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Synthesis of Defective Viral DNA in HeLa Cells Infected with Adenovirus Type 3

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Virus-specific DNA fragments that are shorter than the full-length viral genomes have been isolated from HeLa cells productively infected with adenovirus type 3. A number of predominant size classes could be detected by gel electrophoresis and hybridization, and the array of sizes was similar or identical to the selection in DNA purified from incomplete particles of this serotype (E. Daniell, J. Virol. **19**:685-708, 1976). A large fraction of these short DNA molecules contained long inverted terminal repetitions, as did DNA molecules from incomplete particles. Restriction analysis showed that these subgenomic molecules consist of sequences from the two molecular ends of the normal genome. These results suggest that the predominance of left-hand end fragments seen in packaged incomplete DNAs results from selective packaging, whereas the predominance of certain size classes of intracellular viral DNA is a function of prepackaging events. The incomplete DNAs were generated at all times during viral DNA replication, and the yield relative to complete DNA did not seem to vary significantly with time or multiplicity of infection or when the virus was propagated on different human cell types.

Some of the species of incomplete particles of adenovirus, which are of lower buoyant density than complete particles, have been shown to contain DNA that is virus specific and of shorter length than the complete viral genome (1, 16, 20, 26). Extensive characterization of the DNA contained in the incomplete particles of adenovirus type 2 (Ad2) and Ad3 (2) reveals that many of these short DNA molecules contain extended inverted terminal repetitions and that the sequences of the left-hand end of the normal viral genome are preferentially represented in the DNA extracted from incomplete particles. Somewhat different results were obtained in another study of the DNA in Ad2 incomplete particles (24), in which the bulk of the DNA in incomplete particles was shown to be of cellular origin; host and viral sequences are possibly linked.

The DNA isolated from incomplete particles of Ad2 and Ad3 has been sized on agarose gels (2). Although the molecules are heterogeneous in length, certain size classes predominate, and the pattern of DNA sizes is characteristic of particular serotypes. Since these molecules contain inverted repetitions and consist of specific portions of the viral genome, it appears likely that they arise through errors in viral DNA replication, rather than by fragmentation of completed viral DNAs. A model was presented (2) to account for the generation of these aberrant structures. There is, however, no obvious explanation for the preferential sizes or for the predominance of the left end of the normal genome in the incomplete DNA. Although adenovirus DNA replication is not completely understood, it is generally accepted that both molecular ends of the DNA serve as origins (10) and as termini of replication (21, 25), with extensive strand displacement of either viral DNA strand occurring. It would be expected, in this context, that errors leading to terminal duplications (see model, reference 2) could involve either end of the genome equally.

Selectivity of the packaging system was suggested as a possible explanation for the observed distribution of sizes and sequences in the incomplete DNAs. The present paper describes the detection, isolation, and analysis of short viral DNAs within the cell nucleus by techniques designed to include all DNA, not just that which is packaged. Ad3 was chosen for detailed study as it is a serotype from which DNA-containing defective particles have been easiest to isolate. The results suggest that the selection of one end of the molecule in incomplete particles is dependent on packaging, but that the predominance of specific sizes is not.

MATERIALS AND METHODS

Cells and virus. HeLa cells were obtained from Cold Spring Harbor Laboratory and were cultured on

plastic dishes in Eagle minimum essential medium supplemented with 5% fetal calf serum. Human epithelial cells were a gift of Helene Smith (18). Virus stocks were propagated on HeLa cell monolayers and titrated as described by Williams (28).

Infection of cells and isolation of DNA. Monolayers of HeLa cells were infected with virus at multiplicities of infection of 10 to 20 PFU/cell except where otherwise noted. Virus was absorbed to the cells in 60-mm dishes for 1 h in 0.5 ml of phosphate-buffered saline; 5 ml of Eagle minimum essential medium supplemented with 5% fetal calf serum was then added. Cells were harvested by scraping cells from plates into medium, followed by low-speed centrifugation and several cycles of washing by resuspension in phosphate-buffered saline. The cells were then suspended in buffer for the isolation of nuclei by the method of Honda et al. (9). Isolated nuclei were digested with 1 mg of Pronase per ml and 0.5% sodium dodecyl sulfate, and the DNA was extracted twice with phenol and once with chloroform and dialyzed against 0.02 M Tris-hydrochloride (pH 8.5)–0.002 M EDTA before it was loaded on gels or sucrose gradients. On occasion, as noted, extractions were performed by the method of Hirt (7), which removed cellular DNA and much full-length adenovirus DNA. For the preparation of radioactively labeled DNA, medium was removed at the desired time after infection and replaced by minimum essential medium without phosphate with 100 μCi of ^{32}P (ICN).

Isolation of incomplete particles. The purification of incomplete particles of Ad3 by repeated centrifugation through cesium chloride has been described (2). For the purification of virus particles, cells were harvested at times from 48 to 72 h after infection.

