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Upstream half of adenovirus type 2 enhancer adopts a curved DNA conformation

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ABSTRACT

The putative enhancer domain of human adenovirus type 2 (Ad2) was revealed to contain a bent DNA structure in the upstream half. By using "deletion analysis", this unusual structure was identified experimentally to span from nucleotide 194 to 269 (figures; nucleotide numbers from the left terminal nucleotide of the viral DNA). This region has almost the same nucleotide sequence as the upstream half of the enhancer and packaging region of Ad5 and, therefore, is thought to contain multiple enhancer elements for transcription and the elements required for packaging of Ad2 DNA. The bent DNA structure of this region was further characterized by analyzing temperature-dependent changes in electrophoretic mobility of the DNA fragments used for bending analysis. Ad2 enhancer provides a good system to investigate the relationship between higher order structure of DNA and transcriptional activation.

INTRODUCTION

A fragment from *Leishmania tarentolae* kinetoplast DNA was the first example of naturally occurring bent DNA (curved DNA) (1). This fragment showed anomalously slow electrophoretic mobility in native polyacrylamide gels. Since then, such anomalies in electrophoretic mobility have been the hallmark in detecting bent DNA structure and a number of naturally occurring intrinsic bends have been found in DNA from various sources (1-14), mostly occurring in the region of origin of replication (3-10). Such anomalies in electrophoretic mobility are best understood by presuming that, compared with straight DNA fragment of the same size, the bent DNA fragment encounters much greater friction in snaking through the three-dimensional network of the gel formed by polyacrylamide. In addition to studies using gel electrophoresis, efforts to visualize directly the bent DNA structure by electron microscopy have also been made. Griffith *et al.* have succeeded in the visualization of such structure in a fragment from kinetoplast DNA minicircles of *Crithidia fasciculata* in 1986 (15), and Hsieh and Griffith in the terminal region of replication and transcription of SV40 DNA in 1988 (12). However, the functional significance of bent DNA in processes such as DNA replication, gene expression, and DNA packaging remains obscure.

What confers a curved trajectory on the helix-axis of a DNA molecule is likely to be the distribution of short sequences within it, for almost all (but not all) bent DNA fragments found so far have regularly distributed runs of adenines in their sequences with a periodicity of one run per helical repeat. This observation have prompted several studies to construct models to explain sequence-directed bend (16-21).

Some bent DNAs have been found in the transcriptional control region of both prokaryotic (2,11) and eukaryotic genes (14). An enhancer containing curved DNA structure has been reported, i.e. the E1A enhancer of human Ad5 has been revealed to contain such structure (22). The region containing the enhancer and the element required in *cis* for packaging of Ad5 DNA has been mapped by using various deletion mutants (23,24). This region, existing between nucleotides 194 and 358, can be cut out by digestions with *Rsa*I and *Sst*II. Anderson has shown that the *Rsa*I-*Sst*II fragment of Ad5 migrates anomalously in two-dimensional gel electrophoresis and concluded that a bent DNA domain exists somewhere within the fragment (presumably located between nucleotides 210 and 280) (22). The precise locus of the bend, however, has not yet been determined experimentally. Because *cis* elements for transcriptional activation have been determined in this case, precise description of the curved locus should not only provide new structural information but also clues for speculating a mechanism of enhancement of transcription from the viewpoint of total structural basis of DNA. We have analyzed the bent locus of Ad2 DNA. As Ad2 and Ad5 have almost the same nucleotide sequence, the results obtained in this study are thought to be fully applicable to Ad5.

MATERIALS AND METHODS

Enzymes and DNAs

Restriction endonucleases, nuclease S1, *E. coli* DNA polymerase I large (Klenow) fragment, T4 DNA polymerase and T4 DNA ligase were obtained from Takara Shuzo, Toyobo, or from New England Biolabs. *Eco*RI linker with phosphorylated 5'-ends was from Takara Shuzo. The *Hin*cII digest of phage ΦX174 RF DNA as the size marker was from Toyobo. Human Ad2 DNA was purified as described by Green and Wold (25).

