The Nature of the DNA Associated with Incomplete Particles of Adenovirus Type 2

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SUMMARY

The nature of the DNA in incomplete particles (IP) synthesized by adenovirus type 2 and the ts4 mutant which accumulates such particles were analysed by agarose gel electrophoresis, restriction endonuclease cleavage and blot hybridization techniques. IP DNA consisted of a heterogeneous population of subgenomic-size DNA (IPSD1) and smaller molecules ranging from about 1000 base pairs to 200 base pairs (IPSD2). IPSD1 from ts4 was more heterogeneous than that from wild-type (wt), but both contained sequences from all parts of the viral genome. IPSD2 contained heterogeneous cellular sequences and viral sequences from the left 4.4% of the genome. An endonuclease activity associated with IP and virions was capable of digesting viral or cellular DNA to IPSD2-like fragments suggesting a possible origin for these molecules.

INTRODUCTION

Adenoviruses have been found to form incomplete particles (IP) that contain less DNA than mature virions. The diminished amount of DNA confers on these particles a lower buoyant density than the infectious particles, rendering them easily separable from complete virions by centrifugation in density gradients. The human adenoviruses for which IP have been described include serotypes 2, 3, 7, 12 and 16 (Prage et al., 1972; Burlingham et al., 1974; Daniell, 1976; Tibbetts, 1977; Wadell et al., 1973). Subgroup B (Ad3, Ad7, Ad16) seems to form the largest quantities of incomplete particles. IP from bovine Ad3 have a deletion near one end of the genome (Niöyama et al., 1975) while Ad12-IP have deletions mapping at 16% from the left end of the genome (Mak et al., 1979). Recently, Van Roy et al. (1979) isolated a specific deletion mutant of Ad2 with a buoyant density 0.0025 g/ml lower than mature virions. Unlike most IP of Ad2, this mutant can be enriched by high-multiplicity passages in human cells. This effect also holds in the case of Ad12 (Mak, 1971) and bovine Ad3 (Igarashi et al., 1975). In general, however, there is little or no influence on the quantity and composition of the incomplete particles when the type of cell or its nutrition are altered (Burlingham et al., 1974).

Many viruses assemble their capsids before the insertion of DNA (Casjens & King, 1975). Adenovirus IP have been under scrutiny as possible assembly intermediates. Several types of evidence support IP as precursors to mature virions: (i) pulse-chase experiments show that IP are synthesized before mature virions (Rosenwirth et al., 1974; Khittoo & Weber, 1977; Sundquist et al., 1973); (ii) IP contain precursor proteins (Ishibashi & Maizel, 1974; Winberg & Wadell, 1977; Khittoo & Weber, 1977); (iii) at least two types of particles exist, completely empty or containing DNA fragments, which may be possible intermediates during insertion of

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DNA (Edvardsson et al., 1976; Everitt et al., 1977; Tibbetts, 1977); (iv) H2ts4, H2ts112, H2ts101 and H5ts58 accumulate IP similar to those of wild-type (wt)-IP and block virus maturation (Khittoo & Weber, 1977; D'Halluin et al., 1978; Edvardsson et al., 1978; Boudin et al., 1980); (v) the production of IP is sensitive to arginine deprivation only prior to 20 h post-infection, while the assembly of complete virions is dependent on the continuous presence of arginine (Plaat & Weber, 1979).

Although not infectious, IP from Ad12 are capable of cell killing and induce T-antigen as efficiently as complete virions (Rainbow & Mak, 1970). IP from Ad12 have the equivalent ability of complete virions to induce transformation in primary hamster cells (Schaller & Yohn, 1974) and to induce tumours in newborn hamsters (Mak et al., 1979). These biological properties are peculiar to Ad12-IP, which are deletion mutants, and do not necessarily characterize other IP.

Most studies indicate that IP contain fragments of DNA rather than the complete genome (Prage et al., 1972; Wadell et al., 1973; Burlingham et al., 1974). Restriction endonuclease analysis, end labelling or heteroduplex mapping revealed that IP of Ad3 contain an abundance of the left end of the adenovirus genome (Daniell, 1976). The analysis of DNA from IP of Ad7 revealed that sequences of DNA extending from the left end of the viral genome map by variable lengths towards the right end in proportion to the density of IP (Tibbetts, 1977). Tjia et al. (1977) found that the bulk of the DNA from Ad2-IP is of cellular origin. Two-step filter hybridization studies suggest that some of the viral DNA may be covalently linked to host DNA. Hybridization studies have also shown the presence of host-specific sequences in the IP of Ad16 (Hammarskjold et al., 1977).

