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NUCLEOTIDE SEQUENCE AT THE INVERTED TERMINAL REPETITION OF ADENOVIRUS TYPE 2 DNA

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SUMMARY: We have determined the nucleotide sequence of the inverted repetition present at the termini of adenovirus type 2 DNA. The terminal repetition is 103 nucleotides long. It is exactly identical in sequence at both termini. Adenovirus types 2 and 5 molecules share a perfect homology within this region.

Human Ad 2* contain linear double-stranded DNA molecules with a molecular weight of 20-25 million (2,3). Ad 2 DNA molecules do not have terminal redundancy of the type abc....abc that is present in many bacteriophage DNAs (2). However, Ad 2 DNA possesses a unique inverted terminal repetition of the type a'b'c'....cba, which permits the formation of single-stranded circular DNA after denaturation and reannealing (4,5). The length of this inverted terminal repetition has been estimated at 100 to 140 nucleotides long by the use of specific restriction endonucleases (6).

The terminal sequences are presumably biologically important because of the fact that the origin and termination of DNA replication have been localized in these regions (7-11). All the available data support the notion that Ad DNA replication starts at either one of the two molecular ends, proceeds displacing one of the parental strands and terminates at the other end. The displaced strand is replicated in the opposite direction after completion of the displacement synthesis. A model has been proposed for the replication of the displaced strand involving the region of inverted terminal repetition present in adenovirus DNA (11). There is recent evidence that the initiation of DNA replication occurs within the terminal 75 base pairs (12), although the mechanism of initiation is still unknown.

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*Abbreviations used: Ad 2, Adenovirus type 2; Sma I, Eco RI, and Hind III, the restriction endonucleases from Serratia marcescens (see Ref. 1), E. coli, and Haemophilus influenzae Rd, respectively. SDS, sodium dodecyl sulfate.

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The sequence analysis of the terminal 194 nucleotides at both ends of Ad 5 DNA indicated that the inverted terminal repetition is 103 nucleotides long and is exactly identical in sequence (13). We report in this communication that the sequence analysis of the inverted terminal repetition in Ad 2 DNA is exactly identical in length and in sequence to that present in Ad 5 DNA.

MATERIALS AND METHODS: Ad 2 was grown in suspension cultures of human KB cells and virus DNA was purified as reported earlier (14). EcoRI, and Hind III were purchased from Biolabs (Boston, Massachusetts). Sma I was a generous gift of Dr. Werner Buettner. DNA polymerase I was purified as described by Jovin et al. (15). (α-^{32}P) dGTP (specific activity 2000-3000 Ci/mmole) was purchased from Amersham/Searle. The other labeled dNTP were obtained from New England Nuclear (Boston, Massachusetts). Dimethyl sulfate was obtained from Aldrich Chemical Co., hydrazine (95%) from Eastman Chemicals (Rochester, N.Y.) and piperidine (99%) from Fisher Scientific Co. Acrylamide and bis-acrylamide were purchased from Eastman Chemicals and were purified using an ion-exchange resin (16). Urea was purchased from Schwarz-Mann (Orangeburg, N.Y.).

DNA polymerase I-catalyzed incorporation of labeled nucleotides. The incorporation of labeled nucleotides was catalyzed by DNA polymerase I at 5°C and a salt concentration of 180 mm NaCl equivalents (17). The procedure followed for the nearest neighbor analysis of the labeled DNA was the same as described elsewhere (18). To prepare the labeled DNA for restriction enzyme digestion, the DNA polymerase I reaction was terminated by the addition of 50 μl of a mixture containing 2% SDS, 10 mM EDTA and 10 mM Tris HCl, pH 7.4. The reaction mixture was then extracted with phenol and subsequently dialyzed against TEN (10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 0.1 M NaCl) buffer. The labeled DNA was precipitated with 2.5 volumes of ethanol.

Digestion of terminally labeled DNA by Sma I. The 3' labeled Ad 2 DNA (50-100 μg) was digested with Sma I (19) in a reaction mixture (410 μl) containing 6 mM Tris-HCl, pH 9.0, 6 mM $MgCl_2$, 15 mM KCl, 1 mM dithiothreitol and 50 units of the enzyme. The incubation was carried out at 32°C for 5 hrs and the completion of the reaction was checked by 1.4% agarose gel electrophoresis.