Gel electrophoresis, transfer of DNA to nitrocellulose, and hybridization to viral DNA probe. Electrophoresis of cellular and viral DNA was performed on 0.7% agarose gels as described previously (2), and DNA was transferred to nitrocellulose filter sheets by the method of Southern (22), with the arrangement of gel, filter, and "blotting" paper as described by Ketner and Kelly (14). Specific viral and cellular DNA probes were labeled by the method of nick translation (17) and hybridized to the filters as described previously (2).

Sucrose gradient centrifugation of DNA. [^{32}P]-DNA from infected cells was layered onto 5 to 20% neutral sucrose gradients (0.05 M Tris [pH 7.5–0.02 M EDTA–0.1 M NaCl) and centrifuged at 40,000 rpm in a Beckman SW41 rotor at 15°C for 3 to 4 h. Three- to 5-drop fractions were collected through the bottom of the tube and counted directly by Cerenkov radiation, and portions of selected fractions were analyzed by electrophoresis on 0.7% agarose gels with appropriate markers. Fractions containing complete (V) and incomplete viral DNAs of various sizes were pooled, precipitated directly from sucrose by the addition of 2 volumes of ethanol, and suspended in 0.01 M Tris (pH 7.9)–0.001 M EDTA for further study.

Restriction enzymes and maps. *Hin*dIII and *Bam*HI were purchased from New England Bio Labs, and the buffers used are those prescribed by the company. *Xba* I was the gift of Herb Boyer's laboratory. *Xba* digestions were carried out in 20 mM Tris (pH 7.4)–10 mM $MgCl_2$–7 mM β-mercaptoethanol. The map of Ad3 digested with these enzymes, partially worked out in this laboratory, was generously provided in complete form before publication by Clark Tibbetts. Electrophoresis, staining, and autoradiography were performed as described previously (2).

Electron microscopy. DNA precipitated from sucrose gradient fractions was denatured by the addition of 1/10 volume of 1 N NaOH. After incubation for 10 min at room temperature, it was neutralized by the addition of 1/10 volume 2 N Tris-hydrochloride and stored at 4°C until mounted for electron microscopy by the Kleinschmidt technique, essentially as described by Davis et al. (3). Grids were examined and photographed in a Siemens Elmiskop 1A electron microscope.

RESULTS

Sizes of viral DNA produced in infected HeLa cells. When complete and incomplete particles were purified from a preparation of Ad3-infected cells, the total yield of DNA from the incomplete particles was at most 1% of the amount of full-length DNA in the complete particles. To detect short viral DNAs in the cell nucleus in the presence of this large excess of complete DNAs, a method was devised to first separate the DNA by size and then to detect small amounts of short virus-specific DNA. Figure 1 shows the technique and the primary result. Samples of DNA prepared from infected cells at various times after infection were subjected to electrophoresis on 0.7% agarose gels. Figure 1a is a photograph of such a gel stained with ethidium bromide. A specific band of full-length viral DNA appeared at times after infection when viral DNA synthesis had commenced. The more diffuse band just above it and most of the material trapped at the top of the gel were shown by hybridization and by comparison with mock-infected cells to be cellular DNA. To obtain Fig. 1b, the DNA from this gel was transferred onto a nitrocellulose filter, hybridized with Ad3 probe, and autoradiographed after drying. Only virus-specific sequences in the array are labeled by this technique, and the sensitivity is several orders of magnitude greater than that of staining and UV fluorescence. Figure 1 shows that, as full-length viral DNA appeared in infected cells, there was an array of short DNAs that could be detected and that these DNAs possessed viral sequences. Longer exposures of the same filter showed the distinctive low-molecular-weight bands even at 10 h after infection. Hybridization of identical gels with probes of labeled HeLa cell DNA showed no detectable radioactivity hybridizing in the low-molecular-weight region, except at very long times after infection (greater than 60 h), when the breakdown of cell DNA is expected and

