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# Random cloning of bent DNA segments from *Escherichia coli* chromosome and primary characterization of their structures

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ABSTRACT

A simple method for the selective detection of DNA segments containing a sequence-directed static bend was developed. Two-dimensional polyacrylamide gel electrophoresis performed at two different temperatures (60°C and 10°C) can effectively separate a bent DNA from a mixture of normal DNA. Using this method, a bank of plasmids carrying bent DNA inserts from the E. coli total chromosome was constructed. The primary characterization of a set of bent DNA segments randomly cloned from E. coli was presented.

## INTRODUCTION

The results of recent studies have demonstrated the existence of sequence-directed static bends (curvature) in free double-stranded DNA segments. DNAs containing static bends were originally isolated from kinetoplasts of trypanosomes (1,2). It has been demonstrated that the origins of replication of bacteriophage λ and plasmid pT181 display static bends in DNA (3,4). Bends in DNA have also been found at the simian virus 40 (SV40) DNA replication origin, and at a yeast autonomously replicating sequence (ARS1, a putative replication origin)(5,6). DNAs located upstream of the Salmonella typhimurium histidine tRNA gene and the Escherichia coli rRNA gene display static bends (7,8). These examples imply that static bends in the DNA helix play critical roles in providing local structural alterations that are required for DNA transcription, replication or packaging. There is also growing evidence that a static bend in DNA is important in protein-DNA recognition. Recently, I demonstrated that the upstream region of the ompF gene, which codes for an outer membrane protein of E. coli, contains a static

bend, and that the bending region overlaps the recognition site for a gene-activator protein (OmpR) (9).
Similarly, the nucleotides in contact with the SV40 T-antigen flank a bent DNA which is critical for the binding of T-antigen (5). It has been also demonstrated that the bending region of the yeast ARS1 DNA contains the binding site for a protein factor (ABF1) (6).

The mechanism of DNA bending has been the subject of some debate (10-15). Wu and Crothers have identified the nucleotide sequence responsible for the static bending in kinetoplast DNA (16). The striking feature observed was a regular repeat of A tracts with 10-base pair (bp) periodicity around the centre of the bend. There are two general classes of models for the origin of sequence-directed DNA bending, i.e. the "wedges model" and the "junction bend model". Trifonov and Sussman proposed that the dinucleotide, ApA, would open to form wedges through a combination of tilt and roll, and that the bending of DNA results in co-orientation of periodically spaced wedges between stacked base pairs (17). Koo and Crothers proposed that the bending of DNA results from local structural polymorphism in regions of homopolymeric A tracts, and that the bending occurs at the junctions between the A tracts and adjacent B-DNA (13). In any case, the static bend in DNA can be conveniently observed as anomalously slow mobility on polyacrylamide gel electrophoresis, where the bent DNA presumably encounter higher friction (18).

In order to gain general insights into the structural features and the functional significance of bent DNA, I devised a simple method for the selective identification of DNA segments containing a static bend, by means of novel two-dimensional polyacrylamide gel electrophoresis. Using this method, a bank of plasmids carrying bent DNA inserts from the E. coli total chromosome was constructed. I report here the primary characterization of this set of bent DNA segments.

## METHODS

### Bacteria and plasmids

*E. coli* JA221 *recA* (*hsdR*$^-$, *hsdM*$^+$, *trpE5*, *leuB6*, *lacY*) was used for isolation of total chromosomal DNA (19). *E. coli* JM83

recA (ara, Δlac, pro rpsL, thi, φ80d, laZ15) was used for most experiments (20), such as transformation, plasmid preparation and DNA sequencing. Plasmid pUC19 was used as a cloning vector (21). Plasmid pHF007 carrying the ompF promoter region fused to the tet gene in vector pBR322 was constructed in the previous study (22). Bacteria were grown in L-broth. When required, ampicillin was added at a concentration of 100 μg/ml.