Isolation of 3'-labeled Sma I terminal fragments of Ad 2 DNA. Sma I cleaves Ad 2 DNA into 13 fragments (19) A-M and its characteristic pattern shown in Fig. 1 is different from that of Ad 5 DNA (see Ref. 20). The latter is cleaved by the enzyme into 14 fragments, A-N. The Sma I terminal fragments of Ad 2 DNA, J and K (Fig. 1) are derived from the left and right ends of the DNA, respectively. The fragments were separated by electrophoresis on 1.4% agarose gels. The 3' labeled J and K fragments were purified by hydroxyapatite chromatography as described (16).

DNA sequence analysis. The chemical degradation reactions were carried out exactly as described by Maxam and Gilbert (21). Specific cleavage at guanine residues was achieved by methylation with dimethyl sulfate, at adenine residues by methylation with dimethyl sulfate followed by HCl treatment, at cytosine and thymine residues by hydrazinolysis and at cytosine residues alone by hydrazinolysis in 2 M NaCl, in all cases followed by incubation with piperidine except for adenine cleavage which was incubated with alkali. The reaction products were fractionated on 12 and 20% polyacrylamide slab gels (80 x 22 x 0.075 cm or 40 x 20 x 0.075 cm) containing 50 mM Tris-borate, pH 8.3, 1 mM EDTA, 7 M urea, at 600 - 1200 V for 8 to 72 hrs. The same Tris-borate buffer without urea was used as reservoir buffer. After electrophoresis, the gels were exposed to x-ray film with intensifying screen (Dupont Cronex, Hi-speed) at -20°C in the dark for 3 to 5 days.

RESULTS: Ad 2 DNA served as a substrate for DNA polymerase I-catalyzed repair synthesis. This was shown by the incorporation of two dG residues (one at each 3' end) in the presence of (α-^{32}P) labeled dGTP (Table I, Expt. 1). The nearest neighbor analysis of the labeled DNA showed the presence of dT at both 3' termini of native Ad 2 DNA.

TABLE I

Incorporation of deoxynucleotides at the termini of Ad 2 DNA

Expt.	(α-^{32}P) nucleoside triphosphate added	Time Hrs.	Residues incorporated/DNA molecule	Products found after enzyme digestion 3' nucleotides
1	d$G\overset{*}{T}P$	3.5	1.3	1.9 $\overset{*}{T}p$; less than
		5	2.0	0.1 each of other 3
		6.5	2.1	nucleotides
2	d$T\overset{*}{T}P$	3.5	0.3	0.5 $\overset{*}{A}p$; less than
		5	0.5	0.1 each of other 3
		6.5	0.7	nucleotides

The conditions for the incorporation of nucleotides catalyzed by DNA polymerase I and the nearest neighbor analysis of the labeled DNA were the same as described elsewhere (17). The number of residues incorporated per molecule of Ad 2 DNA were calculated using a molecular weight of 23 x 10^6 for Ad 2 DNA (2) and an absorbance of 6.6 at 260 nm being equal to 1 μ mole nucleotide/ml. The specific activities of deoxynucleoside triphosphates were accurately determined using the DNA polymerase I-catalyzed repair synthesis at the cohesive ends of P2 DNA which are 19 nucleotides long (26,27) as the control.

When the enzymatic repair synthesis was carried out in the presence of (α-^{32}P) labeled dTTP alone (Table I, Expt. 2), the rate was only about one-third of that of the incorporation of dG residues. Labeled dATP or dCTP did not serve as substrate either alone or in the presence of other unlabeled dNTP for the DNA polymerase I reaction (R. Padmanabhan, unpublished observations). The nearest neighbor analysis of dTMP incorporated DNA showed the transfer of (^{32}P) to dA. These data gave the sequence of d(A-T-G-OH) at each 3' end of Ad 2 DNA and hence the complementary sequence, d(C-A-T-) at the 5' end of Ad 2 DNA. These results are consistent with the tetranucleoleotide sequence reported by Steenbergh et al. (22).

For sequence analysis using the procedure established by Maxam and Gilbert (21), Ad 2 DNA was labeled at the 3' end in a DNA polymerase I catalyzed repair synthesis in the presence of (α-^{32}P) dGTP (2000-3000 Ci/mmole). The labeled DNA was digested with Sma I. The terminal J and K fragments (see Fig. 1) were isolated and subjected to the chemical degradation procedure (21). Fig. 2 shows an autoradiograph of a 20% polyacrylamide gel. The sequence that can be unambiguously read off the gel is shown in Fig. 3a. The intensity of the bands in the adenine cleavage reaction (A slots in Fig. 2J & K) are low compared to the other three slots, presumably due to the loss in the recovery of the sample before loading into the gel. As shown in Fig. 2, the sequence is exactly identical for both J and K fragments.