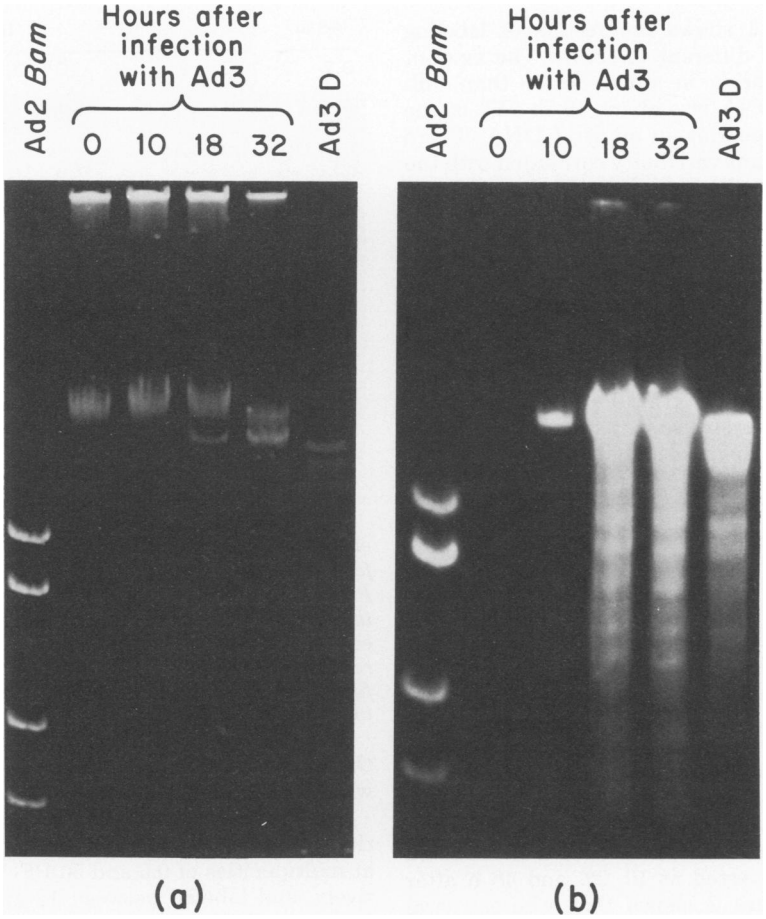


FIG. 1. *Electrophoresis of DNA extracted from the nuclei of HeLa cells infected with Ad3 and detection of viral sequences. HeLa cells were harvested at various times after infection, and DNA was purified from isolated nuclei as described in the text. Sample Ad3 D is DNA extracted from incomplete particles of Ad3 that had been purified by three rounds of differential centrifugation through cesium chloride. The DNA was subjected to electrophoresis through 0.7% agarose for 15 h at 30 V. (a) Photograph of the gel stained with ethidium bromide and illuminated with UV light. (b) DNA in the gel was denatured in situ, transferred to nitrocellulose paper, and hybridized with 30,000 cpm of Ad3 [^{32}P]DNA labeled by nick translation. The resulting autoradiograph shows the location of adenovirus-specific DNAs in the gel.*

becomes apparent; this was totally without a specific pattern.

The rightmost lane in each part of Fig. 1 contains purified "defective" DNA isolated from incomplete particles and characterized previously (2). The autoradiogram shows clearly that the viral DNA isolated from within the nucleus displays the same pattern of size classes as does the DNA that has been purified through packaging. In some subsequent experiments we were able to detect the larger (more plentiful) incomplete DNAs by eye and by photography of stained gels.

Extractions of DNA from whole cells by the method of Hirt (7) were occasionally performed, but this procedure is quite variable. In addition, some of the incomplete DNAs and much of the full-length viral DNA are precipitated with the cell DNA and protein pellet. This method was judged inappropriate for the quantitative analysis of DNA species produced.

Comparative amounts of complete and incomplete viral DNA produced at different times after infection. To ascertain whether aberrant viral DNA molecules are made preferentially at a particular stage of viral replication, we extracted DNA from cells that had been labeled with ^{32}P during different intervals after infection. The DNA was resolved in agarose gels with appropriate markers, and the gels were

sliced. Table 1 shows the results of labeling during several different intervals. The amount of label appearing in sizes shorter than full-length viral DNA was about 15 to 25% of the total label appearing in all viral DNA. There was no significant variability correlated with the time of labeling. We found that HeLa cell DNA synthesis was shut off slowly by Ad3 infection and that some high-molecular-weight DNA continued to accumulate label in periods up to 12 h after infection. Therefore, we did not include counts in DNA larger than the full-length viral genome. Drying and autoradiography of similar gels yielded a pattern of discrete sizes identical to those shown by blotting and hybridization to a specific viral probe, so we assumed that radioactivity in the low-molecular-weight region was predominantly viral.

As a further control, we performed similar gel electrophoresis and quantitation on samples that had been treated with RNase and centrifuged through neutral sucrose. The entire viral peak plus the trailing region (see Fig. 3, V plus I-II) was pooled and precipitated. The distribution of counts in the viral and subgenomic region of such a gel was identical to that of samples in which cell DNA had been loaded directly on the gel after extraction and dialysis.

Effect of multiplicity on production of incomplete DNA. Monolayers of HeLa cells were infected with multiplicities of 20, 2, and 0.2 PFU/cell, respectively, and DNA was extracted from cells harvested at 10, 22, and 48 h after infection. Figure 2 shows that whereas viral DNA in detectable amounts accumulated relatively late in cells infected at a low multiplicity, the incomplete DNAs appeared concomitantly with full-length DNA.

TABLE 1. *Amount of subgenomic viral DNA made during various intervals after infection*[a]

Expt no.	Time of labeling (h post-infection)	S/(S + V)[b]
1	6–26	0.22
	6–44	0.18
2	10–24	0.25
	20–44	0.17
	10–44	0.27
3	10–18	0.22
	24–36	0.18

[a] Cells were infected with Ad3 at a multiplicity of 30 to 40 PFU/cell. The cells were labeled during different intervals and then harvested, and the DNA was purified and analyzed by electrophoresis and slicing of gels (see text).

[b] The fractions represent the ratio between counts (S) in the "subgenomic" region of the gel—DNA from 10 to 90% of the full-length genome—and counts in full-length (V) plus subgenomic DNA.