Preparation of fragments for the deletion analysis

The left terminal *Bbe*I fragment of cloned Ad2 DNA (813 bp), obtained from the plasmid containing the left terminal region of the viral DNA, was digested with *Nsp*(7524)I (cleavage site; at 146), *Acc*I (at 193), *Fok*I (at 242), *Bal*I (at 269), *Sac*II (at 356) or *Dde*I (at 400). The cohesive ends produced by all these enzymes but *Bal*I were altered to be blunt by using Klenow fragment of DNA polymerase I (or T4 DNA polymerase). The resulting fragments were then ligated

to *Eco*RI linkers (5'-$G_2A_2T_2C_2$-3') and digested with *Eco*RI and *Ava*I (at 757). Each deletion fragment was gel-purified and cloned into the *Eco*RI and *Ava*I sites of the plasmid pUC19. The wild type fragment, which has the entire 5'-flanking sequence of EIA gene and the sequence 5'-G_9C-3' between the *Pst*I site (upstream end) and the left terminal end of Ad2 sequence, was also manipulated by almost the same procedure as above and cloned into the same sites of the same plasmid (one additional unit of *Eco*RI linker was left in the construct due to incomplete digestion with *Eco*RI). Each plasmid DNA was propagated and isolated from *E. coli* HB101 by conventional methods, and then digested with *Pvu*II to produce the fragments named Wt (wild type)~⊿400 (numerals designate the number of base pairs which were removed from the left terminal region of Ad2 DNA; see Figure 2). Each fragment had been gel-purified before loading onto the gel.

Gel electrophoresis

7.5% polyacrylamide gel (acrylamide/bisacrylamide = 29/1 (wt/wt)) electrophoresis was performed in 45 mM Tris-borate (pH 8.3) and 1 mM EDTA. Unless otherwise indicated, this was carried out at 20℃ for 14 hr (2.4 V cm^{-1}). After electrophoresis, gels were stained with ethidium bromide.

RESULTS AND DISCUSSION

As shown in Figure 1, the left terminal *Cla*I fragment of Ad2 DNA migrated more slowly in 7.5% polyacrylamide gel than expected from the size of the fragment (1B, lane *Cla*I), and the relative size (RS), which is calculated as the apparent size of the fragment divided by its actual size, showed a very large value (1.68; 1A). These results suggest the existence of pronounced curvature in the fragment. To map the bent region roughly, the *Cla*I fragment was further digested with combinations of *Nsp*(7524)I, *Acc*I and *Pvu*II. The result obtained from the digestion with *Pvu*II clearly shows the existence of the bend in the left terminal fragment of 453 bp (fragment b). Further digestions of this fragment with *Nsp*(7524)I or *Acc*I produced trimmed fragments with RS values of 1.38 (c) or 1.18 (d) respectively. It has been noted by Wu and Crothers that retardation of mobility is most pronounced when the bent sequence is near the center of a fragment and diminished when it is near the end (26). A drastic decrease in RS value (i.e. from 1.38 to 1.18) which was observed by the elimination of 47 bp *Nsp*(7524)I-*Acc*I fragment from 307 bp *Nsp*(7524)I-*Pvu*II fragment (fragment c) clearly shows that the bend exists somewhere in the upstream half region of the *Nsp*(7524)I-*Pvu*II. If the bend were situated to the right of the center of *Nsp*(7524)I-*Pvu*II, digestion with *Acc*I would not be expected to result in as large a change in location of bend relative to the ends of the fragment nor thus in RS value.

Further localization of the bent region was carried out as the next step.

