incomplete particles (IP) of adenovirus have a lighter buoyant density than virions because they are either empty shells or contain pieces of DNA shorter than genome length and, thus, can be separated by centrifugation in density gradients. IP containing subgenomic DNA have been reported for a subgroup A adenovirus serotype 12 (Ad12) (Burlingham et al., 1974), subgroup B serotypes 3, 7 and 16 (Prage et al., 1972; Daniell, 1976; Hammarskjold et al., 1977; Tibbetts, 1977; Hammarskjold & Winberg, 1980) and subgroup C serotype 2 (Burlingham et al., 1974; Daniell, 1976; Khittoo & Weber, 1981). Group B adenoviruses produce large quantities of incomplete particles and analysis of the DNA by gel electrophoresis has shown that it is more heterogeneous in size than the DNA from IP of Ad2 (Daniell, 1976; Tibbetts, 1977; Hammarskjold & Winberg, 1980). Restriction endonuclease analysis and blot hybridization experiments have shown that subgenomic DNA from IP originates from the left end of the genome (Daniell, 1976; Tibbetts, 1977; Hammarskjold & Winberg, 1980; Khittoo & Weber, 1981), whereas DNA from cells infected with Ad2, Ad3 or Ad16 mapped to both left and right ends of the genome (Hammarskjold & Winberg, 1980; Daniell & Mullenbach, 1978; Rajagopalan & Chinnadurai, 1979). This suggested that a sequence at the left end of the genome may be involved in encapsidation. It was subsequently demonstrated, using spontaneous reduplication mutants, that packaging of Ad16 DNA may be dependent on a sequence located 290 to 390 base pairs from the left terminus (Hammarskjold & Winberg, 1980).

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A unique DNA fragment was isolated from Ad2-infected KB cells but was not found in virus particles (Rajagopalan & Chinnadurai, 1979). In our laboratory, analysis of the DNA from IP of Ad2 grown in human Hep-2 cells revealed the presence of discrete subgenomic fragments in some preparations. These fragments were purified and characterized and shown to be different from the species reported previously.

METHODS

Cells and virus. Ad2 and ts4 (an assembly mutant which produces large quantities of IP at 39 °C) were grown in Hep-2 cells as described previously (Khittoo & Weber, 1977). For experiments in which progeny virus DNA was examined for the presence of subgenomic fragments, the cells were infected at 10 to 200 p.f.u./cell.

Purification of DNA from incomplete particles. IP were released from infected cells by six cycles of freeze–thawing. The cell lysates were clarified by low-speed centrifugation, layered on to CsCl gradients (1.2 to 1.5 g/ml) and centrifuged at 100 000 g for 2 h, and in some cases for 16 h. The IP band was collected with a Pasteur pipette and included material between the virion and IP bands (i.e. density less than 1.34 g/ml). In some experiments, the gradients were fractionated through a hole pierced in the bottom of the tube. Fractions above the virion band and including the major IP band were treated individually or were pooled for the extraction of DNA. Samples were diluted with 10 mM-Tris–HCl pH 7-4, 1 mM-EDTA, dialysed overnight against the same buffer, and then deproteinized with 0.5% SDS and endonuclease-free Pronase (Calbiochem, 1 mg/ml) for 1 h at 37 °C. After extraction with phenol (saturated with 100 mM-Tris/HCl pH 7-9) and chloroform–isoamyl alcohol (24:1), the DNA was lyophilized, resuspended in a small volume and precipitated in 70% ethanol containing 0.3 M-sodium acetate and 10 mM-MgCl2 at −70 °C for several hours.

Restriction endonuclease digestion. Restriction enzymes EcoRI, HpaI, HindIII and BamHI were purchased from Bethesda Research Laboratories (Rockville, Md., U.S.A.) and were used according to the conditions specified by the manufacturer.

Isolation of DNA from infected cells. Freeze–thaw extracts of infected cells were layered on to CsCl gradients (1.2 to 1.5 g/ml) and centrifuged at 100 000 g for 2 h. Unpackaged DNA was purified from pooled fractions above the band of incomplete particles (buoyant density 1.30 g/ml) and was centrifuged on a 5 to 20% sucrose gradient (in 50 mM-Tris pH 7.5, 20 mM-EDTA, 100 mM-NaCl) at 180 000 g for 4 h. DNA was precipitated from each fraction and analysed on a 0.7% agarose gel. Intracellular DNA from infected cells was also isolated by the Hirt (1967) method or by a modification of the Hirt method (Rajagopalan & Chinnadurai, 1979) or by Pronase digestion of nuclei as described previously (Daniell & Mullenbach, 1978). DNA was extracted from micrococcal nuclease digests of infected nuclei as described in an earlier report (Brown & Weber, 1980).