We have previously described H2ts4 (ts4), a mutant which at the non-permissive temperature seems to be blocked in the production of mature virions, resulting in the accumulation of large quantities of IP (Khittoo & Weber, 1977). In this study we examine the nature of the DNA present in IP of ts4 and wt. We describe for the first time, a population of small molecules of DNA (IPSD2) specifically associated with IP which contain almost exclusively sequences from the left end of the genome.

**METHODS**

**Cells and virus.** Monolayers of Hep-2 cells were propagated in 9 cm Falcon plastic Petri dishes using Dulbecco's modified essential medium (DMEM) as described previously (Weber, 1976). The wt-Ad2 used in these experiments was the parental strain of ts4 (Khittoo & Weber, 1977). All infections were performed with the same stock of ts4 of third passage (at 33 °C). The multiplicity of infection was maintained at about 10.

**Virus purification.** Unless otherwise stated, all cells and viruses were grown at 39 °C, the non-permissive temperature for ts4 (Khittoo & Weber, 1977). After the incubation periods (37 to 38 h post-infection), the cells were collected from 20 Petri dishes and centrifuged at 500 rev/min for 5 min. The cell pellets were resuspended in 5 ml medium and frozen—thawed six times. The lysates were centrifuged at 2000 rev/min for 15 min to remove the cell debris. The supernatants were layered over a preformed CsCl gradient (1·2 to 1·4 g/ml) and centrifuged for 1 h at 35000 rev/min at 4 °C in the B60 International ultracentrifuge using the SB283 rotor. In some experiments the centrifuge tubes were pierced and the gradients collected dropwise into fractions. For further purification of the viral particles, the viral bands were resuspended in 5 ml 0·05 M-tris–HCl buffer pH 8·1, layered over a solution of CsCl (density 1·4 g/ml) in 12 ml polyallomer tubes and centrifuged for about 15 h at 25000 rev/min and 4 °C. In some experiments the viral particles were centrifuged a third time in a 1·4 g/ml CsCl solution under the same conditions as in the second cycle of centrifugation. The efficiency of the method of purification of IP was assessed by a reconstruction experiment. The wt and ts4 were grown at 39 °C and half the cultures were labelled with 35S-methionine from 16 to 20 h...
post-infection. Thus, both labelled and unlabelled viral particles were produced. After a first cycle of centrifugation, labelled wt virions were mixed with unlabelled wt-IP while labelled wt-IP were mixed with unlabelled wt virions. The mixtures were then centrifuged overnight a second time in CsCl (density 1.4 g/ml). In the case of ts4 the radioactive material was collected and centrifuged a second time in CsCl. The distribution of 35S-methionine-labelled components indicated that after two successive centrifugations in CsCl, there was no significant cross-contamination between wt-IP and wt virions (data not shown). The centrifugation of ts4-infected cell lysates (grown at 39 °C) in CsCl was marked by the absence of mature virions. However, from four to five times more IP than in the case of wt was routinely observed \( A_{260} \) measurements). A second cycle of centrifugation of ts4-IP did not give rise to detectable amounts of mature virions. Therefore, the viral particles of both wt and ts4 were assumed to be pure after two cycles of centrifugation in CsCl.

**Isolation of cellular and viral DNA.** Extraction of cell DNA was essentially as described by Tyndall et al. (1978). The cells were washed twice with 0.05 M-tris pH 8.1 and 5 mM-EDTA. They were then lysed by incubating for 2 h at 37 °C with 1 mg Pronase/ml (Calbiochem; predigested for 1 h at 37 °C), in 0.1 M-NaCl, 10 mM-tris–HCl pH 7.9, 5 mM-EDTA and 0.5% sodium sarcosinate. The lysates were extracted twice with redistilled phenol containing 0.1% hydroxyquinoline saturated with 0.2 M-tris–HCl pH 8, followed by two chloroform–isoamyl alcohol (24:1) extractions. Most of the chloroform–isoamyl alcohol was removed and the solution dialysed overnight against 0.1 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate) at 4 °C. The dialysed solution was digested with RNase at a concentration of 200 μg/ml for 1 h at 37 °C. Pronase (500 μg/ml) was then added and the mixture incubated for another 30 min at 37 °C, then extracted with chloroform–isoamyl alcohol. Most of the chloroform–isoamyl alcohol was removed and the DNA was precipitated by the addition of 2 vol. ethanol at −20 °C. The precipitate was pelleted and dried under a stream of nitrogen then dissolved in 10 mM-tris pH 7.4, 10 mM-NaCl and 1 mM-EDTA and stored at 4 °C.