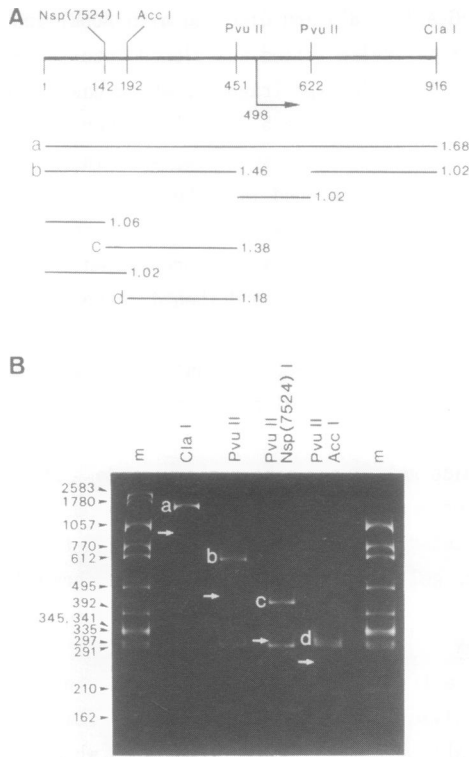

Fig. 1. Electrophoretic behavior of various restriction fragments derived from left terminal region of Ad2 DNA. (A)Restriction map of the left terminal *Cla*I fragment and the fragments tested. Nucleotide sequence numbers designate the first base pair present on the recognition site of each restriction enzyme. The number 498 shows the initiation site of the transcription of EIA gene and the arrow indicates the direction of the transcription. The restriction fragments used are shown by straight lines below the map along with their relative sizes (apparent size/actual size) calculated from the results in (B). (B)7.5% polyacrylamide gel electrophoresis of the restriction fragments. Lane m, marker DNA fragments (size in base pairs) derived from the mixture of a *Hin*cII digest of phage ϕX174 RF DNA and a *Sca*I-*Pvu*II double digest of plasmid pBR 322 (left), or from the *Hin*cII digest alone (right); *Cla*I, gel-purified *Cla*I fragment; The other lanes are indicated according to the name(s) of restriction enzyme(s) used in digesting the *Cla*I fragment. Digested samples were extracted with phenol, washed with ether, precipitated with ethanol, and then loaded onto the gel. Fragments indicated by small letters a, b, c, and d are those which migrated much slower than expected (expected migration; white arrows) and their features are shown in (A).

Two methods are available for the purpose. One is "permutation analysis" and the other is "deletion analysis". Wu and Crothers have developed a method to map the bent center of a fragment. By using the circularly permuted versions of