Extraction of DNA fragments from agarose gels. Fragments were cut from either low-melting agarose (Bethesda Research Laboratories) or from Seakem agarose (Marine Colloids) gels following electrophoresis in Tris–acetate buffer (40 mM-tris–HCl pH 7.6, 5 mM-sodium acetate, 1 mM-EDTA). Phosphate buffers were not suitable since the phosphate was found to precipitate during extraction of the DNA. To extract the DNA from low-melting agarose, the gel slice was melted at 65 °C, 2 vol. electrophoresis buffer were added and the aqueous phase was extracted with phenol followed by chloroform–isoamyl alcohol. To extract DNA from Seakem agarose, the gel slice was placed in a small dialysis bag containing a minimal volume of Tris–acetate buffer and electrophoresed at 50 V for 3 h on a horizontal gel apparatus.

DNA transfer and filter hybridization. DNA was transferred from agarose gels to diazobenzylxoyxymethyl (DBM) paper (Schleicher & Schuell, Keene, N.H., U.S.A.) or to nitrocellulose filters (Schleicher & Schuell) as described by Wahl et al. (1979). Probe DNA
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was labelled with $^{32}$P by nick-translation using ($\alpha$-$^{32}$P)dATP and ($\alpha$-$^{32}$P)dCTP (2000 to 3000 Ci/mmol, Amersham Corp.) in 20 $\mu$l nick-translation buffer (50 mM-Tris–HCl pH 7.4, 10 mM-2-mercaptoethanol, 5 mM-MgCl$_2$, 50 $\mu$g/ml bovine serum albumin) containing 25 $\mu$m-dGTP, 25 $\mu$m-dTTP and 1 unit DNA polymerase I (Boehringer-Mannheim). For later experiments, DNA polymerase I free of endogenous endonuclease activity was obtained from New England Nuclear. The reaction mixture was incubated at 14 °C for 1 h and then extracted with chloroform. Two vol. ethanol were added and the labelled DNA was precipitated at $-70$ °C for at least 1 h. Specific activity of the probes was consistently $1 \times 10^8$ to $5 \times 10^8$ ct/min/µg. SDS (0.2 %) was included in the pre-hybridization and hybridization solutions to decrease non-specific adsorption of the probe to the filter.

RESULTS

Isolation of subgenomic fragments

IP DNA was analysed on agarose gels and three preparations were found to contain discrete subgenomic fragments. One preparation of ts4 IP DNA contained three discrete fragments, 19, 14 and 5 % of the Ad genome, relative to the migration of marker fragments on gels stained with ethidium bromide (Fig. 1 a). Another ts4 IP DNA preparation contained a 37% fragment (Fig. 1 b) and DNA from wild-type (wt) IPs included a discrete 40% fragment (Fig. 1 c). In each case, the input multiplicity was 10 and the incubation temperature was 33 °C.

Mapping of the fragments

The discrete fragments were extracted from the gel, labelled with $^{32}$P by nick-translation and hybridized to restriction endonuclease fragments of Ad2 DNA blotted on to DBM or nitrocellulose filters (Fig. 2). Each of the discrete fragments hybridized exclusively to restriction fragments from the left end of the genome. The hybridization data, summarized in Fig. 3, showed that the 5% fragment mapped within the left 7.9%, the 14% and 19% fragments within the left 17%, and the 37% and 40% fragments within the left 37.3% of the genome. Digestion of the nick-translated 37% fragment with BamHI and with HindIII yielded BamHI B and HindIII B, C, G and I, corresponding to the left 29.0% and 37.3% of the genome respectively (Fig. 4). BamHI D (29-0 to 42-0) was not evident in the digest. A fragment of approx. 8%, which would be expected, was not observed even after longer exposure. The reason for this technical anomaly is unknown. The results confirm the left end origin of the discrete fragments and the HindIII data suggest that the 37% fragment contains the entire left end of the genome as far as coordinate 37.

Failure to isolate discrete subgenomic fragments from infected cells

To determine whether these fragments were synthesized in infected cells or were generated during virus assembly, unpackaged intracellular DNA from those preparations which contained discrete fragments in the IPs was examined. We found that the most efficient source of such DNA was the portion of the preformed CsCl gradient above the IP after a 2 h centrifugation, as described in Methods for the purification of IP. The entire contents of the tube above the major IP band were pooled and the DNA was purified for analysis by sedimentation through a 5 to 20% sucrose gradient. Fractions were collected and the DNA in each fraction was examined by agarose gel electrophoresis. No discrete fragments were evident (results not shown).