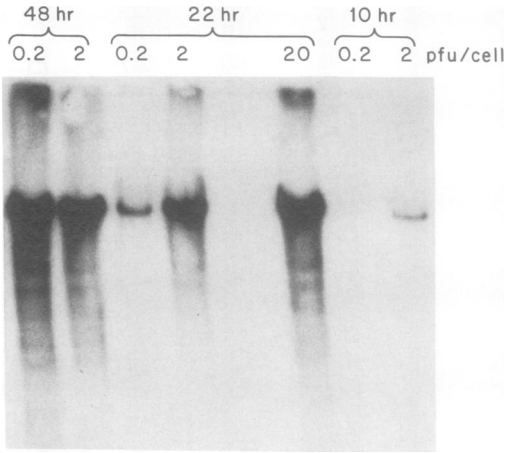


FIG. 2. *Autoradiogram showing the time course of viral DNA synthesis at different multiplicities of infection. HeLa cells were infected with 0.2, 2, or 20 PFU of Ad3 per cell and harvested at the indicated times. Equal total amounts of DNA were loaded onto each slot of a 0.7% agarose gel and, after electrophoresis for 15 h at 25 V, transferred to a nitrocellulose filter, and hybridized to Ad3 [^{32}P]DNA as described in legend to Fig. 1b.*

To represent these results more quantitatively, monolayers of HeLa cells were infected at multiplicities of 0.5 and 50 PFU/cell, respectively, and labeled between 12 and 35 h after infection; DNA sizes were quantitated as described above. In both cases, 20% of the counts were in the subgenomic fraction. Similar results are obtained from measuring the area under the peak and trailing regions of sucrose gradient curves.

Isolation of incomplete DNAs and analysis with restriction endonucleases. Short DNA molecules were separated from full-length viral DNA and from cell DNA by sedimentation through neutral sucrose gradients (Fig. 3). Portions of fractions from different regions of the gradient were characterized in 0.7% agarose gels with appropriate size markers to determine the size of the DNA and its purity from detectable full-length viral DNA and labeled cell DNA. Small amounts of radioactivity that run at the position of cell and genome-size DNA are constant from fraction to fraction and do not obscure the results of restriction analysis.

Fractions of DNA from the virus peak and from more slowly sedimenting regions of the gradient, thus analyzed, were pooled, as indicated in Fig. 3a, and precipitated. The DNA was digested with a variety of restriction endonucle-

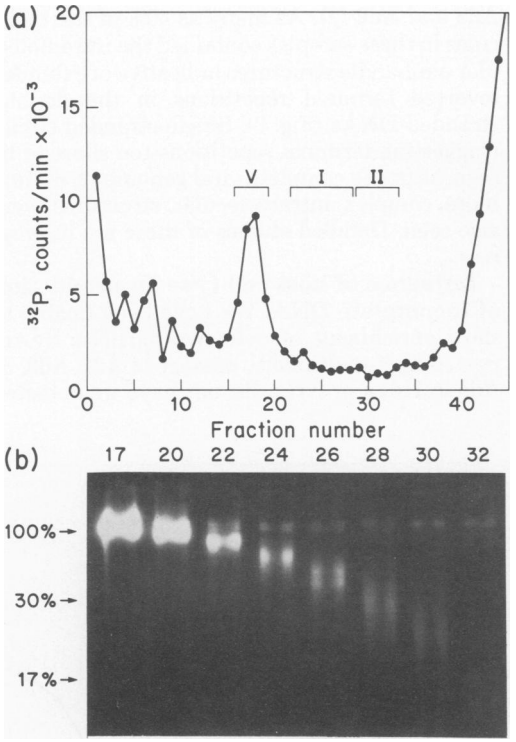


FIG. 3. *Sucrose sedimentation of intracellular DNA. (a) DNA from Ad3-infected cells labeled with ^{32}P from 10 to 44 h after infection was centrifuged through 5 to 20% sucrose gradients. (b) Portions (10 μl) of representative fractions from the gradient were loaded directly onto 0.7% agarose gels and subjected to electrophoresis for 15 h at 30 V, and the gel was dried and autoradiographed. Coelectrophoresis with fragments of Ad2 DNA cut with endonuclease BamHI served as nonradioactive size markers.*

ases to determine the representation of various regions of the normal genome; the restriction fragments generated by digestion of low-molecular-weight DNA isolated from nuclei can be compared, on the one hand, with complete viral DNA and, on the other hand, with the DNA extracted from incomplete particles. Figure 4 shows the results of such a comparative digestion with *Bam*HI. Figure 5 shows the restriction map of Ad3 DNA for three illustrative enzymes.

In the digest of DNA from incomplete particles (I.P. in Fig. 4), the rightward fragments of the restriction map (D, F, C, and E) were markedly depleted relative to the leftward fragments (I, H, and G). In contrast, populations of intracellular DNA molecules (I, II) contained the rightmost fragment (D) in greater yield than the internal fragments C and B. As mentioned above, all DNA samples contained some contaminating full-length DNA, which accounts for the representation of all fragments at a background level.