Viral DNA was extracted from particles that had undergone at least two cycles of CsCl centrifugation and were dialysed against 10 mM-tris–HCl pH 7.4 and 5 mM-EDTA. The viral particles were treated with 1 mg Pronase/ml, and extracted with phenol–chloroform–isoamyl alcohol as above. The viral DNA was precipitated, dried and redissolved as described for cell DNA.

In vivo labelling with 35S-methionine and 32P. In both cases the DMEM was supplemented with dialysed calf serum. Before labelling with 35S-methionine, infected cells were incubated for 1 h in DMEM without methionine. The medium was then replaced with 2 ml medium containing 100 μCi 35S-methionine/ml (about 250 Ci/mmol; Amersham Corporation). The labelling period lasted from 18 to 22 h post-infection. The medium was then replaced by fresh DMEM and incubations continued for another 15 h.

The cells (infected or not) to be labelled with 32P were first incubated for 1 h in phosphate-free medium. This medium was then replaced by medium containing 200 μCi/ml 32P-orthophosphate (New England Nuclear). Incubation usually lasted for 7 h (starting 13 h post-infection in infected cells) after which time the cells were incubated in DMEM for another 17 or 18 h.

**In vitro labelling of DNA.** The **in vitro** introduction of 32P-labelled nucleotides into DNA was performed by the nick-translation reaction of Escherichia coli polymerase (Boehringer, Mannheim) based on the conditions described by Maniatis et al. (1975). About 25 μCi of each of the four 32P-triphosphates (32P-dCTP, 32P-dTTP, 32P-dATP and 32P-dGTP) were used to label 0.2 to 0.3 μg DNA. The reaction was terminated by chloroform extraction. The radioactive DNA was freed from unincorporated deoxynucleoside triphosphates either by passage through a column (0.5 x 20 cm) of Sephadex G-50 equilibrated with 0.01 M-tris pH
8 and 0.01 M-EDTA, or the DNA was precipitated by addition of 2 vol. ethanol in the presence of 0.1 M-NaCl. The specific activity of the final product was usually in the range of $5 \times 10^7$ to $8 \times 10^7$ ct/min/$\mu$g DNA.

**Treatment of DNA with restriction endonucleases.** Both cellular and viral DNA were treated with four different restriction endonucleases during the course of these experiments: *EcoRI*, *BamI*, *HindIII* and *HpaI*. The first three enzymes were purchased as restriction nuclease kits from Miles Laboratories, Elkhart, Ind., U.S.A. *HpaI* was obtained from New England Biolabs, Beverly, Mass., U.S.A. All reactions were carried out in the solutions specified by the manufacturer for 30 min at 37 °C, using 2 units enzyme/$\mu$g DNA. The reactions were stopped by the addition of a saturated sucrose (w/v) solution (containing bromophenol blue and 0.2 M-EDTA) in sufficient quantity to bring the sucrose concentration to 5%. The digested DNA was then immediately loaded on to a gel.

**Agarose gel electrophoresis.** Gel electrophoresis was carried out in vertical and horizontal slab gels. The buffer system for the vertical gel consisted of 0.09 M-tris, 0.09 M-boric acid and 0.0025 M-EDTA (pH 8.3), whilst the horizontal gel was run in 0.04 M-tris, 0.03 M-NaH$_2$PO$_4$ and 0.001 M-EDTA (pH 8.3). All DNA samples were loaded in a solution made of 5% sucrose containing bromophenol blue and 0.001 M-EDTA and run for 15 h at 30 mA. After the runs, the DNA was stained by immersing the gel in ethidium bromide (0.5 gg/ml electrophoresis buffer) for 15 min (Sharp *et al.*, 1973). The gels were photographed through a camron 85 orange filter using polaroid film 55 and u.v. illumination.