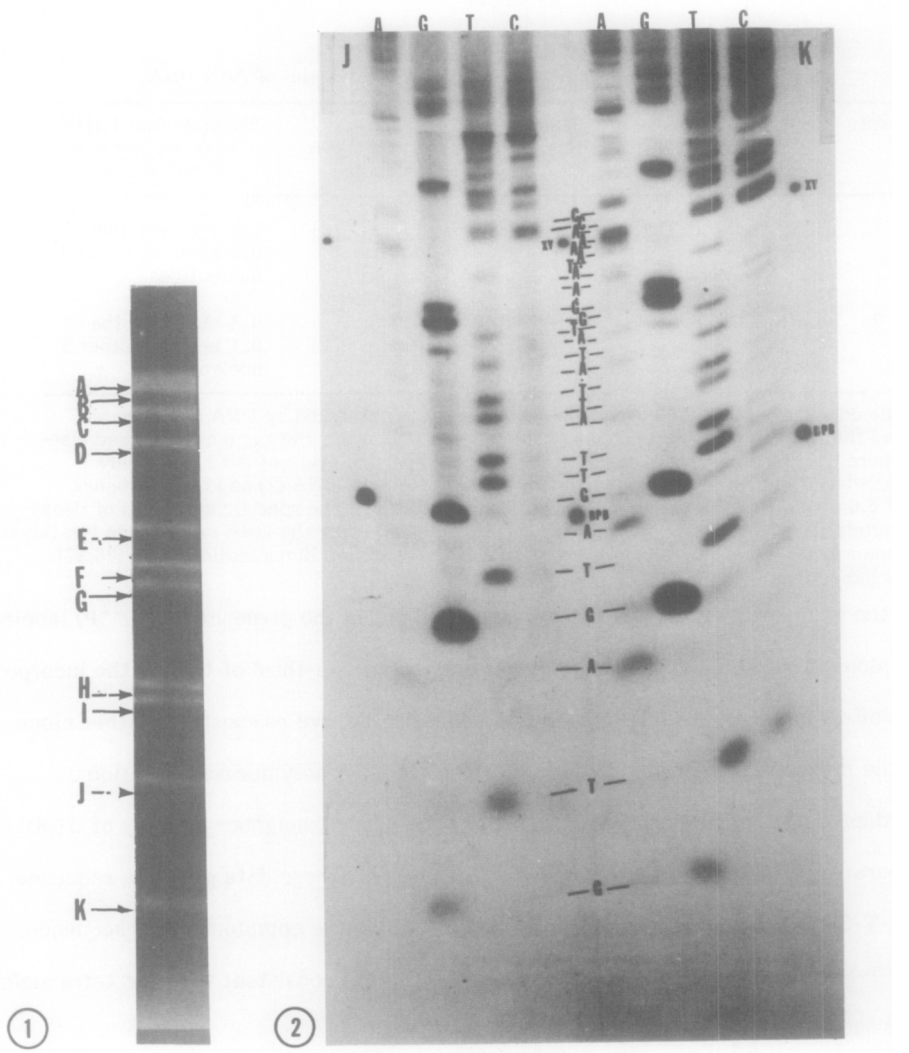


Fig. 1 Cleavage pattern of Ad 2 DNA obtained using Sma I.

Ad 2 DNa was digested with Sma I as described under "Methods". The fragments were dephosphorylated by treatment with E. coli alkaline phosphatase and labeled at their 5' termini using polynucleotide kinase and γ-AT^{32}P as described (16). The 5' labeled fragments were separated by gel electrophoresis on 1.4% agarose and detected by autoradiography. The small fragments L and M ran out of the gel under the conditions of electrophoresis. In another Expt. the separated Sma I fragments of Ad 2 DNA were detected by their ethidium bromide fluorescence (28).

Fig. 2 DNA sequencing gel (21) of the terminal Sma I-J and K fragments.

Ad 2 DNA was labeled at its 3' termini using DNA plymerase I-catalyzed repair synthesis as described in the text. The 3' labeled DNA was digested with Sma I and the terminal J and K fragments (19) were separated (see Fig. 1) and purified (16). They were subjected to DNA sequence analysis by chemical degradation (21). A 20% polyacrylamide gel containing 7 M urea was used. The positions of Xylene Cyanol (XY) F.F. and bromophenol blue (BPB) dyes are marked. The sequence that is read from this gel is shown in Fig. 3a.