DNA from infected cells was also examined on sucrose gradients following extraction by the Hirt method (Rajagopalan & Chinnadurai, 1979; Hirt, 1967) and by Pronase digestion of infected nuclei (Daniell & Mullenbach, 1978). Again, no discrete fragments were observed
Fig. 1. Gel analysis of IP DNA. The *ts*4 and wt IP were purified and the DNA extracted as described in Methods. (a) *ts*4 IP (lane 2); (b) *ts*4 IP (lane 1); (c) wt IP (lane 2). Marker fragments were formed from digests of wt Ad2 DNA using the following restriction endonucleases: (a) *HpaI* (lane 1); (b) *EcoRI* (lane 2), *HpaI* (lane 3), *HindIII* (lane 4) and *BamHI* (lane 5); (c) *HpaI* (lane 1). Discrete subgenomic fragments are indicated (.). The smear of small material at the bottom of lane 1 in (b) consists of fragments from the left end of the adenovirus genome, as identified previously (Khittoo & Weber, 1981). The numbers refer to the length of each fragment as a percentage of the Ad2 genome, where 1% is equivalent to 366 base pairs.

visually. Nevertheless, the DNA from discrete regions of the gel, corresponding to the fragment sizes observed in IPs, was eluted, labelled and hybridized to filters bearing standard Ad2 DNA restriction fragments. These probes hybridized to all regions of the genome (results not shown). We concluded that discrete subgenomic fragments could only be isolated from IPs.

The origin of subgenomic fragments

Discrete subgenomic fragments were found in only three IP DNA preparations out of thirty-two examined over a period of 1 year. They could not be induced by measures normally used in other virus systems to generate defective particles, such as high multiplicity of infection and undiluted serial passage. Virus stocks whose progeny IP contained discrete fragments failed to produce such fragments in subsequent infections.

Inapparent contamination of adenovirus stocks with adenovirus-associated virus (AAV) can give misleading results concerning discrete fragments. A 13% fragment was found in micrococcal nuclease digests and in Hirt extracts of cells infected with two adenovirus stocks which were later shown by electron microscopy to be contaminated with AAV. Because the encapsidated AAV genome is protected from micrococcal nuclease, this also provides for a very sensitive and rapid assay for AAV using agarose gel electrophoresis of the resistant DNA. When isolated from nuclei treated with micrococcal nuclease, the 13% fragment did not hybridize to Ad2 DNA. However, when the fragment was isolated from Hirt extracts without micrococcal nuclease treatment, hybridization to Ad2 restriction fragments was detected due to co-migration of subgenomic Ad2 DNA with the AAV fragment. The identity of the 13% band was confirmed as AAV DNA by restriction endonuclease patterns of the nick-translated DNA, which were consistent with published patterns (results not shown) for AAV-2.

DISCUSSION

The presence of subgenomic DNA fragments in particles with buoyant density between 1.30 and 1.34 g/ml in CsCl (Fig. 1c) is consistent with previous reports that the buoyant density of adenovirus particles is proportional to the size of the DNA contained within them.
Fig. 2. Hybridization of nick-translated subgenomic DNA fragments to blots of restriction endonuclease digests of wt Ad2 DNA. The restriction endonucleases used to cleave the wt DNA were: (a) EcoRI; (b) HindIII; (c) HpaI; (d) BamHI. Lane 1 in (a) to (d) was hybridized to nick-translated DNA prepared from purified virus. The length of each subgenomic fragment used as a probe (expressed as a percentage of the Ad2 genome) is as follows. (a) Lane 2, 40; lane 3, 19; lane 4, 14; lane 5, 5. (b, c) Lane 2, 40; lane 3, 37; lane 4, 19; lane 5, 14; lane 6, 5. (d) Lane 2, 40; lane 3, 37. The position of fragments A to J are shown (see also Fig. 3).
Fig. 3. Summary of hybridization data from Fig. 2. Hybridization of subgenomic DNA fragments to particular restriction fragments is indicated by a heavy black line. For each subgenomic fragment, the right-end limit of the region within which it maps is indicated by a vertical dotted line.

(Burlingham et al., 1974; Daniell, 1976; Hammarskjold et al., 1977; Tibbetts, 1977; Hammarskjold & Winberg, 1980).