DNA techniques

The conditions used for DNA-manipulating enzymes, such as restriction endonucleases, T4 ligase and the Klenow fragment of E. coli DNA polymerase I, were those recommended by the suppliers. Other recombinant DNA techniques were carried out according to the conventional laboratory manual of Maniatis et al. (23). DNA sequences were determined by the dideoxy chain termination method of Sanger et al.(24), using a commercially available sequencing kit.

Two-dimensional polyacrylamide gel electrophoresis

Gel electrophoresis (first dimension) was done in a cylindrial glass tube (3 mm diameter, 14 cm length) containing 6% polyacrylamide gel (30:0.5, monomer/bis). The running buffer comprised 40 mM Tris acetate (pH7.4), 5 mM Na acetate, 1 mM EDTA. The applied voltage was 7 V/cm. During the electrophoresis, the temperature was maintained at 60°C in a temperature-controlled incubator. Then, the gel was laid on a slab gel (14 cm X 18 cm, 2 mm thick). The compositions of the polyacrylamide gel and the running buffer for the second dimension were the same as those of the first dimension, whereas the running temperature was maintained at 10°C. DNA fragments on gels were detected by staining with ethidium bromide.

Plasmid construction for a circular permutation test

Plasmids containing a tandem dimer of a cloned DNA insert were constructed as follows. A small HindIII-EcoRI fragment isolated from a plasmid of the pBENT-series (see Fig. 2) was digested with either SmaI or HincII. The HindIII-SmaI fragment and the HincII-EcoRI fragment were isolated and mixed, and then ligated together with the large HindIII-EcoRI fragment of pUC19.

## RESULTS

### Simple detection of bent DNA segments by two-dimensional polyacrylamide gel electrophoresis

A hallmark of bent DNA is an anomalously slow electrophoretic mobility on polyacrylamide gel electrophoresis. It is also known that a bent DNA tends to migrate more normally at higher temperature (18). Based on these properties of bent DNA, I devised a two-dimensional polyacrylamide gel electrophoresis method for the selective detection of a bent DNA in a mixture of DNA fragments. Electrophoresis of a mixture of DNA fragments was firstly performed on a cylindrial polyacrylamide gel at a high temperature (60°C). The gel was then subjected to a second slab polyacrylamide gel electrophoresis at a low temperature (10°C). After the two-dimensional gel electrophoresis, most of the DNA fragments were found along a diagonal line on the gel. On the other hand, DNA fragments containing a bent DNA were expected to migrate off and above the diagonal line, since the electrophoretic mobility of a bent DNA would be reduced on gel electrophoresis at the low temperature.

To evaluate this simple and novel method for the detection of a bent DNA in a mixture of DNA fragments, it was used for analysis of a plasmid DNA containing the previously reported bent DNA, which was found in the upstream region of the *ompF* promoter of *E. coli* (9). The plasmid (pHF007) carries a 519-bp bent DNA derived from the *ompF* gene. I have previously shown that the 519-bp *Eco*RI-*Bgl*II fragment shows an anomalously slow gel electrophoretic mobility, and demonstrated that the fragment contains a static bend. After digesting the plasmid DNA (5.1-Kb) with *Eco*RI, *Bgl*II and *Hin*fI, the mixture of restriction fragments was analyzed by the novel two-dimensional gel electrophoresis method. As shown in Fig. 1, most of the DNA fragments formed spots along a diagonal line but the 519-bp *Eco*RI-*Bgl*II fragment was located off and above the diagonal line. Bacteriophage λ DNA (approximately 50-Kb), the replication origin of which has been reported to contain a bent DNA (3), was subjected to the same analysis (Fig. 1). The majority of DNA fragments were found on the diagonal line, whereas several fragments were clearly seen

