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DNA bending induced by Cro protein binding as demonstrated by gel electrophoresis

(protein–DNA interaction/DNA bending)

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ABSTRACT **We report an approach for studying protein-induced DNA bends in solution that is based on measuring the sizes of circular DNA molecules by using two-dimensional gel electrophoresis. These circular fragments are obtained by ligating short synthetic oligonucleotides containing a protein-recognition region in the presence of protein. Oligonucleotides 21-base-pairs-long containing the O_R3 recognition site were synthesized and ligated in both the presence and the absence of the *Cro* repressor from λ phage. We show that in the presence of Cro protein, circular DNA molecules are formed with substantial frequency. No circular molecules are observed in the DNA samples ligated in the absence of Cro. These experiments clearly demonstrate that DNA bending is induced by Cro in this operator site. The sum of inherent plus Cro-induced bending is estimated as 45°.**

Cro protein belongs to the family of regulatory proteins that play an important role in the bacteriophage λ life cycle. It competes with repressor protein for binding to the specific 17-base-pair (bp) operator sites O_R1, O_R2, and O_R3, which switch λ phage growth from the lysogenic to the lytic mode (1). Understanding the mechanisms by which such proteins act is of fundamental importance in gene regulation.

The three-dimensional x-ray crystallographic structure of Cro protein was obtained almost a decade ago by Anderson *et al.* (2). After this, a model for the sequence-specific interaction of Cro protein with its DNA operator region was suggested (3, 4). This model now appears to be sufficiently general to accommodate also a number of other sequence-specific repressor–operator DNA interactions (5, 6) that follow a similar helix–turn–helix motif. Very recently, Brennan *et al.* (7) have reported the crystallographic structure of the Cro protein–operator DNA complex. In this work, it is clearly demonstrated that the operator DNA is bent by the bound Cro protein. Bending occurs in two kinked regions adjacent to the pseudodyad, and the crystallographic bending angle is given as 40°. It is now widely suspected that bending of operator DNA in helix–turn–helix association complexes may be widespread and may be a factor in the specific architectures of larger, multiprotein complexes.

NMR studies can, in principle, also obtain detailed information not only on specific protein–DNA contacts but also on binding-induced DNA and protein structural distortion, and a number of recent studies have directly addressed these questions (5, 6, 8–10). Comparative gel electrophoresis techniques have also been used to obtain information on protein-induced DNA bending in solution. Crothers and coworkers (11–14) suggested a method for determining the magnitude of protein-induced DNA bends in the catabolite activator protein (CAP)–operator complex relative to a set of standard adenine tract bends. Almost the same approach was used by Landy and Thompson (15, 16), who estimated the CAP-induced bend by comparing the relative mobility of the complexes to that of poly(A) tracts. Both of these methods are similar, but neither leads to a direct evaluation of the bend angle. By using the cyclic permutation method, Kim *et al.* (17) have estimated bending angles for a number of specific binding proteins including Cro, and Zinkel and Crothers (18, 29) determined a CAP-induced bend, in semiquantitative agreement with the results of a crystallographic study on the CAP–operator complex (19). Another promising approach has recently been reported (20, 21) in which the ability of CAP protein in the presence of cAMP to induce ring closure in fragments containing the CAP binding site was investigated.

In this paper, we report a method for studying protein-induced bends in DNA; it has the virtue of relative simplicity and permits, in favorable cases, a direct measure of bending angles. This method appears to be of general utility for those cases in which the operator sequence of a protein–DNA operator complex can be reproduced in a ligation element having nearly integral helical periodicity. In this approach, we have measured the dimension of circular DNA molecules obtained from the ligation of short synthetic oligonucleotides containing a protein recognition site in the presence of the protein. In the specific study reported here, we ligated synthetic 21-base-pair (bp) DNA fragments that contained the 17-bp-long O_R3 operator region for Cro protein binding. This fragment was first ligated in the presence of Cro, and the number and sizes of circles were determined by two-dimensional gel electrophoresis. Similar ligations in the absence of protein were also performed as controls. The circumference of the smallest circle formed in the presence of the protein was found to be 147 bp. The maximum in the distribution of circle sizes occurs at 168 bp, or eight ligamers. The latter size leads to an estimated overall bending angle of about 45° in each operator region. No evidence of short circle formation was observed with ligation in the absence of Cro protein. Although the present work was completed without prior knowledge of the crystallographic results of Brennan *et al.* (7), the agreement between the two studies on the bending angle is excellent and this work provides an important solution confirmation of their crystallographic bending-angle value.