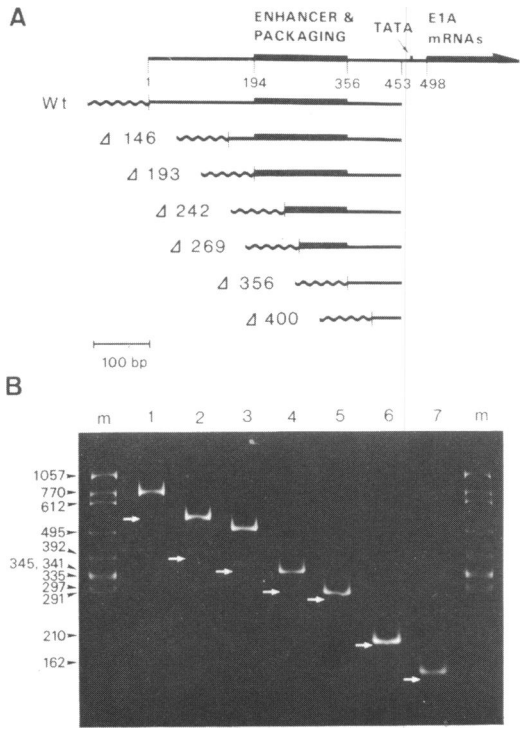

Fig. 2. Determination of the bent DNA region by deletion analysis. (A)Schematic view of the left terminal region of Ad2 genome and deletion fragments. Homology to the enhancer/packaging region which has been identified by Hearing and Shenk (23) and TATA box are shown by solid rectangles. Also shown is the start site (nucleotide sequence number 498) and the direction of the transcription of EIA gene. The wavy line in each fragment designates the linear fragment derived from the vector plasmid and the linker. Its length is 94 bp in Δ146~Δ400, and 113 bp in Wt (Wt has an extra linear fragment between *Eco*RI linker and the left terminal base pair of Ad2 DNA). The sizes of the resulting fragments are: Wt, 566 bp; Δ146, 401 bp; Δ193, 354 bp; Δ242, 305 bp; Δ269, 278 bp; Δ356, 191 bp; Δ400, 147 bp. (B)Electrophoretic mobilities of deletion fragments. Lane m, marker DNA fragments (size in base pairs) derived by digestion of phage ΦX174 RF DNA with *Hin*cII; 1, Wt; 2, Δ146; 3, Δ193; 4, Δ242; 5, Δ269; 6, Δ356; 7, Δ400. White arrows in the gel indicate the expected mobilities of these fragment.

the kinetoplast DNA fragments, they succeeded to map the locus of the center (26). This is the origin of "permutation analysis". A useful alternative for detecting bends is deletion analysis, developed by Diekmann and Wang, who were able to localize strong determinants of the abnormal gel electrophoretic mobility in a kinetoplast DNA by constructing deletion fragments (27). Snyder *et al.* have also succeeded in localizing the bent DNA in a yeast autonomously

Table 1. Temperature-dependent changes in the relative sizes of DNA fragments used for bending analysis

Fragment	Relative size		
	4.5℃	20℃	35℃
Wt	1.63	1.36	1.13
Δ146	1.72	1.41	1.17
Δ193	1.81	1.43	1.19
Δ242	1.21	1.15	1.08
Δ269	1.04	1.04	1.03
Δ356	1.02	1.02	0.99
Δ400	1.02	1.02	0.99

Electrophoreses were carried out at indicated temperatures. Fragments; see Fig.2 for details. Relative size is calculated as the apparent size of the fragment divided by its actual size.

replicating sequence by using this method (8). We have applied the latter analysis. A series of recombinant plasmids carrying Ad2 deletion fragments were constructed. By digesting these plasmids with *Pvu*II, we obtained a series of deletion fragments named Δ146～Δ400 (the numbers mean the base pairs deleting from the left terminal of Ad2 DNA) (Figure 2A). Each fragment contains a 94 bp linear fragment derived from the vector plasmid, attached to the deletion end of the viral DNA (shown by wavy line in each construct). Although it is usually difficult to detect curvature at the end of a fragment by electrophoresis, during deletion analysis, bent DNA structure, even if it (or part of it) is at the end of Ad2 DNA in the course of making deletions, is positioned at an inner site of the fragment to be tested and can be detected in native polyacrylamide gel electrophoresis. As shown in Figure 2B, Wt (lane 1), Δ146 (lane 2) and Δ193 (lane 3) migrated very slowly as compared with white arrows which indicate expected mobilities (RS values: 1.36 (Wt), 1.41 (Δ146) and 1.43 (Δ193); see Table 1). Slow migration is also observed in Δ242 but the degree of retardation was considerably diminished in this case (lane 4; RS value, 1.15). Furthermore, in Δ269 (lane 5), Δ356 (lane 6) and Δ400 (lane 7), the electrophoretic anomaly disappeared almost completely. Thus, it is evident that bent DNA exists in Δ193, is partly present in Δ242, and is almost absent in Δ269, leading to the conclusion that the bent DNA extends from nucleotide 194 to 269.