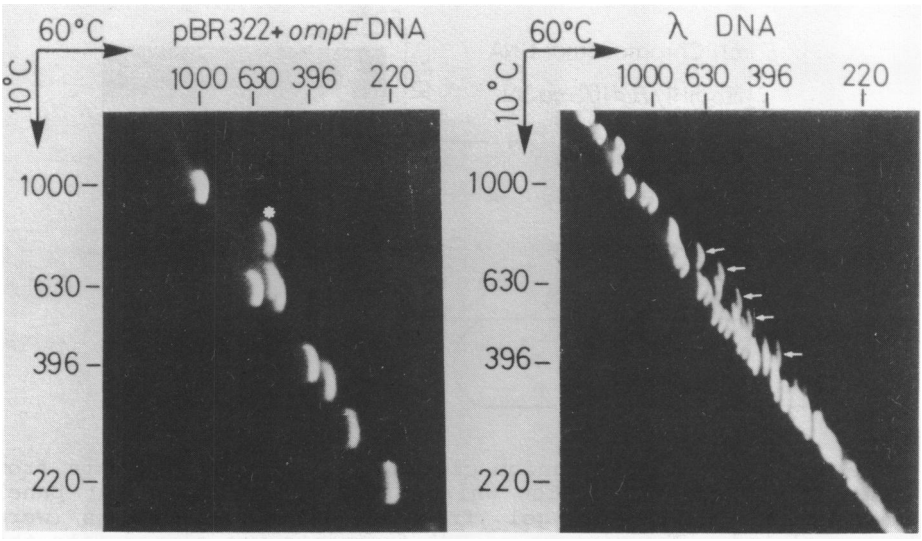



Figure 1. Two-dimensional polyacrylamide gel electrophoresis. Plasmid pHF007 (pBR322+519-bp ompF DNA) was digested with EcoRI, BglII and HinfI (left panel). Bacteriophage λ DNA was digested with HapII (right panel). Each mixture of restriction fragments was subjected to two-dimensional polyacrylamide gel electrophoresis at two different temperatures (60°C and 10°C). A white asterisk in the left panel indicates the 519-bp EcoRI-BglII ompF DNA. White arrows in the right panel indicate suspected bent DNAs found in λ DNA. The sizes of the restriction fragments of pHF007 are known, thus some of them are indicated in bp as size markers.

off and above the diagonal line. These DNA fragments exhibiting temperature-dependent anomalous gel electrophoretic mobilities were considered to be possible bent DNA fragments. Thus, it was demonstrated that the novel two-dimensional gel electrophoresis technique can effectively separate suspected bent DNA fragments from the majority of normal DNA fragments.

Random cloning of bent DNA segments from the E.coli total chromosome

In the hope of finding some clue as to the general structural features and functional roles of static bending of DNA, I attempted to randomly clone DNA segments containing a bent DNA from the E. coli total chromosome. This was easily achieved, as schematically summarized in Fig. 2. E. coli chromosomal DNA

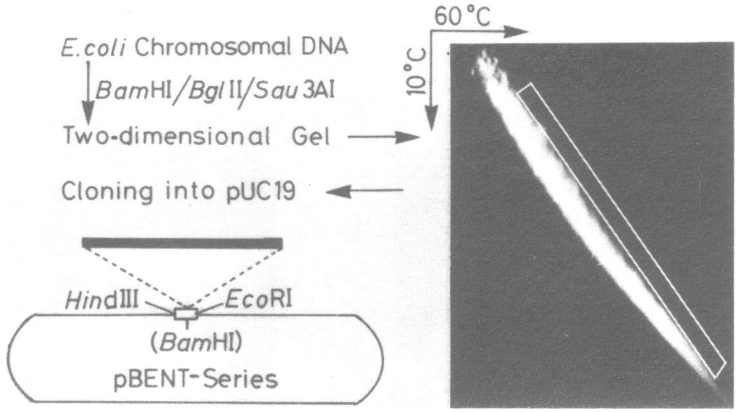



Figure 2. Strategy for random cloning of bent DNA segments from E.coli chromosomal DNA. The white rectangle in the right panel indicates the area of gel from which DNA fragments were electro-eluted. The mixture of DNA fragments was cloned into the BamHI site in the polylinker region (51-bp HindIII-EcoRI) of pUC19. The resultant plasmids were designated as the pBENT-series.