**Elution of DNA from the agarose gels.** The DNA was localized by viewing the ethidium bromide-stained gel under u.v. light, and carefully cut out and transferred to a 10 ml syringe which was then frozen for a few hours at −20 °C. After thawing, the gel was driven through the syringe a few times, transferred to a 50 ml tube and suspended in about 2 vol. ammonium acetate (0.5 M in 0.01 M-tris pH 7.5 and 1 mM-EDTA). The tube was shaken for about 15 h at 37 °C. The agarose was pelleted by centrifugation (5000 rev/min in a Sorvall RC-3 at 4 °C for 30 min) and the DNA extracted as described for viral and cellular DNA.

**Blotting and hybridization.** The DNA in agarose gels was denatured by immersing the gels in 0.2 M-NaOH, 0.6 M-NaCl for 30 min at room temperature. After washing three times with water, the gels were submerged in 1 M-tris–HCl pH 7.4, 0.6 M-NaCl for another 30 min. Transfer of the DNA from the gel to the nitrocellulose (Millipore) was accomplished as described by Southern (1975) and modified by Botchan *et al.* (1976). After blotting, the nitrocellulose sheets containing DNA were washed with 2 × SSC, dried at room temperature and heated in an oven at 80 °C for about 3 h. They were then soaked in 5 × Denhardt (1966) solution (0.02% Ficoll, bovine serum albumin, polyvinylpyrrolidone) in 6 × SSC, 0.1 M-sodium phosphate pH 7 and 0.001 M-EDTA for about 6 h at 68 °C in sealed polyethylene bags. The probe was denatured adding 0.2 M-NaOH for 5 min at room temperature, neutralized by equivalent amounts of HCl and tris–HCl pH 7.4. The soaking solution was then replaced and 0.2 to 2 $\mu$g denatured probe was added. The polyethylene bags were heat-sealed and shaken at 68 °C (Birg *et al.*, 1977). About 18 h later, the nitrocellulose sheets were washed for about 6 h (with frequent changes) in a solution of 1 × SSC, 0.1 M-sodium phosphate, 0.5% SDS and 5 × Denhardt solution. The filters were then air-dried, taped on a sheet of 3MM paper, heated at 80 °C for 10 min, allowed to reach room temperature and subjected to autoradiography at −70 °C using a lightning plus screen (Dupont) to boost the exposure on the RP-1 Kodak film.

**RESULTS**

**Electrophoretic mobility of IP DNA**

IP were purified by equilibrium sedimentation in CsCl and the DNA extracted and analysed by agarose gel electrophoresis (Fig. 1). Principally, two classes of DNA molecules
were observed: (i) large mol. wt. DNA ranging in size from subgenomic to genome size or 15S to 31S as determined by sedimentation analysis (IP-specific DNA or IPSD₁) and (ii) small mol. wt. DNA of approx. 200 to 1000 base pairs (IPSD₂). The relative proportion of IPSD₁ was always greater in wt-IP whereas that of IPSD₂ was always greater in ts4-IP. These two types of DNA were also detected in IP of Ad3 and Ad5 (data not shown). In addition to IPSD₁ and IPSD₂, two other DNA fragments were also observed in some preparations of DNA from wt virions, wt-IP and ts4-IP under conditions of overloading. The larger of the two fragments was usually located slightly ahead of fragment B of HindIII. This corresponds to about 13% of the Ad2 genome. Similar estimations suggest that the smaller DNA fragment corresponds to about 6% of the Ad2 genome. Blotting experiments coupled with hybridization have shown these two fragments to be of viral origin (data not shown). The larger fragment was digested by HindIII.

**IP do not contain RNA**

The small size and heterogeneous nature of IPSD₂ prompted us to check for the presence of RNA in IP. Samples of nucleic acid extracted from complete virions and IP were incubated with electrophoretic grade RNase and DNase. Yeast RNA was included as control. The
resistance of both IPSD₁ and IPSD₂, from wt and ts4 particles, to RNase compared to their sensitivity to DNase strongly suggests that these molecules do not contain significant amounts of RNA.