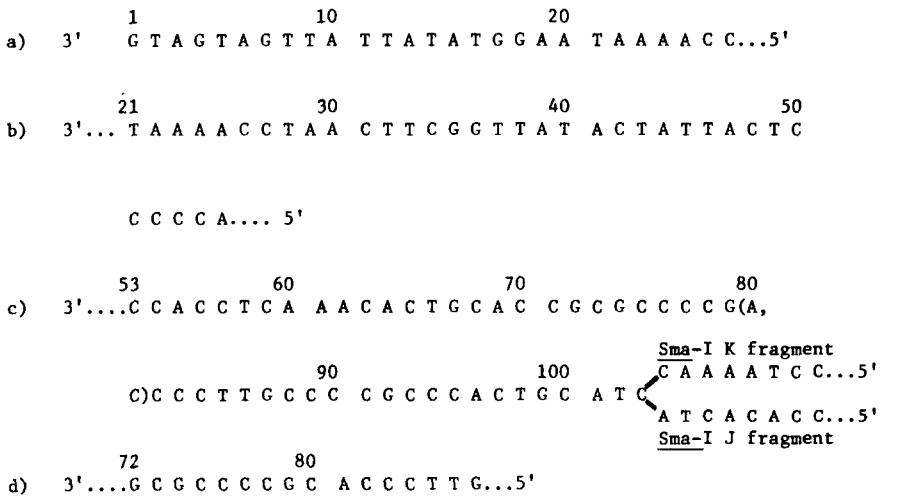
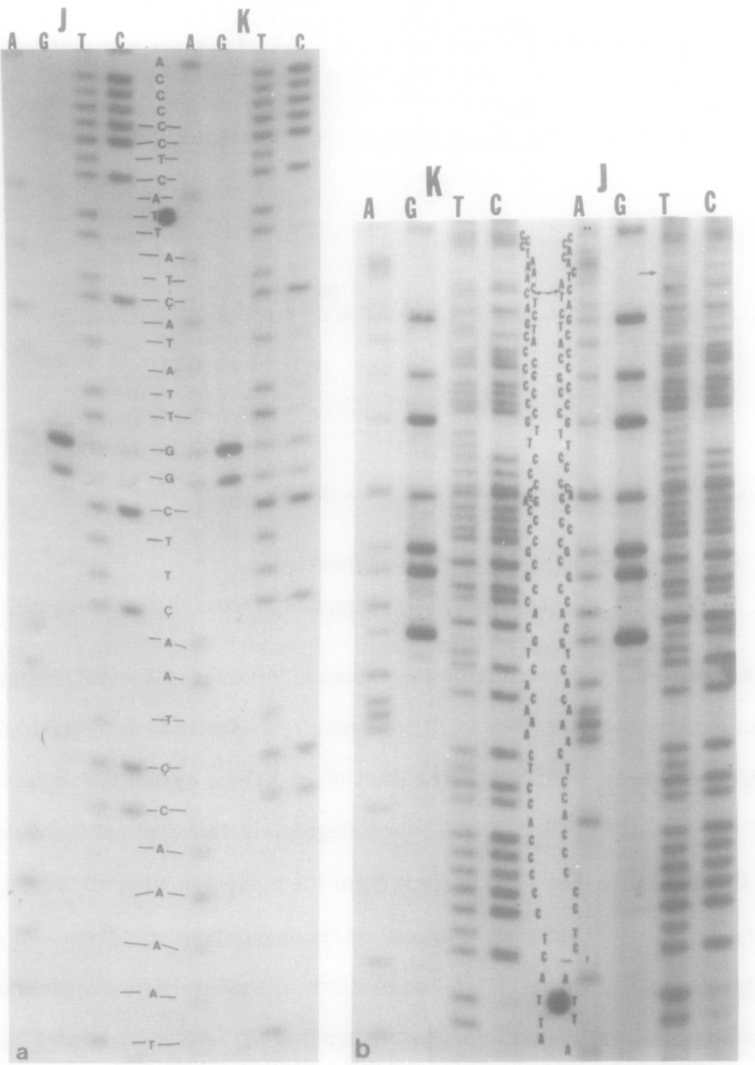


Fig. 3 Sequence derived from Maxam-Gilbert gels.
DNA sequence that is read unambiguously from (a) Fig. 2, (b)-(d), Fig. 4, a - c.

Another set of samples derived from the chemically degraded J and K fragments were loaded into a 12% gel (80 x 22 x 0.075 cm) and electrophoresed until the bromophenol blue dye just ran out of the gel. The position of Xylene cyanol F.F. dye is shown as a dark dot in the center of Fig. 4. The autoradiograph of this long gel is shown in two parts, in Fig. 4a and b. Fig. 4a is derived from the bottom portion of the gel and Fig. 4b is from the top. The sequence that can be read unambiguously from Fig. 4 (a & b) is shown in Fig. 3b and 3c, respectively. The patterns of base specific chemical degradation were once again identical upto nucleotide number 103 (shown by arrows in Fig. 4b) from the original 3' ends of both J and K fragments.