was successively digested with BamHI, BglII and Sau3AI to yield relatively small DNA segments. The restriction fragments were subjected to the two-dimensional gel electrophoresis. The majority of the DNA fragments formed an ethidium bromide stained diagonal line on the gel (Fig. 2). However, I assumed that bent DNA segments, which presumably comprise a minor population, must migrate above the diagonal line. Thus, a piece of gel was cut out from above the diagonal line, taking care that the stained area of the gel was avoided (Fig. 2). DNA fragments isolated from the gel piece were cloned into the unique BamHI site located in the polylinker region of a cloning vector, pUC19. Most of the plasmids thus isolated were found to contain E. coli DNA inserts of various sizes (100-bp to 1000-bp). Moreover, many of the HindIII-EcoRI fragments from the plasmids, i.e., the cloned DNA plus the 51-bp polylinker region (see Fig. 2), were found to exhibit temperature-dependent anomalous gel electrophoretic mobilities to various extent. Five hundred plasmids were subjected to such gel mobility assaying, and 20 of them were selected as representatives for further characterization in this study. As shown in Fig. 3A and B, these cloned DNA fragments

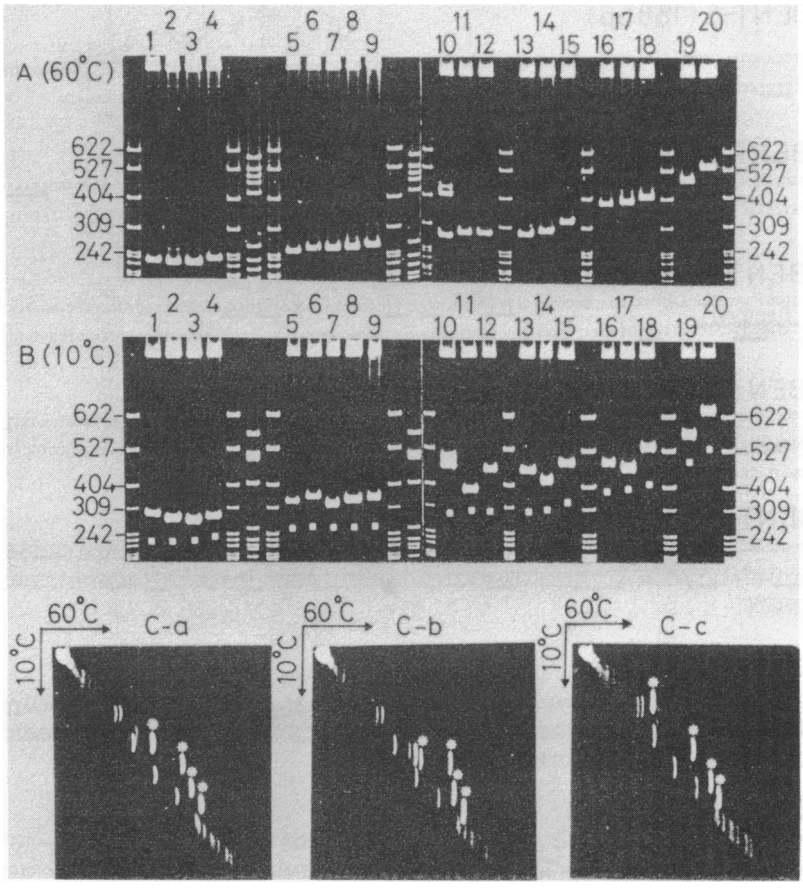
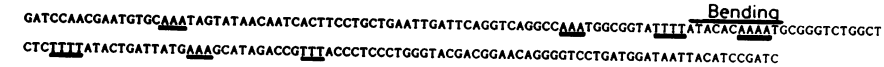
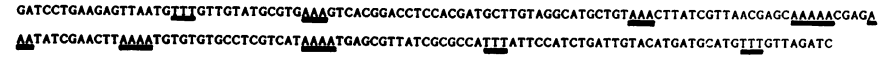