**Restriction endonuclease analysis of DNA from IP**

DNA from wt complete virions, wt-IP and ts4-IP were separately digested with several restriction endonucleases and analysed on agarose gels. Comparison of these cleavage patterns revealed that IPSD₁ from both wt and ts4 were sensitive to HindIII. In the case of wt-IP, the IPSD₁ was cleaved to give the typical restriction pattern obtained when the complete Ad2 genome is digested by HindIII. In the case of ts4, IPSD₁ was cleaved into a series of atypical fragments. This suggests that either ts4-IPSD₁ consists of a very random selection of viral DNA fragments or the viral sequences are unusually arranged. IPSD₂ of wt and ts4 seemed to be unaffected by HindIII. Similar results were obtained when restriction endonucleases BamI and EcoRI were used.
A denovirus incomplete particle DNA

Fig. 3. Detection of viral sequences in IPSD1 and IPSD2 using in vivo labelled probes. In vivo 32P-labelled DNA was extracted from wt-IP and ts4-IP and run on a 1% agarose horizontal slab gel. IPSD1 and IPSD2 of wt and ts4 were located by ethidium bromide staining and cut out and the DNA extracted. The four different probes obtained (wt-IPSD1, wt-IPSD2, ts4-IPSD1 and ts4-IPSD2) were denatured and hybridized to nitrocellulose strips bearing restriction fragments of Ad2 DNA. These strips were obtained by blotting gels similar to those described in Fig. 4. The numbers designate the restriction endonucleases used to cleave Ad2 DNA before blotting: 1, EcoRI; 2, BamI + EcoRI; 3, HpaI; 4, HindIII.

All parts of the viral genome are present in IP DNA

Our experimental strategy consisted of hybridizing both in vivo and in vitro labelled (by nick-translation) IP DNA to strips of nitrocellulose bearing restriction fragments of Ad2 DNA (Southern, 1975). Comparison of the endonuclease restriction and hybridization patterns (Fig. 2) showed that all restriction fragments were represented in IP DNA. The separate analyses of in vivo labelled IPSD1 and IPSD2 (Fig. 3) of both wt and ts4 show that IPSD1 contained sequences from all parts of the viral genome, without the enhancement of any specific region. By contrast, the hybridization pattern of ts4-IPSD2 reflects an enhancement of fragments from both ends of the genome (EcoRI, A and C; BamI/EcoRI, A and E; HpaI, D and E; HindIII, F and G) with particular emphasis on the left-hand end.

In vitro labelled probes with high specific activities made it possible to increase the sensitivity of the hybridization experiments. Furthermore, all packaged DNA, including molecules that did not replicate were now labelled. Fig. 4 shows the result of hybridizing the nick-translated IP DNA probes to restriction endonuclease fragments of Ad2 DNA. It is clear that in the case of ts4-IP all the DNA fragments arising from the left end of the genome were strongly intensified. These results indicate that the left end of the viral genome is enriched in ts4-IP. Since fragments F and G of HindIII digests co-migrated, this enzyme was not useful for determining which end of the genome was preferentially encapsidated.

The separate analysis of IPSD1 and IPSD2 of wt and ts4 led to the surprising finding that there seemed to be no over-representation of any particular region of the viral genome in IPSD1 of both wt and ts4 (Fig. 5). However, IPSD2 of both wt and ts4 were enriched for both ends of the viral genome with a particularly marked abundance of the left end (Fig. 6). The
fact that fragment C (adjacent to the left end fragment E) of the \textit{HpaI} digests was not enhanced suggests that the enhancement of the left end region does not extend beyond 4 to 5\% of the viral genome. In many experiments of this type we noted an under-representation in the probe of the region of the genome corresponding to fragments \textit{HindIII}-I or \textit{BamI}-D (see Fig. 4). We have no explanation for this observation.

\textit{IP} also contain cellular DNA

Our approach was essentially the same as the previous hybridization experiments. Cellular DNA was digested with restriction enzymes, electrophoresed through an agarose gel and transferred to nitrocellulose strips by blotting. DNA obtained from wt virions, wt-IP and \textit{ts4-IP} were labelled by nick-translation and used as probes to hybridize with the nitrocellulose strips bearing the cellular DNA. The abundant presence of cell DNA sequences was clearly demonstrated in IPSD$_2$ of wt and \textit{ts4} (Fig. 7). IPSD$_1$ did not appear to contain significant amounts of cellular DNA. Similar results were obtained in the case of IPSD$_1$ and IPSD$_2$ of Ad3 and Ad5.

