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The Length of the Terminal Repetition in Adenovirus-2 DNA

(exonuclease III/avian myeloblastosis virus DNA polymerase/restriction endonuclease)

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ABSTRACT **Adenovirus-2 DNA was end-labeled by partial digestion with *Escherichia coli* exonuclease III and resynthesis with the DNA polymerase from avian myeloblastosis virus and α-^{32}P-labeled deoxyribonucleoside triphosphates. This end-labeled DNA was cleaved with several specific endonucleases and the terminal fragments were characterized by gel electrophoresis and pyrimidine tract analysis. Two endonucleases gave identical fragments from both ends, presumably from cleavage within the inverted terminal repetition, while all other endonucleases gave dissimilar fragments from the two ends. From the sizes of these fragments it is estimated that the inverted terminal repetition is between 100 and 140 nucleotide pairs long.**

Adenovirus-2 DNA is linear with a molecular weight of about 23×10^6 (2). The molecule possesses an inverted terminal repetition, as shown by its ability to form single-stranded rings upon denaturation and renaturation at low DNA concentrations (3, 4). The DNAs of adenovirus serotypes 1, 3, 7, 18, and 31 (3) and of adeno-associated virus (5, 6), which is a small defective human virus that requires a helper adenovirus for productive infection, have the same structure. Several authors (3, 4, 7) have suggested a role for this structure in DNA replication and recently a circular DNA–protein complex has been seen by electron microscopy (7). Such a complex would require interaction between the terminally repetitious sequences and a binding protein.

The terminal repetition in adenovirus-2 DNA was estimated to be more than 350 nucleotides long (3), as judged by the effect of exonuclease III digestion on ring formation. Another estimate of 200 to 500 nucleotides (4) was based on heat stability and the absence of a duplex projection visible in electron micrographs of single-stranded rings. This seems inconsistent with the finding of a visible projection only 40 to 100 nucleotide pairs long in an adeno-associated virus DNA (6).

We have made use of specific endonucleases to examine directly the terminal regions of the adenovirus-2 genome. This analysis takes advantage of intrinsic properties of an inverted terminal repetition as illustrated in Fig. 1. The structure requires that cleavage within the terminal repetition produce identical terminal fragments, whereas cleavage beyond the terminal repetition would be expected to produce dissimilar fragments from each end. Analysis of terminal fragments, as described below, generated by a number of specific endonucleases suggests that the inverted terminal repetition is between 100 and 140 nucleotides long.

Abbreviations: AMV, avian myeloblastosis virus; Ad-2, adenovirus 2; SV40, simian virus 40; EDTA, ethylenediaminetetraacetate. Restriction endonucleases are named in accordance with the proposals of Smith & Nathans (1).

MATERIALS AND METHODS

Enzymes. Exonuclease III from *Escherichia coli* (8, 9) was obtained as a by-product of DNA polymerase I preparations (10). Contaminating endonuclease was removed by chromatography on phosphocellulose and DEAE-Sephadex. Avian myeloblastosis virus (AMV) DNA polymerase was purified by a modification of the procedure of Kacian *et al.* (11), using chromatography on DEAE-cellulose and phosphocellulose and sucrose gradient centrifugation. One unit of activity is defined as the amount of enzyme that incorporates 1 nmol of TMP into trichloroacetic acid-precipitable material in 10 min at 37° when assayed with poly(rA)·oligo(dT). Endonucleases *Eco*RI (12), *Hpa* I (13), *Hpa* II (13), *Hind*II/III (14), and *Hae* III (Endonuclease Z) (15) were purified as described previously (12–15). *Hae* II, a second endonuclease from *Haemophilus aegyptius* (R. J. Roberts, J. B. Breitmeyer, N. F. Tabachnik, and P. A. Myers, to be published); *Hha* I, an endonuclease from *Haemophilus haemolyticus* (R. J. Roberts and P. A. Myers, to be published); and *Hph* I, an endonuclease from *Haemophilus parahaemolyticus* (J. H. Middleton, P. V. Stankus, M. H. Edgell, and C. A. Hutchison III, personal communication) were also used. One unit of each endonuclease is the amount required to completely digest 1 μg of adenovirus-2 (Ad-2) DNA in 1 hr.