More quantitative information on the representation of segments of the normal genome in the subgenomic DNA was obtained by scanning photographs of the stained gels with a densitometer. Figure 6 shows graphically the yields of the various *Bam*HI fragments from digests of defective DNAs relative to their representation in complete DNA. The representation of a fragment in complete DNA was proportional to the

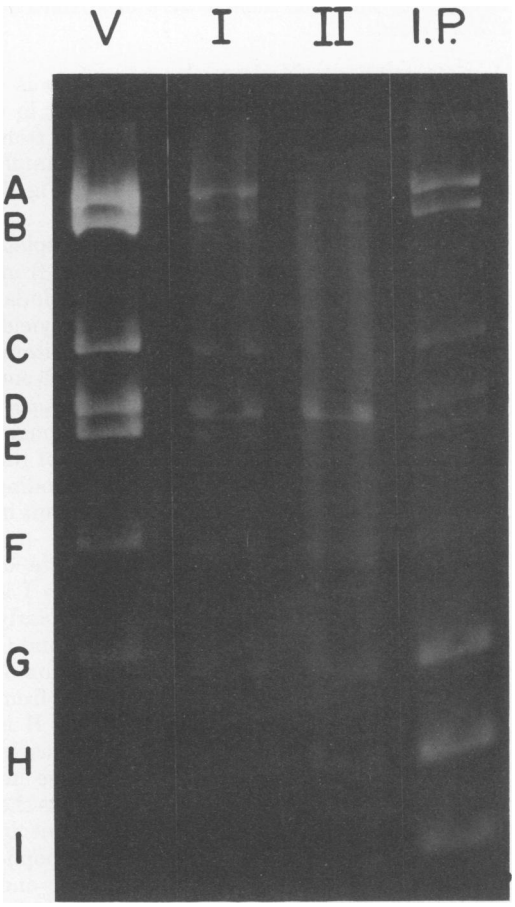


FIG. 4. *Restriction endonuclease digestion of full-length (V) and subgenomic (I, II) DNAs from infected cells and from incomplete particles (I.P.). Samples of each pool of DNA from the sucrose gradient shown in Fig. 3 were digested with restriction endonuclease BamHI and analyzed by gel electrophoresis through 1.4% agarose gels for 4 h at 50 V. One microgram of DNA from a preparation of total incomplete particles of Ad3 was also digested (leftmost gel lane). This preparation of DNA contained a considerable amount of full-length viral DNA (see lane labeled Ad3 D in Fig. 1a). The gel was stained and photographed under UV illumination.*

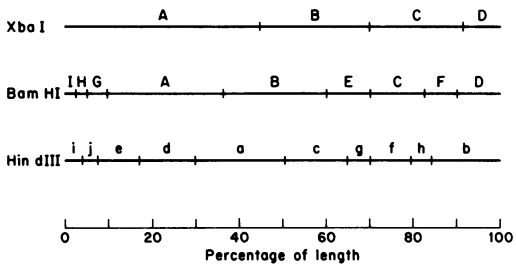


FIG. 5. *Sites of cleavage of three restriction endonucleases on Ad3 DNA. Specific data on sites of cleavage of Xba and BamHI were determined by Clark Tibbetts.*

length of the fragment; this ratio, then, was a measure of the molar yield of a fragment in a given sample. In Fig. 6, a sample of DNA from incomplete particles and an intracellular sample of similar size distribution (see legend to Fig. 6) were compared.

The data show that in DNA from incomplete particles there is a gradient of molar yield from left to right, whereas in subgenomic intracellular DNA the central fragments are in lowest yield.

The absolute values of the peaks in densitometer scans show that the right- and left-end sequences are present in roughly equal proportions. For example, fragment D is equal in length to the sum of I, H, and G at the left end of the molecule, and the amount of material banding as the latter fragment adds up to the amount in band D.

A similar analysis of a different isolate of intracellular DNA with endonuclease *Xba* I is shown in Table 2. This enzyme is particularly illustrative because internal fragments B and C are very close in size, and a visual inspection of the digested DNAs shows that in DNA from defective particles the yield of fragment B is greater than that of fragment C, whereas in intracellular subgenomic DNA the relative intensity of the bands is reversed. Table 2 lists the fractional representation of each fragment in digests from purified viral DNA, from a population of intracellular subgenomic DNA, and from DNA from incomplete particles. In the subgenomic intracellular DNA, the central fragment B, extending from 0.43 to 0.69 fractional map unit, is the fragment in lowest yield; in the incomplete particle DNA there is again a gradient in representation of fragments from left to right.

Electron microscopy of DNA from sucrose gradient fractions. DNA in subgenomic fractions from sucrose gradients was denatured and neutralized as described above to look for evidence of the long inverted terminal repetitions observed in packaged incomplete DNAs of Ad3 and Ad2 (2). As many as 40% of the molecules in these samples contained the intramolecular panhandle structures indicative of extended inverted terminal repetitions in the double-stranded DNAs (Fig. 7). Single-stranded circles (suggesting terminal repetitions too short to be seen, as in the complete viral genome [2, 6]) and more complex intramolecular structures were also seen. Detailed studies of these are in progress.