Figure 3. Mobility analyses of the restriction fragments displaying DNA bends. Plasmids, pBENT-series (pBENT-1 to pBENT-20), were digested with HindIII and EcoRI, and the resultant restriction fragments were subjected to one-dimensional polyacrylamide gel electrophoresis at either 60°C (panel A) or 10°C (panel B). The column numbers (1 to 20) correspond to the plasmid numbers, pBENT-series. The white squares in each column of panel B indicate the positions where the restriction fragments migrated to at 60°C. The unmarked columns are size markers (HapII or HaeIII digests of pBR322). The sizes of pBR322 HapII marker fragments are given in bp. A mixture of the HindIII and EcoRI digests of pBENT-series was subjected to two-dimensional polyacrylamide gel electrophoresis; panel C-a, (pBENT-1, -6, -12 and -18); panel C-b, (pBENT-3, -8, -10 and -16); panel C-c, (pBENT-4, -9, -15 and -20). HapII digests of pBR322 were also applied concomitantly as internal standards. Note that the HindIII-EcoRI fragments of the pBENT-series plasmids indicated by white asterisks are above the diagonal line, whereas the HapII digests of PBR322 gave spots on the diagonal line.

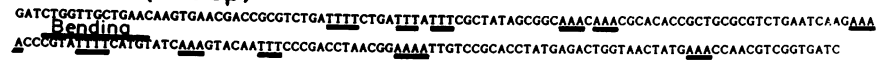
## BENT-5 (188 bp)



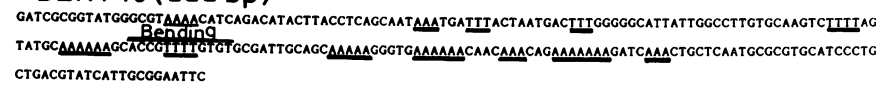

## BENT-6 (195 bp)



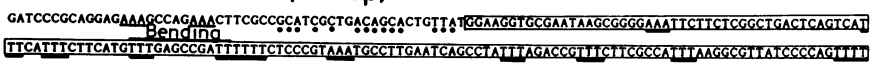
## BENT-9 (196 bp)