\textit{IP} have DNase activity

An endonuclease activity has been reported to be associated with purified mature adenovirions (Burlingham \textit{et al.}, 1971). The presence of fragments of DNA in IP prompted us to check for DNase activity in these particles. Our experimental approach consisted of incubating $^{32}$P-labelled viral or cellular DNA with purified wt virions, wt-IP and \textit{ts4-IP}. The extent of digestion was then determined by electrophoresis in agarose gels. Fig. 8 shows a

![Fig. 4. Comparison of viral sequences in DNA of wt virions (wt-V), wt-IP and \textit{ts4-IP} using in vitro labelled probes. DNA from the three types of particles were labelled with $^{32}$P-triphosphates by nick-translation. These were then used as probes and hybridized to nitrocellulose strips bearing restriction fragments of Ad2 DNA. The restriction endonucleases used to cleave Ad2 DNA before blotting were: 1, \textit{HindIII}; 2, \textit{HpaI}; 3, \textit{BamI}; 4, \textit{BamI} + \textit{EcoRI}; 5, \textit{EcoRI}.](image)
**Adenovirus incomplete particle DNA**

Fig. 5. Detection of viral DNA sequences in IPSD. DNA was extracted from wt-IP and ts4-IP and run on a 1% agarose gel. IPSD of wt and ts4 were located by staining with ethidium bromide. Both IPSD regions were cut out and the DNA extracted. The IPSD samples thus obtained were labelled with 32P-triphosphates by nick-translation and hybridized to nitrocellulose strips bearing HindIII and BamH1/EcoRI restriction fragments of Ad2 DNA. The nitrocellulose strips were then subjected to autoradiography. The probes used are indicated at the top while the different restriction enzymes used to cleave the Ad2 genome (before blotting) appear immediately above the hybridization patterns.

**DISCUSSION**

The nature of the DNA in IP synthesized by Ad2 and the ts4 mutant (Khittoo & Weber, 1977), which accumulates these particles, was characterized. The DNA within IP can be separated by agarose gel electrophoresis into a heterogeneous population of large molecules (IPSD1) which co-migrate with genome size DNA and smaller molecules ranging from about 1000 base pairs to 200 base pairs (IPSD2).
Fig. 6. Detection of the viral sequences in IPSD₂ of wt and ts4. Purified wt-IPSD₂ and ts4-IPSD₂ were labelled with ³²P-triphosphates and hybridized to nitrocellulose strips bearing restriction fragments of Ad2 DNA. The probes used are indicated at the top. The restriction endonucleases used to generate the Ad2 DNA fragments were: 1, HindIII; 2, HpaI; 3, BamI; 4, EcoRI.

Fig. 7. Detection of cellular DNA sequences in IPSD₁ and IPSD₂. Cellular DNA was treated with restriction endonucleases and transferred to nitrocellulose filters by blotting. Nick-translated IPSD₁ and IPSD₂ of wt and ts4 were hybridized to the nitrocellulose filters bearing the cleaved cellular DNA. Nick-translated viral and cellular DNA were also hybridized to similar nitrocellulose filters for comparison. The probes used were: (a) cellular DNA; (b) Ad2 viral DNA; (c) ts4-IPSD₁; (d) wt-IPSD₁; (e) ts4-IPSD₂; (f) wt-IPSD₂. The restriction endonucleases used to cleave the cellular DNA before blotting were: 1, HindIII; 2, HpaI; 3, BamI; 4, untreated cell DNA.
Cleavage of wt-IPSD$_1$ with restriction endonucleases usually yielded normal fragments in molar ratios typical of viral DNA, while cleavage of ts4-IPSD$_1$ generated mostly atypical fragments. This suggested that ts4-IP packed more DNA of unusual molecular topography than wt-IP. IPSD$_2$ was too small to be affected by restriction endonucleases. To our knowledge IPSD$_2$-like DNA has not been reported before. Possibly, because of its small size, it was electrophoresed out and consequently missed. When IP DNA was nick-translated and hybridized to Ad2 restriction fragment-bearing filters under non-saturation conditions, two conclusions could be drawn: (i) all sequences of the genome were represented and (ii) ts4-IP contain more sequences from the genomic left end. The separate analysis of IPSD$_1$ and IPSD$_2$ revealed that all parts of the viral genome were equally represented in IPSD$_1$ of both wt and ts4. This agrees with the restriction endonuclease analysis discussed above. A very different result was obtained with IPSD$_2$; both molecular ends were enriched with a particularly marked abundance of the left end (Fig. 6). The hybridization pattern of IPSD$_2$ to the viral HpaI fragments shows that the enrichment of the left end of the viral genome in IPSD$_2$ is confined to the E fragment, which represents 4.4% of the genome. Consequently, IPSD$_2$, which is about 1 to 3% of the genome, is largely derived from the left 3% of the genome. This agrees with the observed resistance to cleavage by EcoRI, BamI and HindIII, none of which have cleavage sites in the HpaI–E fragment. The slight increase in right end sequences could be due to hybridization within the 102 base pair inverted terminal repetition (Arrand & Roberts, 1979). The efficient nick-translation of IPSD$_2$ shows that it consists of double-stranded DNA.