Viral DNAs. Ad-2 was grown in suspension cultures of human KB cells. Both unlabeled and ^{32}P-labeled DNA were prepared from purified virus as described previously (16, 17). The DNA was stored at 4° in 0.01 M Tris·HCl (pH 7.9), 0.1 mM Na_2 ethylenediaminetetraacetate (EDTA). Simian virus 40 (SV40) DNA was a gift from B. Sugden.

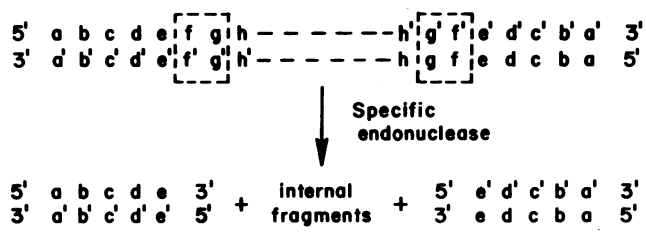

FIG. 1. A possible structure for the inverted terminal repetition in adenovirus-2 DNA (4). Cleavage by a specific endonuclease at a recognition site within the terminal repetition (indicated by boxes) generates an identical fragment from each end. It is important to note that this is an intrinsic property of an inverted terminal repetition and does not require a palindromic recognition site.

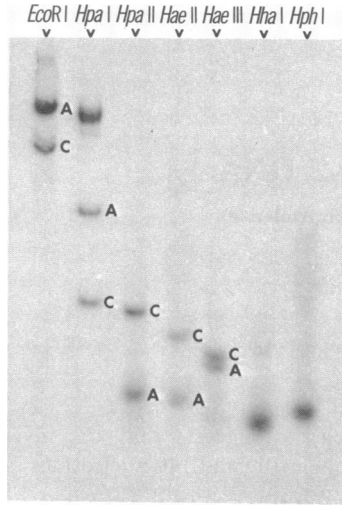

FIG. 2. Agarose gel electrophoresis of specific endonuclease digests of end-labeled adenovirus-2 DNA. Ad-2 DNA was resected with exonuclease III for 3 min and repaired with AMV DNA polymerase as described in *Materials and Methods*. Aliquots were then treated with various specific endonucleases, the digests were fractionated on 1.4% agarose gels, and the terminal fragments were located by autoradiography. The endonuclease used for each digest is indicated above the channel and labeled to indicate its origin: A, from the *Eco*RI "A" end; C, from the *Eco*RI "C" end. The upper band in the *Hpa* I digest is due to incomplete digestion.

End-Labeling of Adenovirus-2 DNA. (*a*) Partial digestion with exonuclease III. Reaction mixtures contained, in a total volume of 60 μl: 0.01 M Tris·HCl (pH 7.9), 2 mM $MgCl_2$, 12.5 μg of Ad-2 DNA, and 3 units (9) of exonuclease III. The extent of reaction was controlled by incubation for various times at 37° and was stopped by heating the mixtures at 65° for 5 min.

(*b*) Resynthesis. Reaction mixtures contained, in a total volume of 100 μl: 0.06 M Tris·HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiothreitol, 10 μM of three unlabeled deoxyribonucleoside triphosphates (Schwarz/Mann), 5 μM [α-^{32}P]deoxyribonucleoside triphosphate (specific activity approximately 100 Ci/mmole; New England Nuclear Corp.), 10% glycerol, 2.5 units of AMV DNA polymerase, and 12.5 μg of exonuclease III treated Ad-2 DNA [from part (*a*)]. The mixtures were incubated at 37° and the reaction was monitored by withdrawing 1-μl aliquots at various times and measuring the amount of radioactivity that became trichloroacetic acid-precipitable. The reactions were usually complete after 2 hr.

Endonuclease Digestion. Reaction mixtures contained, in a total volume of 50 μl: 6 mM Tris·HCl (pH 7.9), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 2 μg of unlabeled Ad-2 DNA, 20,000 cpm end-labeled Ad-2 DNA (about 0.02 μg), and 2 units of endonuclease. Incubation was carried out at 37° for 1 hr and terminated by the addition of 5 μl of 0.1 M Na_2 EDTA, 5% sodium dodecyl sulfate.