MATERIALS AND METHODS

Two complementary 21-bp single strands containing the O_R3 operator site and having the following overall sequences

5′–TATCACCGCAAGGGATAAATA–3′

3′–TGGCGTTCCCTATTTATATAG–5′

were synthesized on an Applied Biosystems model 380A oligonucleotide synthesizer and were purified by HPLC as described elsewhere (22). Single-stranded end overhang was 4 bases for precise ligation and ring closure. Single-stranded

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samples were radioactively labeled with ^{32}P by using T4 polynucleotide kinase (New England Biolabs) in a buffer containing 50 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, and 10 mM dithiothreitol. Complementary strands were mixed, heated to 80°C, and then slowly cooled to 4°C. After annealing, DNA duplexes were ligated in 50 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, and 10 mM dithiothreitol, using 400 units of T4 DNA ligase (New England Biolabs). The reaction was allowed to proceed at 0°C overnight.

Cro protein samples used in this study were kindly provided by A. Kurochkin (Institute of Molecular Biology, Moscow). The amount of the native protein was no less than 90%. The binding reaction of Cro with the 21-bp DNA duplexes was carried out for 10 min at ambient temperature in the ligase buffer. The DNA–Cro complex was then ligated overnight using 400 units of T4 DNA ligase at 0°C. After ligation was complete, the Cro protein was removed from the DNA substrate by adding SDS to 0.5% and heating at 37°C for 15 min.

The analytical gel electrophoresis procedure for the detection of ring closures was the same as described in ref. 23. Separation in the first dimension was performed in 4% polyacrylamide gel in TBE (90 mM Tris/90 mM boric acid/2 mM EDTA, pH 8.3). Separation in the second (perpendicular) dimension was in 10% polyacrylamide gel in TBE with chloroquine phosphate (50 μg/ml) in the gel and buffer. Electrophoresis was carried out at ambient temperature. The acrylamide to *N'*,*N'*-methylenebisacrylamide weight ratio of the gel was 29:1. For denaturing gels, urea was added to a final concentration of 7 M. The gels were run at room temperature in TBE buffer as described above; field strengths were 10 V/cm for nondenaturing gels and 30–40 V/cm for denaturing gels. Autoradiography was performed by exposing gels to x-ray films (Eastman Kodak) at room temperature. To quantitate circle sizes, several spots were sliced from the two-dimensional gel, extracted, and analyzed under denaturing conditions using the procedures described in refs. 23 and 24.

RESULTS AND DISCUSSION

In this study, the 17-bp specific binding recognition region for the Cro protein was placed in a 21-bp ligation element. The length of this element ensures close helical periodicity for the operator site in longer fragments deriving from this element by ligation. If the protein induces a permanent bend in each ligation element and all bends are in phase with each other, then the ligation product will be a flat loop. At a definite contour length, the ends will be nearly in juxtaposition and ligase will circularize the molecule with high efficiency. This is demonstrated graphically in Fig. 1 for a circle of eight ligated oligomers. Efficient closure is facilitated in spite of possible minor nonintegral helical repeats in the ligation element by using 4-base single-stranded ends. In the cyclization, the greater the bend in each ligation element, the smaller the circles that are formed. Thus, from the predominant size of the circular molecules formed, one can easily calculate the bending angle per binding site. Similar protocols have been used previously to estimate the AA/TT wedge angle in DNA (23) and to measure the bending angle at TT photodimer sites (24).