Influence of host cell type on production of incomplete DNA. We have been unable to show enrichment of defective particles by repeated high-multiplicity passage of Ad2, Ad3, or Ad5 in HeLa or KB cells, nor have we detected

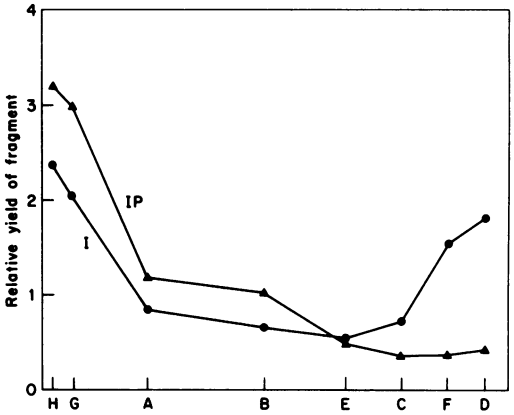


FIG. 6. *DNA from incomplete particles, complete virions, and subgenomic intracellular isolates was digested with BamHI and subjected to electrophoresis, as described in the legend to Fig. 4. The gel was stained and photographed with Polaroid type 55 film, and the negative was scanned with a densitometer (Joyce-Loebl). Peaks were cut out of the tracings and weighed, or the area under the peaks was measured with a Numonics graphics calculator. The fraction of the total DNA comprised by each fragment was determined. The graph shows the ratio between this value for each fragment in the incomplete DNA preparations and the value for the same fragment in the complete virion DNA. The positions on the ordinate of the graph are the midpoints of the given fragments in the restriction map. The distributions of size classes of DNA in the two subgenomic samples were determined by densitometer tracings of photographs of gels containing undigested DNA. The two samples analyzed here had similar distributions: 13% of the subgenomic DNA ranged in size from 0.58 to 0.95 fractional unit; 40% ranged from 0.40 to 0.58; 33% ranged from 0.30 to 0.40; 14% ranged from 0.25 to 0.30. The incomplete particle sample displayed a peak of full-length viral DNA amounting to 13% of the total subgenomic DNA; the intracellular sample showed only 4.5% contamination with full-length DNA. IP, Incomplete particle DNA; I, intracellular DNA.*

TABLE 2. *Fractional yield of DNA in the four restriction fragments from Xba digestion of Ad3 DNA from complete virions, incomplete particles, and subgenomic intracellular isolates*[a]

Fragment	DNA from complete virions	Intracellular subgenomic DNA[b]	Intracellular/V[c]	DNA from incomplete particles[d]	Incomplete/V
A	0.41	0.36	0.89	0.48	1.2
B	0.28	0.10	0.36	0.28	1.1
C	0.21	0.14	0.67	0.17	0.81
D	0.10	0.40	4.0	0.06	0.6

[a] Unlabeled DNA of each type was digested with *Xba* and subjected to electrophoresis, and the gel was stained and photographed with Polaroid type 55 film. The negative was scanned with a densitometer (Joyce-Loebl), and peaks were cut out of the tracing and weighed to ascertain the percentage of total DNA in each fragment. All of the area above background was included in some fraction; there was somewhat more diffuse material between fragment peaks in the incomplete preparations than in the virion DNA. Values for fragment lengths calculated from these measurements on virion DNA agree (within 2%) with independent length measurements.

[b] This preparation consists of DNA of lengths from 30 to 70% of full genome length.

[c] Ratios between the percent representation of a given fragment in the indicated incomplete DNA preparation and of its representation in full-length DNA are a measure of the molar yield of the fragment.

[d] This preparation contains DNA ranging in size from 10 to 100% of the full genome.

interference by incomplete particles (2). However, we have found that Ad5, a serotype that produces few if any defective particles in human cells, synthesizes a large proportion of short viral DNAs during DNA replication in (semipermissive) monkey cells (Daniell and Mullenbach, manuscript in preparation). Holland et al. (8) report that HeLa cells are unable to replicate or produce defective particles of vesicular stomatitis virus, although in other cell types vesicular stomatitis virus produces a variety of truncated RNAs. We studied Ad3 viral DNA synthesis in other human cell types that grow more slowly than do HeLa, yet also produce virus. A human diploid epithelial cell line from fetal intestine and two strains of human fibroblasts not established as a line (about passage 10 in culture) were used to grow Ad3. Low levels of viral DNA were produced; there were no detectable changes in the fractional yield or pattern of incomplete DNA from that in infection of HeLa cells.

DISCUSSION

We have shown that subgenomic viral DNAs can be isolated from Ad3-infected cells and that they comprise over 15% of the viral DNA synthesized. This DNA is made at all times during viral DNA replication, at a rate that apparently remains steady relative to the synthesis of complete DNA. We found no evidence of a precursor-product relationship between these short DNAs and full-length genomes. The distribution of sizes of incomplete DNA, while heterogeneous, yields a distinct pattern of bands in agarose gels; the pattern is identical to that observed in DNA samples extracted from incomplete particles of Ad3. These findings are consistent with the hypothesis that the predominance of specific size classes is independent of the packaging of subgenomic DNAs into the incomplete particles. We have obtained further supporting evidence for this model through a study of Ad5 grown on (semipermissive) monkey cells. A substantial fraction of the viral DNA molecules made in this system are subgenomic in length and fall into a characteristic array of sizes, although little of the viral DNA in this system is ever packaged (Daniell and Mullenbach, manuscript in preparation).