Gel Electrophoresis. Agarose gels (1.4%) were prepared as described by Sharp *et al.* (13) except that 20 cm × 20 cm × 0.3 cm slabs were used (B. Sugden, personal communication). Electrophoresis was carried out on polyacrylamide slab gels as described by DeWachter and Fiers (18). Samples were adjusted to 10% sucrose–0.025% bromophenol blue before loading onto the gels. Radioactive bands were located by radioautography.

Pyrimidine Tract Analysis. Radioactive fragments were eluted from gels electrophoretically, purified by treatment with phenol, and precipitated with ethanol. They were redissolved in 200 μl of 0.01 M Tris·HCl (pH 7.9), 1 mM Na_2 EDTA, and depurinated by the procedure of Ling (19). The pyrimidine tracts were fractionated by electrophoresis in the first dimension on cellulose acetate at pH 3.5 and homochromatography (20) in the second dimension using a 3% "homomix d" (21).

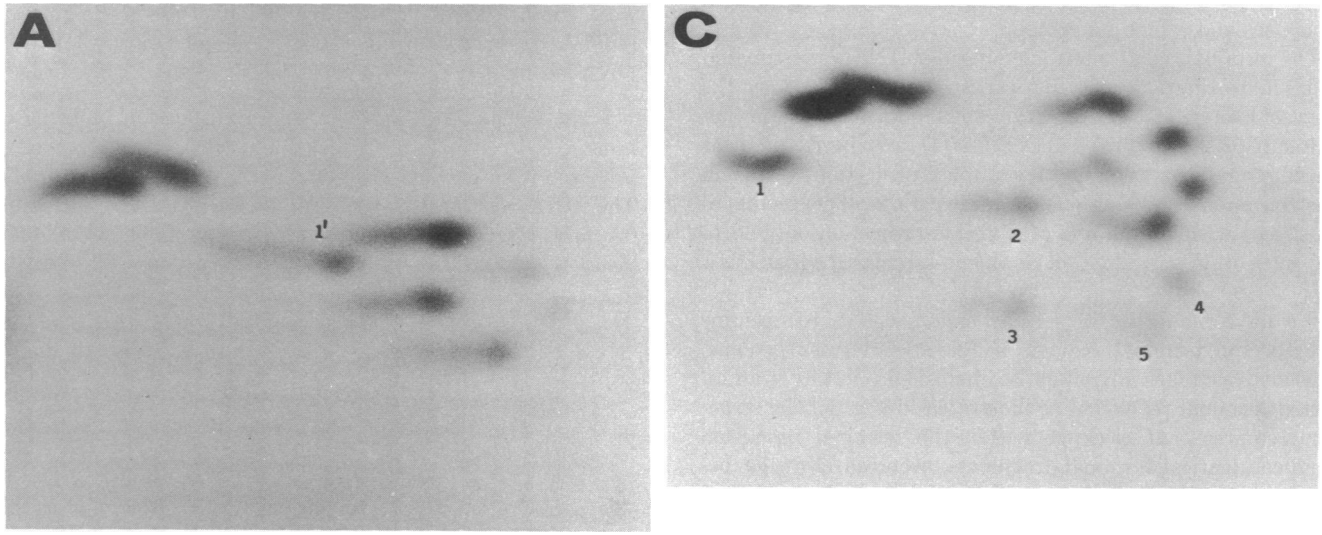

FIG. 3. Pyrimidine tracts from the terminal region of the adenovirus-2 genome. Terminal fragments of the Ad-2 genome were prepared with the specific endonucleases *Eco*RI, *Hpa* I, *Hpa* II, *Hae* II, and *Hae* III, and fractionated as described in the legend to Fig. 2. They were then eluted, and the pyrimidine tracts were prepared as described in *Materials and Methods*. Two distinct patterns were obtained: (A) the typical pattern from the fragments labeled A in Fig. 2 and originating from the A end; (C) the typical pattern from the fragments labeled C in Fig. 2 and originating from the C end. The actual patterns shown came from *Hpa* II terminal fragments. In both cases [α-^{32}P]thymidine triphosphate was used to label the adenovirus-2 DNA.