In this work, controls are provided by the curvature of the DNA duplex alone (i.e., in the absence of protein). Synthetic duplexes containing the *Cro* operator site were ligated without protein, and the product was coelectrophoresed with *Hpa* II restriction fragments of pBR322 as markers to measure the retardation factor R_L, defined as the ratio of apparent to actual molecular weight (see, for example, ref. 22). The results are shown in Fig. 2. The data clearly demonstrate the relative straightness of the duplex: R_L values are near unity at all ligation product sizes, with $R_L = 1.10 \pm 0.05$ (mean ± SD). No circles of circumference less than 300 bp were visible

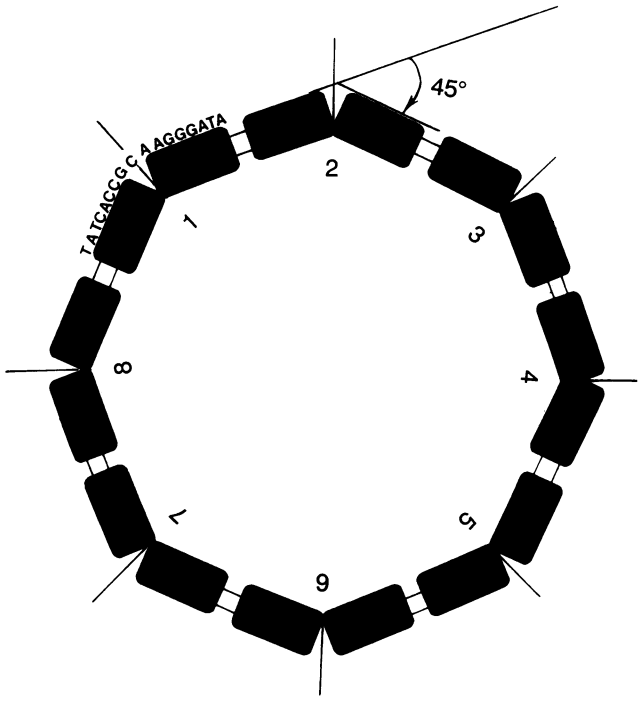

FIG. 1. Schematic illustration of Cro protein-induced bending of its operator site and the ligation of Cro-bound 21-bp oligomers into a circle. An octamer circle is shown, corresponding to the most probable size (see Fig. 4). The dark rectangles represent bound Cro monomers.

on the second-dimension gels in the absence of Cro protein (data not shown).

Fig. 3 shows the second-dimension gel electrophoresis for the ligation of the same 21-bp ligation elements in the presence of Cro protein. In addition to the linear ligation products on the diagonal, the off-diagonal spots corresponding to the closed circular molecules show substantial circularization at the heptamer and higher levels. To quantitate circle sizes, DNA samples from several spots, corresponding to different circles on Fig. 3, were eluted from the gel and electrophoresed under denaturing conditions (23, 24). By using this method, the smallest circle clearly visible on Fig. 3 corresponds to the heptamer of the basic 21-bp repeat (i.e., its circumferential length is 147 bp). Positions and intensities of all off-diagonal (closed circle) spots were quantitated by using computerized scanning densitometry.

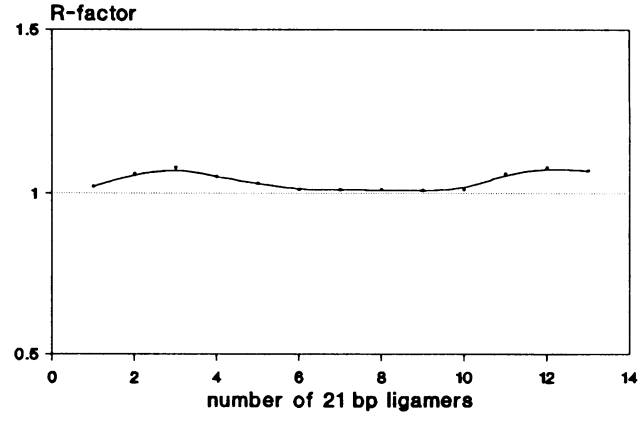

FIG. 2. Dependence of the retardation factor, R_L, of the 21-bp ligation element on the length of ligamers in the absence of Cro protein.