The fact that IPSD$_1$ did not show an under-represented left-end region suggests that IPSD$_1$ and IPSD$_2$ do not originate from the same molecules. Furthermore, it is not possible to determine from the results if IPSD$_1$ and IPSD$_2$ are contained in separate or the same capsids. The work of Tibbetts (1977) would suggest that lighter particles might contain IPSD$_2$ and heavier particles IPSD$_1$. 

Fig. 8. An endonuclease activity associated with virus particles. About 2 μg samples of cellular and viral DNA were incubated for 6 h (at 37 °C) with ts4-IP (2 A$_{260}$), wt-IP (2 A$_{280}$), wt virions (4 A$_{280}$) and for 20 min with 5 μg DNase I. After the incubation periods, the various samples were analysed on a vertical agarose slab gel. The substrate DNA (cell DNA or virus DNA) used is indicated at the top. 1, ts4-IP; 2, wt-IP; 3, wt virions; 4, DNase I; 5, either undigested cellular DNA (left lane) or viral DNA (right lane).
Several authors have reported the presence of cell DNA sequences in IP (Hammarskjold et al., 1977; Tjia et al., 1977). Our data indicate that as much as half the DNA in IP could be of cellular origin. The question of whether particular sequences of cell DNA were preferentially present in IP was not pursued exhaustively. However, the data suggest that they are heterogeneous (Fig. 7).

The presence of subgenomic viral DNA in IP and particularly IPSD$_2$ suggested the possibility of endonuclease involvement in the generation of these fragments. Several authors have described the association of an endonuclease activity with adenovirions and adenovirus-infected cells (Reif et al., 1977a, b; Burlingham et al., 1971; Burlingham & Doerfler, 1972; Marusyk et al., 1975). More recently Padmanabhan et al. (1979), showed that an endonuclease purified from Ad2-infected KB cells produces limit digest fragments of 140 to 240 base pairs. Our results show that IP can cleave both cellular and viral DNA into fragments quite similar in size to IPSD$_2$.

The results presented here and those reported previously may be combined to suggest the following model to account for IP and the mechanism of DNA encapsidation. At least four types of DNA molecules may be observed in IP, namely (1) IPSD$_1$ or large heterogeneous molecules, (2) IPSD$_2$, or molecules in the range of 200 to 1000 base pairs, (3) molecules with long inverted terminal repetitions and (4) heterogeneous cellular DNA. From quantitative considerations alone, all four types of molecules cannot reside within the same IP, thus there must be subpopulations of IP containing a limited selection of these molecules. The IP which contain molecule types (3) or (4) are unlikely to be assembly intermediates, while those containing types (1) and (2) are good candidates. The kinetics of assembly of IP, the presence of precursor proteins and the existence of ts mutants which accumulate IP (such as ts4) are factors which strongly suggest that a subpopulation of IP are true DNA encapsidation intermediates, while the remaining IP must be defective particles. The viral DNA or nucleoprotein complex might associate with pre-assembled empty particles via a recognition signal located within the left 4% of the molecule to initiate encapsidation. Such a signal could explain the preponderance of left end sequences. The data suggest that this step is rate limiting which would permit the fragmentation of DNA through endonuclease cleavage or mechanical shear during extraction and purification. However, it is also possible that some of the fragments of DNA arise due to errors in DNA replication.

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