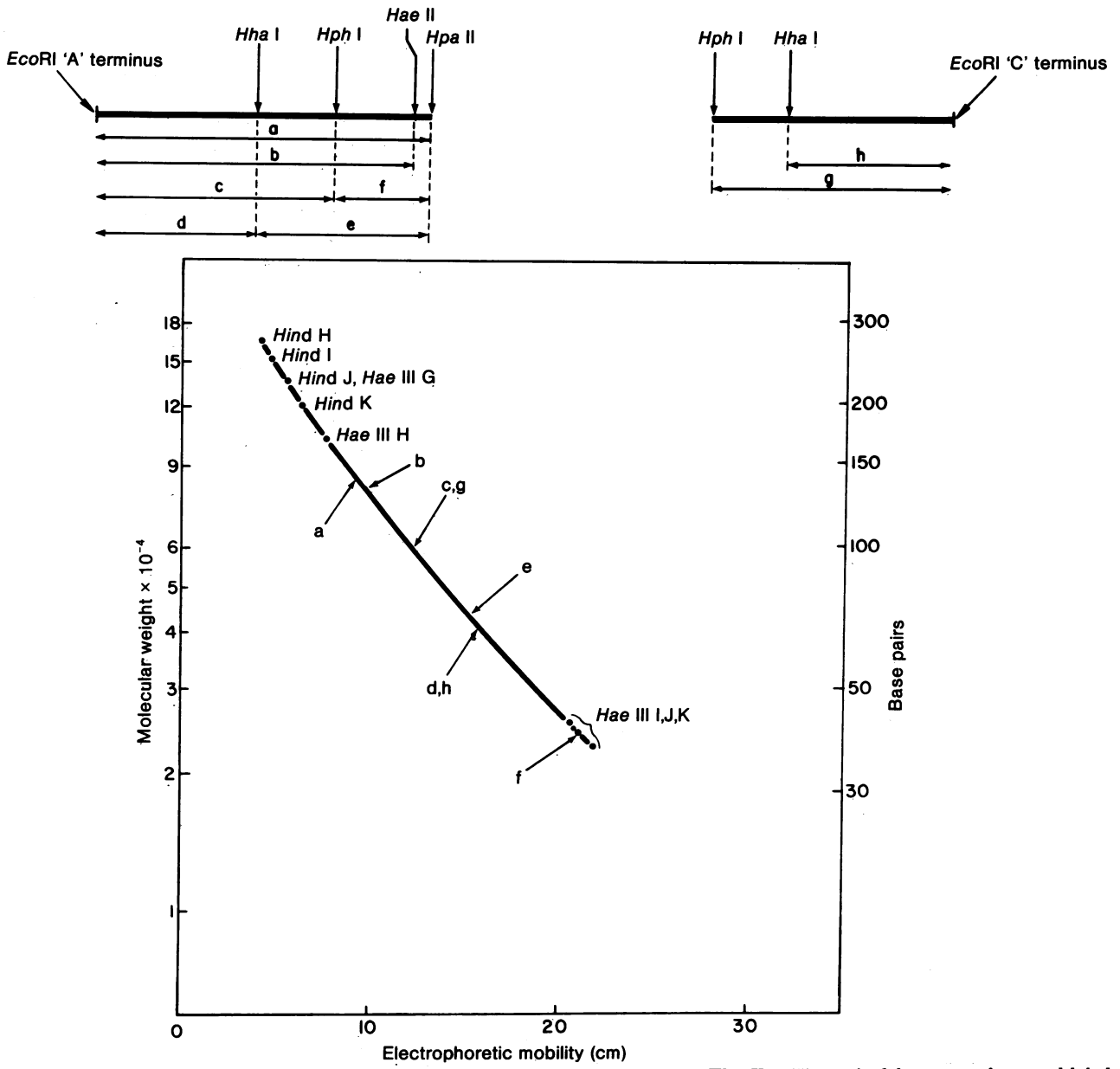

FIG. 4. Estimated sizes of some terminal fragments from the adenovirus-2 genome. The *Hpa* II terminal fragments from end-labeled adenovirus-2 DNA were prepared as described in *Materials and Methods*. They were then redigested with the specific endonucleases *Hha* I and *Hph* I and fractionated on a 10% polyacrylamide gel. Unlabeled fragments of SV40 DNA, prepared by digestion with *Hae* III or with *Hin*dII/III, were run alongside as markers and their position located by staining with ethidium bromide (13). In the lower part of the figure, relative electrophoretic mobilities are plotted against the log of the molecular weights of the various fragments for each gel system. The upper part of the figure indicates the origin of the various fragments within (A), the *Eco*RI A terminal region, and (C), the *Eco*RI C terminal region.