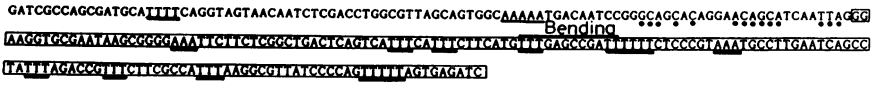

## BENT-10 (222 bp)



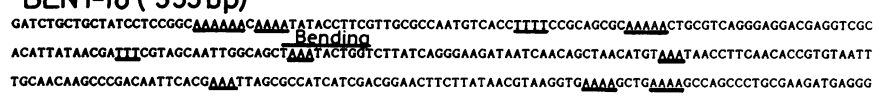

## BENT-12、BENT-13 (210 bp)



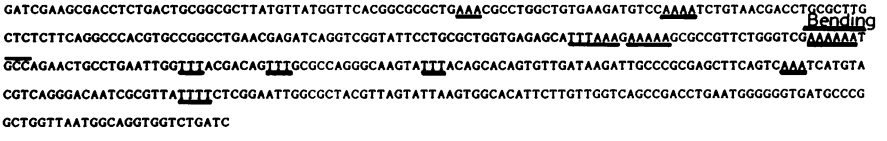

## BENT-15 (255 bp)



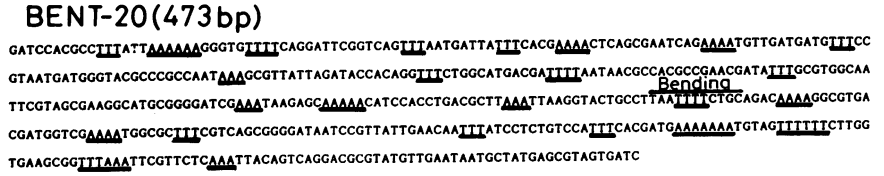

## BENT-18 (353 bp)




## BENT-19 (426 bp)




## BENT-20 (473 bp)



exhibited anomalous gel electrophoretic mobilities, although they differed as to the degree of the anomaly. For example, the apparent size of one of the cloned DNA was estimated to be 260-bp on the gel at 60°C (Fig. 3A, lane-6), while the same DNA migrated to a position corresponding to that of a 345-bp fragment at 10°C (Fig. 3B, lane-6). Similarly, another cloned DNA migrated to a position corresponding to that of a 275-bp fragment at 60°C (Fig. 3A, lane-10) but migrated to one corresponding to that of a 455-bp fragment at 10°C (Fig. 3B, lane-10). Such anomalous gel electrophoretic mobilities of these cloned DNA fragments can be seen more clearly on the two-dimensional gel electrophoresis (Fig. 3C). Considering the fact that an anomalous gel electrophoretic mobility is a hallmark of a bent DNA, I concluded that these cloned DNA represent a set of bent DNA segments derived from the E. coli chromosome.

Nucleotide sequences of the set of cloned DNA segments

To characterize the structural features of the set of cloned DNA segments, their nucleotide sequences were determined (Fig. 4, but the data for some of them are not shown). All of the cloned DNA segments were confirmed to migrate 20-60% more slowly than predicted from the determined DNA sequences at 10°C. For example, the 257-bp HindIII-EcoRI fragment of BENT-12 migrated to a position corresponding to that of a 440-bp fragment at 10°C, i.e. 57% more slowly than anticipated (Figs. 3 and 4). The 257-bp fragment, however, migrated to a position corresponding to that of a 280-bp fragment at 60°C.

Figure 4. Nucleotide sequences of a set of bent DNA segments from E. coli. The nucleotide sequences of Sau3AI inserts in pBENT-series plasmids are shown. The inserts are denoted by the respective plasmid (BENT-series) numbers, and the sizes of these inserts are indicated in parentheses. It should be noted that in the case of BENT-10, the insert appeared to have an EcoRI site, thus pBENT-10 generated two fragments upon HindIII and EcoRI digestion (see Fig. 3). The small fragment exhibiting an anomaly of gel electrophoretic mobility was subjected to sequence determination and further characterization. The stretches of An and Tn ($n \geq 3$) found in the determined nucleotide sequences are underlined. The approximate bending locus determined in a circular permutation test is also indicated (see Fig. 5). The bending locus of BENT-6 has not been determined. Identical and highly homologous sequences in BENT-12 and BENT-15 are boxed and dotted, respectively.