The temperature dependence of relative size was also tested (Table 1). It is known that the anomalous migration originating from bent structure decreases

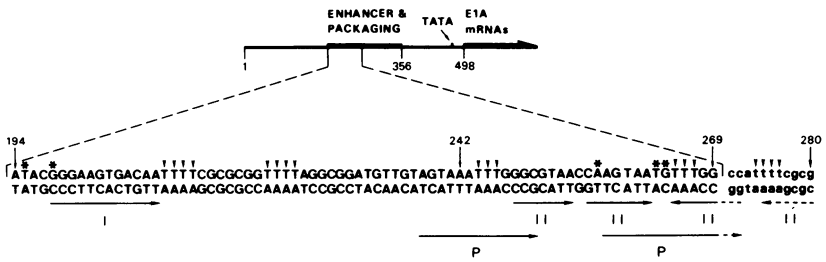

Fig. 3. DNA sequence of the bent DNA region. The base pairs differing between Ad2 and Ad5 are marked with asterisks. $(T\cdot A)_{3-4}$ stretches are shown by consecutive arrow heads. I, II, and P designate elements homologous to the repeated sequence elements required for enhancer function (I and II) or packaging (P) which had been revealed in Ad5 by Hearing and Shenk (23) or Hearing et al. (24), respectively. Nucleotide sequence between position 270 and 280 are also shown by small letters as the putative constituent element of the bent DNA structure.

with increasing temperature (16,27,28). RS values observed for Wt, Δ146, Δ193 and Δ242 changed dramatically in a temperature dependent manner, thus displaying properties of bent DNA. On the other hand, Δ269, Δ356 and Δ400, all of which lack the region spanning nucleotides 194~269, showed almost the same value of around 1.0 independently of the temperature. The existence of the bent structure between nucleotides 194 and 269 can well account for all these results.

We conclude that the region extending from nucleotide 194 to 269 has a bent DNA structure. This is consistent with the predictions of computer modelling of Ad5 (29). Except for five different base pairs, indicated by asterisks in Figure 3, the nucleotide sequences of this region are the same between Ad2 and Ad5. The enhancer and packaging region in Ad5 genome is known to have multiple sequence elements which are critical for transcriptional activation or DNA encapsidation. This region contains two types of enhancer elements; one activates the transcription of EIA gene specifically (element I) and the other enhances transcription in *cis* across the entire viral chromosome (element II). Furthermore, element I was revealed to exist at positions 200 and 300 repeatedly and four units of element II to nest between the element I repeats (23). The sequence elements required for the packaging of the viral DNA are also contained repeatedly in this region (24). The bent region of Ad2 described in this study contains most of these elements (Figure 3). Runs of $(T\cdot A)_{3-4}$ also exist repeatedly in this region (indicated by arrow heads in Figure 3), but these runs do not lie so regularly as seen in the cases, for example, of kinetoplast DNA (26), replication origin region of λ phage (3), or yeast ARS1 (8).

Nevertheless, if 10.5 base pairs are counted off in each directions from the position between nucleotides 243 and 244, a remarkable symmetry of $(T \cdot A)_{3-4}$ runs is observed with such runs occurring at or very close to positions 21 and 31.5 in both directions. Although $(T \cdot A)_{3-4}$ runs are missing at positions 10.5, the existing runs show good phasing which may be crucial for determining bent structure. This region also contains one or more short stretches of alternating purine/pyrimidine interspersed between runs of $(T \cdot A)_{3-4}$. Such stretches are often observed within enhancers (30). In addition, bent DNA derived from the origin region of plasmid pT181 replication was shown to contain such a stretch, which was thought to be a possible determinant of the curved structure (9). Thus, in the bent DNA within the Ad2 enhancer, alternating purine/pyrimidine stretches may assist well phased $(T \cdot A)_{3-4}$ runs over a region of about 70 bp in producing pronounced curvature of DNA.

The observation of bent DNA within the Ad2 enhancer is provocative of some role for such structures in both transcriptional activation and DNA packaging. Such a possible role for bent DNA is being investigated with *in vitro* transcription assays using HeLa cell extract. Moskaluk and Bastia previously showed that bending of the bovine papillomavirus enhancer is induced by binding of a truncated E2 protein (31). Although their report described protein-induced bending of an enhancer, it and our results strongly suggest the participation of bent DNA structure in some aspect of transcriptional activation.

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