RESULTS

Endonucleolytic Cleavage of End-Labeled Adenovirus-2 DNA. The RNA-dependent DNA polymerase from avian myeloblastosis virus will catalyze the repair of DNA whose 3′ ends have been removed by *E. coli* exonuclease III (22). We have used this reaction to end-label adenovirus-2 DNA previously digested with exonuclease III under conditions that led to the removal of about 40 nucleotides from each end. The ends were repaired by AMV DNA polymerase in the presence of [α-^{32}P]deoxyribonucleoside triphosphates. The end-labeled DNA was cleaved by a number of specific endonucleases, and the digests were fractionated by agarose slab gel electrophoresis. Radioautography revealed the labeled bands and permitted the isolation of terminal fragments. The labeled terminal fragments produced after digestion with *Eco*RI and *Hpa* I could be assigned to well resolved bands by comparison of the radioautograms with stained gels. The results for seven restriction endonucleases are shown in Fig. 2. Five of these endonucleases (*Eco*RI, *Hpa* I, *Hpa* II, *Hae* II, and *Hae* III) gave dissimilar fragments from the two ends, whereas two (*Hha* I, *Hph* I) gave either identical or very similar fragments. Sizing by electrophoresis of the *Hha* I and *Hph* I fragments on a 10% polyacrylamide gel failed to resolve the two ends, and we conclude that they are identical and result from cleavage within the inverted terminal repetition.

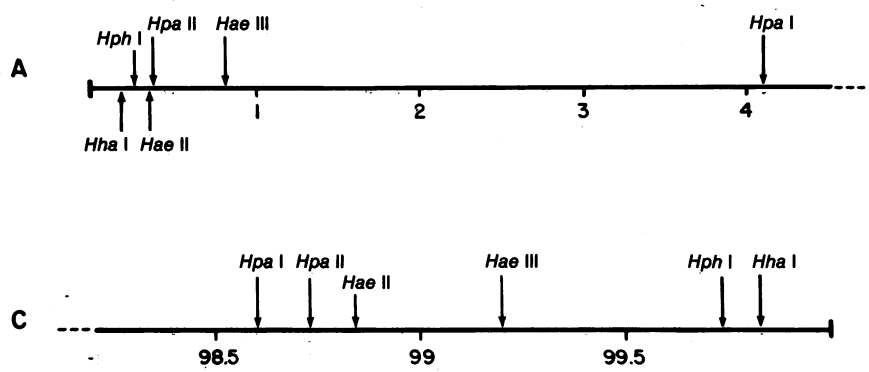

FIG. 5. Cleavage sites of six specific endonucleases close to the termini of the adenovirus-2 genome. The data shown in this figure are a composite of those obtained in Figs. 2 and 4. Sizes of the larger fragments were estimated from their electrophoretic mobilities on agarose gels, as compared with fragments from adenovirus-2 DNA of known size—the *Eco*RI fragments (17) and the *Hpa* I fragments (R. J. Roberts and P. A. Sharp, unpublished). (A) location of sites at the A end; (C) location of sites at the C end. The scales are percent of the genome, based on a molecular weight of 23×10^6 (2).

Pyrimidine Tract Analysis. After treatment with each of the five endonucleases yielding dissimilar terminal fragments, we sought to assign each fragment to its appropriate end by analysis of pyrimidine tracts in fragments labeled after removal of approximately 150 nucleotides. The method of labeling ensured a similar length of labeled DNA within each fragment so that despite the differing lengths of the various fragments direct comparison of the labeled pyrimidine tracts was possible. Two distinct families of patterns were observed, and an example of each is shown in Fig. 3. There is at least one oligonucleotide unique to each fingerprint, and it can be used to assign each fragment as originating from either the A end* or the C end* of the adenovirus-2 DNA. This information is included in Fig. 2. This evidence is consistent with the idea that the *Hpa* II site at the A end lies just outside the terminal repetition; however, further sequence analysis will be necessary to rigorously prove it.