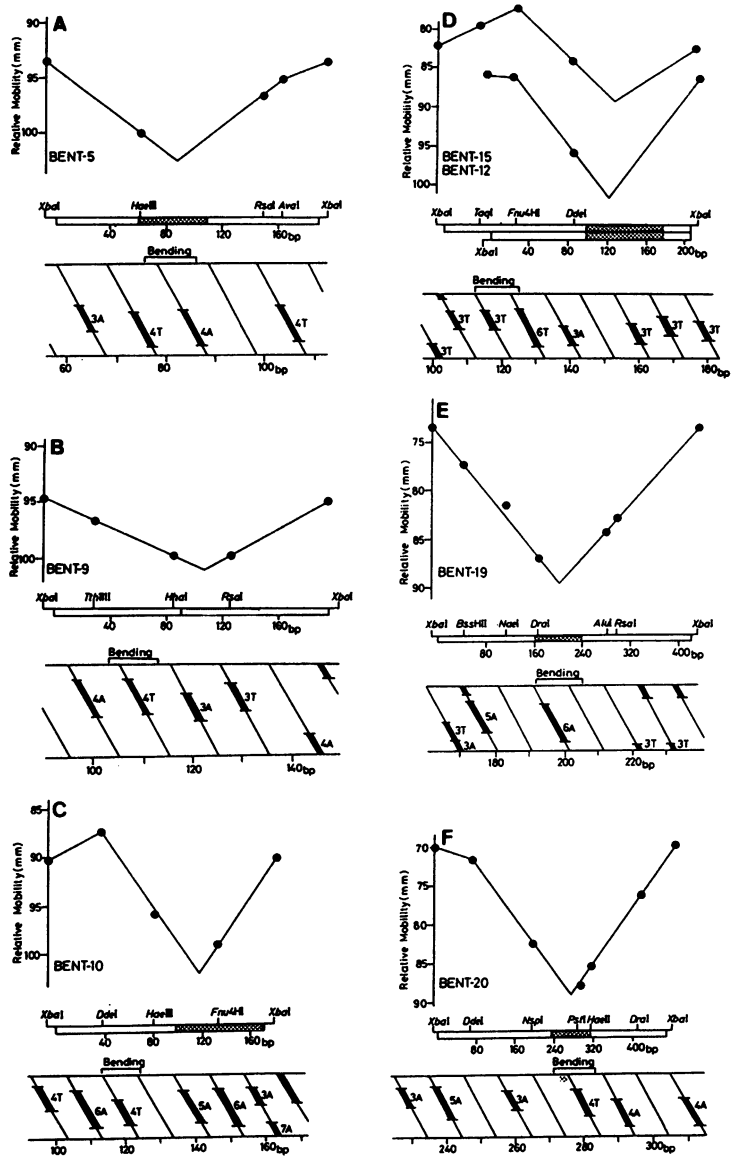



Figure 5. Circular permutation analyses. A tandem dimer of a BENT-series fragment (A, BENT-5; B, BENT-9; C, BENT-10; D, BENT-12 and BENT-15; E, BENT-19; F, BENT-20) was isolated. It should be noted that in the case of BENT-10, the insert appeared to have a Sau3AI site at the position of nucleotide 170 (see Fig. 4), thus the 170-bp Sau3AI fragment of BENT-10 was recloned in pUC19 and used for the circular permutation analysis. The DNA

fragment was digested with the indicated enzymes, and followed by one-dimensional polyacrylamide gel electrophoresis at 10°C. The relative mobilities of the permutated fragments on the gel are plotted against the position of the restriction site in one unit of the tandem dimer. The nucleotide numbers on one unit of the tandem dimer correspond to the numbers given in Fig. 4. The DNA structure of the shaded region, which contains the determined bending locus, was depicted on a cylindrical projection with 10.5-bp per turn, and stretches of An and Tn ($n \geq 3$) existing in the region were plotted. Only one of the strands of the DNA helix is shown for clarity.

DNA sequence analyses also revealed that two of the cloned DNA segments (BENT-12 and BENT-13) are identical. More interestingly, the DNA sequence of BENT-15 showed extensive sequence homology to that of BENT-12. As indicated in Fig. 4, the nucleotide sequences of the 157-bp regions which cover the 3'proximal regions of BENT-12 and BENT-15 are identical The identical regions are flanked by short sequences which are not identical but highly homologous. The nucleotide sequences covering the 5'proximal regions of these DNAs are totally different. These results suggest that the particular sequence found in both BENT-12 and BENT-15 may be present repetitively in the E. coli chromosome.

Since many nucleotide sequences of DNA segments from the E. coli chromosome have been reported, a computer-aided search was made for sequences homologous to the cloned bent DNA segments, using the IDEAS program ( the GenBank, Genetic Sequence Data Bank). None of the DNA sequences in the data base showed homology to any of the cloned DNA segments.

Identification of the bending locus of the cloned bent DNA segments

It has been proposed that stretches of An and Tn ($n \geq 3$), periodically phased, are the major cause for the static bending indicated by gel electrophoresis experiments (13). Therefore, searches were made for such stretches of An and Tn in the determined nucleotide sequences, as shown in Fig. 4. Although many stretches of An and Tn were found scattered throughout the sequences, little information was obtained with respect to specific DNA sequences causing the DNA bending. In order to define the sequences responsible for the DNA bending more