Many of the incomplete viral DNA molecules isolated either from incomplete particles or from infected cell nuclei have extended inverted terminal repetitions. Thus, they are neither cleavage products generated from completed viral genomes nor intermediates in normal viral DNA replication.

Molecules in the intracellular subgenomic population that do not contain inverted terminal repetitions may be fragments generated during extraction, perhaps as breakage products from fragile replicating forms. Since replication of viral DNA starts at the ends of the molecules (see below), such products could contribute to the observed predominance of terminal fragments.

This speculation requires the assumptions that newly replicated pieces of DNA would be broken off at the replication fork and that the remaining piece of the replication complex, which contains single-stranded DNA, would be removed from our subgenomic preparations by electrophoresis or sedimentation. We think it unlikely that such fragments are primarily responsible for the overrepresentation of molecular ends in our samples, since heteroduplex anal-

ysis shows that a large percentage of these molecules contain inverted repetitions.

We infer from these data that incomplete DNAs are formed by errors in DNA replication that occur at a constant rate throughout replication and that are governed by factors that we have not yet been able to define. Any model that purports to explain the appearance of these aberrant viral DNAs must take into account what is known about normal adenovirus DNA replication.

Although we are now rid of the problem of explaining why replicative errors generate incomplete DNAs preferentially containing one end of the viral genome, we are confronted with another problem: what event can produce specific size classes of incomplete DNA with the characteristics that we observe? The picture that emerges from these data is that incomplete molecules of a particular size may contain either the right-hand end sequences or left-hand end sequences. Sequence-specific errors (such as "stuttering" spots for a polymerase) would not be expected to yield such a distribution, since the normal viral genome is not symmetric; yet the sizes are reproducible and serotype specific and are not altered by exhaustive plaque purification of virus stocks. Closely related serotypes such as Ad2 and Ad5 yield similar patterns of defective molecules that are distinctly different from that exhibited by Ad3 (Daniell and Mullenbach, manuscript in preparation).

The generally accepted scheme for adenovirus replication is that both molecular ends of the viral genome can serve as origins and as termini (10, 11, 21, 25, 27). The large amount of single-stranded DNA produced by strand displacement during replication (19) consists of sequences from both strands of the virus in roughly equal amounts (15, 23). Flint et al. (5) report that replication intermediates of Ad2 and Ad5 contain excess single-stranded DNA homologous to the right end of the *r* strand and to the left end of the *l* strand of the genome; there is a gradient of excess sequences increasing toward the end of the DNA.

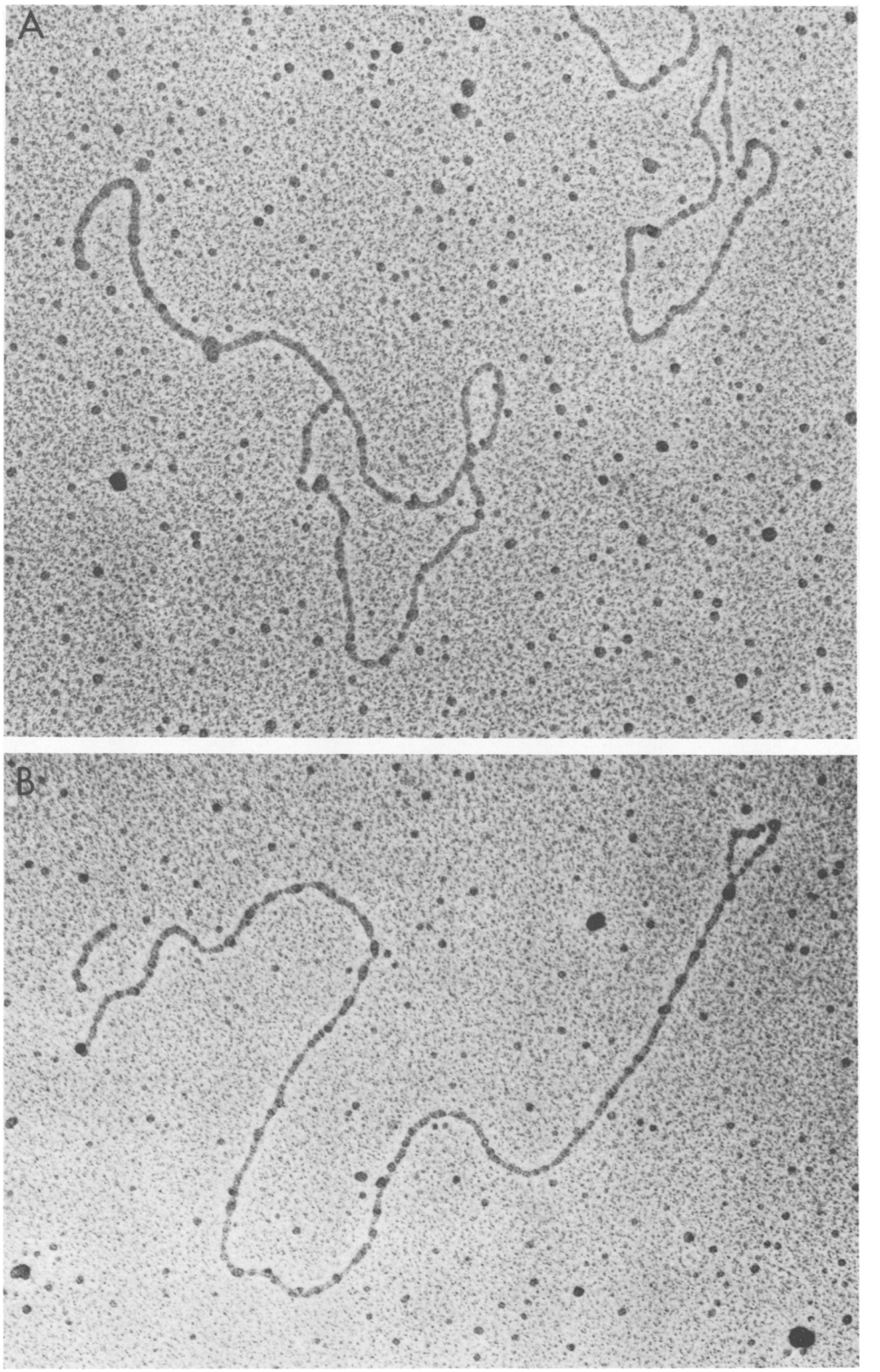
Our general model for the generation of incomplete viral DNAs is that normal replication is somehow interrupted and that inverted duplications of a partially displaced strand may occur (see Fig. 21 in reference 2). The finding that both ends of the normal genome are overrepresented in incomplete DNAs makes it easier to reconcile this model with the data that implicate both ends of the genome as origins of replication.