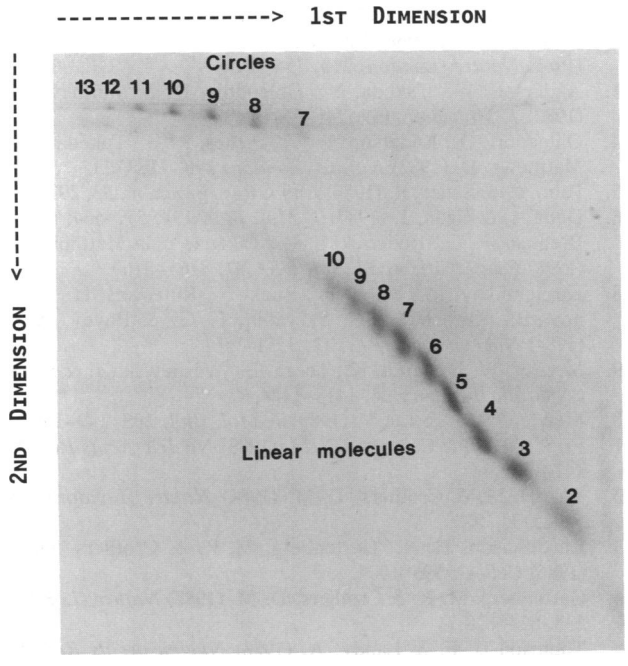

FIG. 3. Analysis of ligation products of the 21-bp ligation element containing the 17-bp Cro protein recognition region. The ligation mixture of circles and linear molecules was separated first on a 4% polyacrylamide gel (first dimension) and then on a 10% polyacrylamide gel containing chloroquine phosphate (50 μg/ml) (second dimension). The length of the molecules in each spot was determined by extracting the DNA from the gels and analyzing it by denaturing gel electrophoresis. The doublets in each circle group correspond to nicked or open (upper) and covalently closed (lower) circles (23, 25).

Fig. 4 shows the results of an optical density scan of off-diagonal spots in the second gel dimension corresponding to various circle sizes. It is not normalized for the number of radioactive phosphates (ligamers) in each spot, so the actual distribution is more symmetric than is shown. It is clear that the distribution of circular molecules in size is fairly narrow. Most of the density falls within the octamers and nonamers, with a pronounced maximum at the 8-mer level (168 bp). This overall circle size corresponds to a bend angle of 45° in each 21-bp-long repeat (i.e., per each operator site). However, this figure is a lower limit estimate because some dissociation of the protein from the DNA may occur during ligation. A

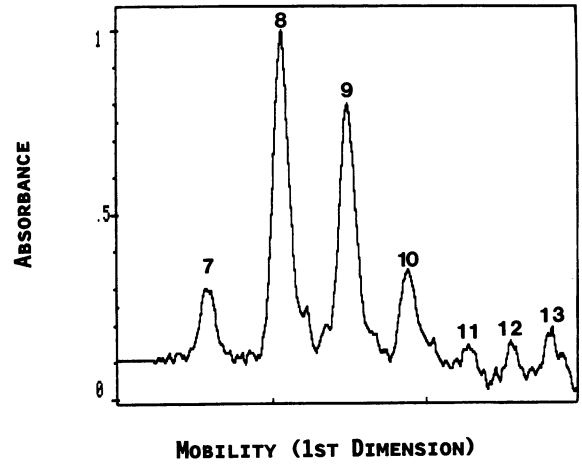

FIG. 4. Computerized densitometric scans of closed circles from Fig. 3.

narrow size distribution of circular DNA molecules suggests good torsional alignment of the ends during ligation. Mismatching of the DNA ends due to *overall* nonintegral helical repeat in the Cro-bound oligomers could affect ring closure probabilities and cannot be completely excluded, but it evidently does not exert a major effect on the ligation process in the present case. Additional lines of evidence also support this conclusion. Summation of the dinucleotide twist angles obtained by Kabsch *et al.* (26) for the 21-bp oligomer predict an almost perfect helical repeat of 10.5 bp per turn. Ring closure ligations performed at 37°C have been obtained and compared to the results at 0°C in Fig. 4. These show a slight decrease in the yield of circular molecules with increasing temperature (data not shown). If the binding constant of Cro to its operator does not change drastically over this temperature interval, this suggests that Cro binding does not lead to a major change in the overall DNA twist in the operator site, although local variations within the 21-bp oligomer are certainly not ruled out by this type of analysis. These results are highly preliminary, however, and site-specific DNA twisting induced by Cro protein binding is a special and very interesting question that is beyond the scope of the present work.