specifically, I used the circular permutation test developed by Wu and Crothers (16). I cloned the tandem dimer of the bent DNA segment, and the isolated tandem dimer was cleaved with a variety of enzymes which cut only once within the sequence, to produce a family of circularly permutated molecules with an identical base composition. The mobility of identically sized DNA fragments is dependent upon the position of the bending locus relative to the ends of the fragments (16). The results of such a circular permutation test for some of the bent DNA segments are presented in Fig. 5. Such analyses allowed me to localize the bending centre within the respective nucleotide sequences of BENT-5, -9, -10, -12, -15, -18, -19 and -20 (Figs. 4 and 5). Then, I investigated the features of the nucleotide sequences around the bending locus more closely. As shown in Fig. 5, the DNA structure was depicted on a cylindrical projection with 10.5-bp per turn, and stretches of An and Tn ($n \geq 3$) existing around the determined bending locus of an individual nucleotide sequence were plotted on the cylindrical projection. In most cases tested, a striking pattern observed was a regular repeat of stretches of An and Tn with periodicity, i.e., several stretches of An and Tn were located along one face of the DNA helix. This characteristic feature, however, was less clear in the case of BENT-19.

## DISCUSSION

Based on the known property of anomalous gel electrophoreic mobility of a bent DNA, I succeeded in the selective cloning of a set of bent DNA segments from the *E. coli* total chromosome. In addition to the set of bent DNA segments characterized in this paper, a hundred bent DNA inserts on plasmids have been obtained (data not shown). The finding of static bending in natural DNA sequences, so far described, has mainly been due to the fortuituous observation of anomalous electrophoretic mobilities of DNA fragments on polyacrylamide gel. The use of the novel method described here appeared to be feasible for a systematic survey and molecular cloning of bent DNA segments from any DNA source. Recently, we constructed a bank of bent DNA segments from an eukaryotic cell, yeast (T. Mizuno, et al., unpublished

results). Such approaches should shed light on the general structural features and functional roles of naturally occurring bent DNAs.

A striking feature observed in the nucleotide sequences of the bent DNA segments examined was a regular repeat of An and Tn tracts ($n \geq 3$) with about 10-bp periodicity around the bending locus (Figs. 4 and 5). This feature appeared to be similar to that observed for the bending locus of naturally occurring bends in DNA, for example, kinetoplast DNA (16) and bacteriophage λ DNA (3). A new approach taken by Koo et al. has highlighted further the crucial importance of phased An tracts with 10- or 11-bp periodicity (13). In contrast with the cases of kinetoplast DNA and DNA, in which An tracts were only observed along one strand of the DNA helix, the set of bent DNA segments examined exhibited periodic phasing of mixtures of An and Tn tracts around the bending locus. This feature is consistent with the recent observation of Koo et al (13). They investigated the symmetrical properties of the bending locus through synthesis of (A6-T6)n, in which every other A6 element is inverted to T6, demonstrating that this molecule shows nearly as anomalous electrophoretic mobility as that of (A6-N4)n. Thus the periodic phasing of mixtures of An and Tn tracts is thought to produce the static bending. Given the importance of the An and Tn tracts in the bending, it might be expected that the extent of bending would depend on the flaking and spacing sequences (13). Statistical analyses of the flanking and spacing sequences found in the set of bent DNA segments will provide further information on the sequence requirements for pronounced static bends in natural DNA sequences.

Roles of DNA bends in such processes as gene expression, initiation of DNA replication and DNA packaging have been proposed but not established (25,26). In connection with a possible function of bent DNA, the homologous sequences found in BENT-12 and BENT-15 are particularly interesting, because they were suggested to be present repetitively, at least at two different loci, in the *E. coli* chromosome. Nothing more is known about the functions of the set of bent DNA segments at present. Where did these bent DNA segments come from, from a coding region

of a gene or from a non-coding region? What roles do they play at their original positions in the E. coli chromosome? Cloning of the flanking sequences of the set of bent DNA segmetns and determination of the DNA (gene) structures, in which the bent DNA is located, are of interest and necessary to answer the above questions. Experiments along these lines are currently underway in our laboratory.

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