The sequence of nucleotide, G(A,C)C, from 79 - 82 shown in Fig. 4b could not be derived accurately as the bands were not well resolved. In order to clarify the sequence in this region, we repeated the experiment under identical conditions, except that the duration of the electrophoresis run on a 12% polyacrylamide gel was longer in order to improve the resolution of these bands. The autoradiograph of the gel showing the sequence of this region is shown in Fig. 4c. The sequence between nucleotide 79 - 82 is clearly G-C-A-C and is identical for both J and K fragments.

DISCUSSION: The presence of an inverted terminal repetition was suggested by the observation using electron microscopy, that denaturation of native adenovirus DNA followed



by renaturation at low concentration leads to the formation of single-stranded circles (4, 5). From the sequence analysis, Ad 2 is shown to contain an inverted terminal repetition of 103 base pairs exactly identical to that in Ad 5 DNA reported by Steenbergh et al. (13).

Ad 2 and Ad 5 are closely related and belong to Group C adenoviruses. They share greater than 92% homology estimated using an electron microscopic heteroduplex technique (23). They differ in their cleavage patterns obtained using certain restriction enzymes. Ad 2 DNA used in this study gave the characteristic cleavage patterns reported elsewhere when Eco RI (24), Hind III (25) and Sma I (see Fig. 1 and Ref. 20) were used.

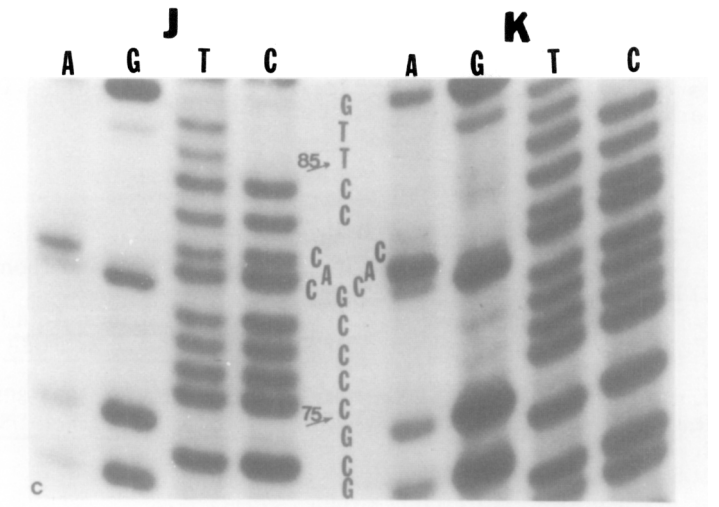


Fig. 4 DNA sequencing gel of the terminal Sma I-J and K fragments of Ad 2 DNA. The conditions used were the same as in Fig. 2 except that a 80 x 22 x 0.075 cm long gel (12% polyacrylamide) was used as the sequencing gel. Fig. 4a is the bottom and 4b is the top portion of this gel, the chain lengths increasing from the bottom towards the top. The position of the Xylene Cyanol F.F. (XY) dye is shown as a dark dot (40 cm from the origin) in Fig. 4b. Fig. 4c, in another experiment the sequencing gel similar to 4a & 4b, was run longer to achieve better resolution (XY dye moved 64 cm from the origin). The nucleotide sequence derived from Fig. 4a - c is shown in Fig. 3b - d.

The termini of Ad 2 were labeled in the DNA-polymerase I catalyzed repair synthesis in the presence of (α-^{32}P)dGTP due to the incorporation of one dpG residue per 3'-end. Donelson and Wu (29) have shown that at 5^{o}C and 180 mM salt concentration, the 3' 5' exonuclease activity of the DNA polymerase I is sufficiently inhibited. Under these conditions, the nucleotide interchange reaction takes place only with the 3' terminal nucleotide at a rate about one-third of the rate of polymerization. Our data on the rates of incorporation of dTMP and dGMP (Table I, Expts. 1 and 2) may suggest that dpG residues were added to the 3' termini complementary to the 5' protruding dC residues by the polymerizing activity of the DNA polymerase I. However, we have not ruled out the alternative possibilities for the observed incorporation of dpG residues at the 3' termini. Further experiments are in progress to resolve this question.

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Note added in proof: Drs. Arrand and Roberts recently communicated their results to us, which are in agreement with our data, except for a deletion of one A residue in their sequence at position 8 from the 5' terminus of Ad 2 DNA. The basis of this difference is not clear at present.

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