The factors that govern the production of defective DNAs and incomplete particles in adenovirus infection are not understood. Different rates of synthesis of viral DNA relative to viral transcription and translation may control differences between serotypes, as well as the minor variations we see from preparation to preparation. Systematic study of the effect that altered cell growth conditions have on viral DNA replication may augment our understanding of this phenomenon. Treatment of cells with cycloheximide inhibits protein synthesis and cellular DNA replication, but allows adenovirus DNA replication to proceed (12). Experiments are in progress to determine whether the presence of cycloheximide (at a time that shuts off late viral protein synthesis) alters the yield and/or array of incomplete DNAs in Ad3-infected HeLa cells.

The most obvious difference between intracellular and packaged incomplete genomes is that packaging has somehow selected against molecules that are missing the left end of the genome. Sequences in this region are perhaps required for the initiation of packaging or for the stability of completed particles. Another contrast is a quantitative one: at most, 1% of the total packaged DNA is subgenomic, whereas we find that over 15% of the intracellular DNA is subgenomic. It may be that incomplete DNA is less efficiently packaged than is complete DNA. In addition, incomplete particles are more fragile, and we may in fact destroy a greater fraction of incomplete particles than complete particles during repeated purification.

The size of the pool of incomplete DNAs generated by a particular serotype may correlate with the percentage of total virus particles that end up as incomplete species. Ad3 generates a high fractional yield of subgenomic DNAs relative to serotypes 2 and 5, and Ad3 particle preparations have a relatively large proportion of incomplete particles (2, 20). This possibility can be explored by inspecting the intracellular viral

FIG. 7. *Electron micrographs of typical "panhandle" structures seen in preparations of subgenomic DNA from Ad3-infected cells. A sample of DNA (average length as judged by electrophoretic mobility = 0.6 fractional map unit) purified by sedimentation through sucrose was denatured and allowed to renature at a very low concentration so that intramolecular homologies could be detected. Forty percent of the molecules in this preparation showed visible panhandle structures or were single-stranded circles (see text). Lengths (expressed as fractions of the complete viral genome) of double-stranded regions (ds), single-stranded loops (ss), and total (in single-strand units) DNA are as follows: (A) ds = 0.10, ss = 0.22, total = 0.42; (B) ds = 0.27, ss = 0.03, total = 0.57. The single-stranded circle in the upper panel is the single-stranded DNA of phage fd, used as a length standard in the preparations.*


A
B

DNA produced by other serotypes, such as Ad16 (26), known to yield high levels of incomplete particles.

Incomplete particles that are intermediates in viral assembly (and can be chased into complete particles) have been described (4, 13). Some classes of these "immature virions" contain DNA. The incomplete particles containing aberrant short DNAs are isolated late after infection (2). It seems likely that, at the step in maturation when DNA becomes associated with immature virions, a fraction of the particles will accumulate short DNAs from the intracellular pool. These may then be blocked in maturation because they do not contain a full complement of DNA and become the stable DNA-containing defective particles that can be isolated late in infection.

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