Sizes of the Small Terminal Fragments. Estimates of the length of the inverted terminal repetition were obtained by sizing the smallest fragments produced by these seven specific endonucleases. For this purpose we chose the terminal fragments produced by *Hha* I and *Hph* I, originating from within the terminal repetition, and those produced by *Hae* II and *Hpa* II, coming from outside the repetition. The strategy adopted was to first cleave end-labeled adenovirus-2 DNA with *Hpa* II, separate the two terminal fragments, and then redigest samples of each fragment separately with *Hha* I and *Hph* I. To measure sizes, all the fragments were then subjected to electrophoresis on 10% polyacrylamide gels, with *Hin*dII/III and *Hae* III fragments of SV40 as markers (23, 24). The results of this analysis are shown in Fig. 4 and indicate that the *Hha* I and *Hph* I terminal fragments are approximately 70 and 100 nucleotide pairs long, thus serving to define 100 nucleotide pairs as the minimum length for the inverted terminal repetition. A maximum length is provided by the *Hae* II and *Hpa* II fragments from the A end which are about 140 nucleotide pairs long. Because of the absence of markers close to the size range of interest, these fragments were also run on 6% polyacrylamide gels in order to obtain a better estimate of the sizes of the *Hpa* II and *Hae* II fragments from the A end. Identical estimates for the sizes of these fragments were obtained.

In order to rule out the possibility of artifacts arising from the *in vitro* labeling procedure, we have also used adenovirus-2 DNA uniformly labeled *in vivo* with ^{32}P to prepare these terminal fragments. In this case we first prepared the *Hpa* I terminal fragments and then redigested them with *Hpa* II, *Hha* I, and *Hph* I. Electrophoresis on a 10% polyacrylamide gel gave results identical to those obtained with the end-labeled material. In all cases the fragments labeled *in vivo* and the end-labeled fragments exhibited identical electrophoretic mobilities (data not shown).

Attempts to redigest the small *Hpa* II fragment with *Hae* II usually resulted in incomplete cleavage. Consequently the size of this *Hae* II fragment was estimated in a second experiment in which intact adenovirus-2 DNA was cleaved separately with *Hpa* II and *Hae* II and then fractionated on a 10% polyacrylamide gel. Under these conditions the *Hae* II terminal fragment consistently ran slightly ahead of the *Hpa* II fragment, a result suggesting that the cleavage site for *Hae* II is slightly closer to the terminus than the *Hpa* II site is. The mobility difference was too small to allow an accurate estimate of size difference; however, we would judge it to be less than seven base pairs (5% of the size of the *Hpa* II fragment, which is probably the limit of our accuracy). The failure of *Hae* II to cleave this *Hpa* II fragment may be a result of the proximity of the recognition sequence to the end of the fragment.

A composite diagram showing the various sites of cleavage adjacent to the adenovirus-2 termini is shown in Fig. 5.

DISCUSSION

The data presented in this paper show that the recognition sequence for *Hha* I occurs about 70 nucleotide pairs in from each end of adenovirus-2 DNA. It is unlikely, but of course possible, that these sequences would occur by chance within an otherwise dissimilar sequence. The finding of a second identical stretch, the *Hph* I recognition sequence, some 100 nucleotides from each terminus considerably strengthens the argument that both recognition sequences occur within the inverted terminal repetition and therefore serve to define a lower limit to its length.

* The ends of the adenovirus-2 genome are termed A-end and C-end as determined by the *Eco*RI fragments within which each is contained.

An upper limit for the length of the terminal repetition is provided by the finding that two further specific endonucleases, *Hpa* II and *Hae* II, both cleave adenovirus DNA within a few nucleotide pairs of each other at about 140 nucleotide pairs from the A end. Neither of these enzymes cleaves the C end at similar sites. Therefore, these sites must lie beyond the terminal repetition.

The exact lengths of these fragments could be in error because of their small size, which would magnify the effect of base composition on electrophoretic mobility in polyacrylamide gels (25). We have not attempted to improve our data at this stage because we are at present deducing the complete sequence of the *Hpa* II fragment from the A end. From an analysis of the terminal sequences present at both ends it will then be possible to define exactly the extent of the terminal repetition and also to define the exact lengths of these terminal fragments. Furthermore, as the number of available restriction enzymes increases, other cleavage sites close to the termini will be found, and it will be possible to use this kind of analysis to quickly examine the DNA of many adenovirus serotypes for the extent of the terminal repetition and also for their homology with adenovirus-2 DNA.

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