Since the 21-bp ligation element may have some intrinsic curvature in addition to that induced by Cro binding, it is of interest to estimate the net protein-induced bending effect

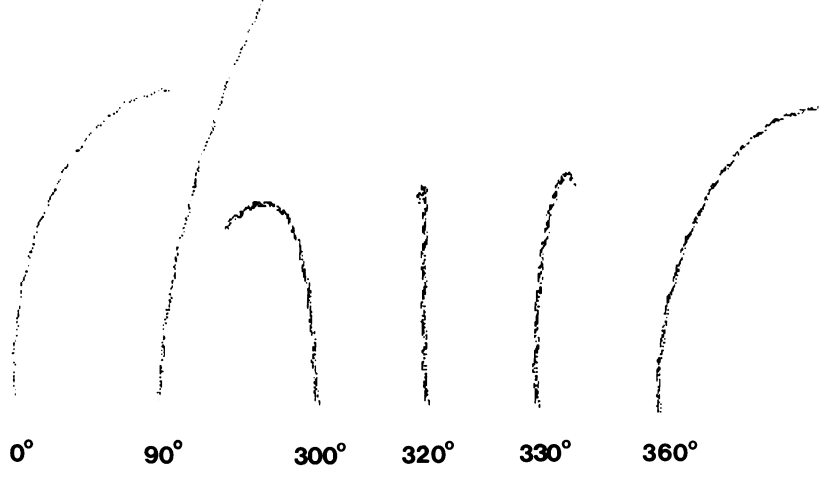

FIG. 5. Computed DNA trajectory of a heptamer ligamer of the 21-bp ligation element. Calculations of curvature are based upon dinucleotide wedge angle data from ref. 27. Different views correspond to rotations by the indicated angles with respect to the structure on the left. Rotations are made with respect to the lowest base pair (5′ end of the sequence). Because of this, the various plots seem to suggest differences in fragment length; these apparent differences are due entirely to dissimilarities in perspective among the plots.

(i.e., that part of the overall bending angle per 21-bp repeat that can be attributed to the protein binding alone). This is obtained by subtracting the inherent curvature of the 21-bp multimer. We have used a special computer program to simulate inherent curvature in the DNA helix geometry by using the recently determined experimental wedge angles of DNA (27). In Fig. 5, several projections are shown of a heptamer of the 21-bp ligation repeat element (147 bp), which correspond to the shortest circle visible in Fig. 3. It is clear from Fig. 5 that, according to this criterion, the 21-bp element has a slight planar curvature (i.e., the helical axis lies in a single plane, and the inherent curvature of each element is ≈11°). Thus, because the helical repeat of this element is very nearly integral, we can estimate the contribution of the Cro protein binding to the overall bending angle as ≈34° by subtraction. Again, as noted above, this estimate represents a lower limit to the protein-induced bending of the operator site, and it furthermore assumes that the overall curvature of each ligation element is roughly planar.

The above experiments and calculations cannot provide the location for the protein-induced bend in the operator site. Earlier NMR results (8) have suggested that the bend is near the middle of the O_R3 region, at the GC dinucleotide pair at position 9. More recent data (10) have placed the bend into two sites several bases away from the pseudodyad center. Analysis of a large number of CAP protein binding sites (which also follow the helix–turn–helix motif) have implicated the YR dimer (Y = T or C and R = G or A) located about 5 bp away from the pseudodyad (28) as a likely site for protein-induced DNA bending in the operator region. It has recently been suggested (25) that stereochemical kinks may form at CA or TA dinucleotide elements. There are several such sites in the Cro binding site that are immediately flanking the sequence elements discussed above. We plan to perform further experiments with specific point mutations in the operator region in order to attempt to localize Cro-induced bending sites more precisely.

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