In view of the findings of Hammarskjold & Winberg (1980), that packaging of Ad16 DNA is dependent on a sequence at the left end of the genome, it is not surprising that the discrete fragments from IP contain only left end sequences. Tibbetts (1977) and Khittoo & Weber (1981) suggested that IP-containing left end fragments may be assembly intermediates whose unprotected DNA was digested during purification, leaving the left end sequences protected within the capsid. An alternative theory is that subgenomic fragments are generated in infected cells from both ends of the genome and that the left end fragments are selectively packaged (Daniell & Mullenbach, 1978). A model for generation of such genomic fragments during DNA replication has been proposed by Bodnar & Pearson (1980) and a modified, updated version is outlined in Fig. 5. If the 37% fragment was generated in such a way, the terminal repeat must be small since restriction endonuclease digestion of the fragment showed that it contained the left 37.3% of the genome. The other fragments were not analysed in the same way because they were degraded during nick-translation by an endogenous endonuclease activity in the DNA polymerase. This did not affect their ability to hybridize.

The fact that discrete fragments were not found in DNA from infected cells is consistent with the hypothesis that such fragments are generated during virus assembly, but may also reflect the low proportion of such fragments relative to the total pool of unpackaged DNA.
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The latter explanation is supported by the fact that Daniell (1981) failed to detect subgenomic fragments of Ad2 DNA in infected human cells. However, when more sensitive detection methods were used, it was shown that DNA from Ad2-infected human cells contained sequences from both ends but not the middle of the virus genome (Rajagopalan & Chinnadurai, 1979). This provides evidence that subgenomic fragments of Ad2 DNA may be generated by errors in DNA replication (see Fig. 5; Bodnar & Pearson, 1980). However, such a mechanism would be expected to generate subgenomic fragments with a heterogeneous size distribution. It is possible that certain fragments are generated preferentially during replication, or that certain fragments are preferentially amplified within the cell. The 37% and 40% fragments reported here may in fact be the same; the size of the 40% fragment was determined relative to HpaI restriction fragments of Ad2 DNA, the largest of which is 29.1% and thus is only approximate. Interestingly, these two fragments (37% and 40%) appear to
Fig. 5. Model for generation of subgenomic DNA fragments. (1) Ad2 genome before replication. (2) Type I replication. (3) Single strand is displaced. (4) Type II replication. (5) Type I and type II replication on the same molecule; type I replication is faster than type II. (6) Single-stranded fragment is displaced. (7) Illegitimate base pairing and repair synthesis. (8) Subgenomic fragment is copied. (9) Double-stranded subgenomic fragment with origin of replication at both ends. O, 55,000 dalton terminal protein; ●, 87,000 dalton precursor to the terminal protein (Challberg et al., 1980). The model is based on the original proposal of J. Sambrook (Daniell, 1976) and incorporates recent developments (Bodnar & Pearson, 1980; Challberg et al., 1980).

be very similar to one of the subgenomic species from Ad2 IP reported by Daniell (1976). This is noteworthy since the 37% and 40% fragments reported here were from ts4 and wt preparations respectively, grown in Hep-2 cells, and the IP examined by Daniell (1976) were from a wt Ad2 preparation grown in HeLa cells. In our laboratory, discrete DNA fragments were found in only three out of thirty-two preparations examined and two of these contained fragments of approximately equal size. Similarly, Daniell (1981) reported that subgenomic fragments of Ad3 DNA were not always produced. Thus, it appears that although the generation and/or amplification of such fragments is under the control of factors which are not yet identified, the size of the fragment may be determined by the serotype of the virus.

In experiments designed to look for subgenomic fragments of Ad DNA, it is important that the Ad stocks be free of AAV. DNA preparations from cells infected with two of our Ad stocks contained a dominant 13% fragment similar to the 13% fragment from Ad2-infected cells reported by Rajagopalan & Chinnadurai (1979). In our laboratory, the 13% fragment was protected during micrococcal nuclease digestion of infected nuclei and it did not hybridize to Ad2 DNA. The two adenovirus stocks in question were subsequently shown by electron microscopy to contain AAV, and restriction endonuclease analysis of the nick-translated 13% fragment gave patterns characteristic of AAV type 2 genomic DNA. Hybridization to Ad2 DNA restriction fragments and/or restriction endonuclease analysis of the nick-translated fragment is not sufficient proof of adenovirus identity since subgenomic Ad
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DNA can co-migrate in sucrose gradients and in agarose gels with AAV genomic DNA. Consequently, a probe prepared from such a fragment may be a mixture of Ad and AAV DNA. In the light of results obtained in our laboratory, it is possible that the 13% fragment from Ad2-infected cells, reported by Rajagopalan & Chinnadurai (1979), is AAV DNA. This would be consistent with the atypical patterns obtained by restriction endonuclease digestion of the fragment and the fact that the fragment was not found in Ad particles (Rajagopalan & Chinnadurai, 1979).

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REFERENCES


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