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Plasmid Vectors for the Analysis of Protein-Induced DNA Bending

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Summary

Bending is not only required to accommodate DNA within the cell but also is a mechanism used by proteins to initiate DNA replication, transcription, and recombination. Determining the angles by which regulatory DNA segments deviate from linearity upon binding of proteins is a necessary step toward a better understanding of a large number of essential biological functions. The pBend plasmids contain duplicate sets of restriction sites and, when combined with “gel shift” experiments, allow the straightforward determination of the bending angle in a DNA molecule. The steps for successfully carrying out a binding/bending experiment are described. They include the cloning of the protein-binding site into the chosen pBend vector, the isolation of a series of DNA fragments with identical in length but variable placing of the protein-binding site, and the gel electrophoretic analysis of the free and protein-bound fragments.

Keywords

DNA bending; Protein–DNA complexes; Replication; Recombination; Transcription

1. Introduction

Bending of DNA by proteins plays an important role in transcription initiation, DNA replication, and recombination. The degree of protein-induced DNA bending can be simply and conveniently determined by combining gel electrophoresis of DNA–protein complexes with the use of special plasmid vectors carrying the bendable DNA sequence (1, 2). The vectors contain duplicate sets of restriction sites in a direct repeat order as well as cloning sites for insertion of protein-binding sequences between the two sets. Restriction enzyme digestion readily generates fragments which are identical in size but differ in the location of the binding site (*see* Fig. 1). The mobility of a DNA fragment is less when a bend is located at the center of the fragment than when the same bend is located toward one or the other end (3, 4). The bending angle α is defined as the angle by which a segment of the rod-like DNA

⁵If many transformants are obtained, but none contains the protein-binding site, the pBend DNA might not be fully linearized. Alter the DNA-enzyme ratio in favor of the enzyme and confirm complete digestion of an aliquot by electrophoresis on an agarose gel.

bends away from linearity. Thus, the bending angle is 0° for a straight DNA fragment. α can be estimated by measuring μ_M (mobility of the complex with the protein bound at the middle of the fragment) and μ_E (mobility of the complex with the protein bound near the end of a DNA fragment) using an empirical relationship $\mu_M/\mu_E = \cos(\alpha/2)$ (4, 5). To carry out a bending experiment, the protein-binding site is inserted into the bending vector. Next, a series of DNA fragments with the binding site located either in the middle or toward the end is generated by digestion of the vector with different restriction enzymes. Finally, DNA–protein complexes formed with the series of DNA fragments are analyzed by gel electrophoresis to determine the bending angle α . As an example, we illustrate the bending at the *lac* promoter by cyclic AMP receptor protein (CRP) (4, 6). However, the method is easily adapted to other protein–DNA complexes if they can be separated from free DNA by gel electrophoresis. General considerations for successful complex formation and for the selection of restriction sites are discussed in **Subheading 4**. pBend3, 4, and 5 (Figs. 1 and 2) are different versions of the bending vector pBend2 (4). pBend2 and pBend3 contain the same 236 base pair *EcoRI*–*HindIII* fragment with 17 duplicated restriction sites. Because of the higher copy number of pBend3, preparation of plasmid DNA is more efficient. The remainder of the digested vector DNA is less likely to comigrate with protein–DNA complexes; therefore, high molecular weight DNA-binding proteins that might introduce substantial bending can be analyzed without the tedious purification of individual DNA fragments containing the protein-binding site. pBend4 and pBend5 contain an additional *HpaI* cloning site to facilitate the insertion of DNAs with blunt ends. The promoters for the T₃- and T₇ RNA polymerases have potential use for analyzing the bending of double-stranded RNA or RNA/DNA hybrids by proteins such as transcription factor TFIID (7). Details of the properties of pBend3, 4, and 5 are described in the legends to Figs. 1–3.

2. Materials

2.1. Insertion of the Protein-Binding Site into pBend

1. pBend vector (available from the ATCC at <http://www.atcc.org/>. pBend3 accession number: 87121; pBend4: 87122; pBend5: 87123; pBendBlue (8): 87690).
2. Oligonucleotides or DNA-fragments containing the investigated protein-binding site, compatible with the cloning sites of pBend (see Notes 1 and 2).
3. *Restriction enzymes*. *HpaI*, *EcoRI*, *HindIII*, and *MluI* at about 10 U/ μ L with 10 times concentrated digestion buffers as specified or provided by the vendors.

¹Protein-binding sites can be inserted into the pBend vectors using restriction fragments or synthetic oligonucleotides. Restriction fragments should not be considerably larger than the protein-binding site to be tested. Oligonucleotides are normally available with blunt ends; therefore, the *HpaI* sites of pBend4 or pBend5 (see Fig. 2) should be used. Newly synthesized oligonucleotides might be designed with “sticky” ends such that they are compatible with the *XbaI* and the *Sall* site; they can be cloned efficiently and inserted into the DNA in a single orientation.

²Insertion of the protein-binding site may not occur if the oligonucleotides are of poor quality. In this case, they should be purified and checked by polyacrylamide gel electrophoresis. If the transformation efficiency with supercoil control DNA is high, yet very few transformants are obtained with the annealed oligonucleotides, reduce the amount of insert DNA. Multiple insertion of the binding site can occur and is detected by gel electrophoresis and sequencing. For sequencing use primers named T₃, T₇, M13–20, or reverse primer (Stratagene). Do not use the SK and KS primers (Stratagene) because they are not fully complementary to pBend3, 4, and 5.

4. *500 mM EDTA of pH 8.* Mix 93 g of disodium ethylene diamine tetraacetate-2H₂O in 400 mL water, adjust pH to 8.0 by adding about 20 g of NaOH pellets (only then will EDTA dissolve completely), adjust volume to 500 mL, autoclave.
5. *TE.* 10 mM Tris-HCl of pH 7.5, 1 mM EDTA, mix 1 mL of 1 M Tris-HCl of pH 7.5 and 200 μ L of 500 mM EDTA of pH 8. in a total volume of 100 mL of water. Store at 4°C.
6. *7.5 M ammonium acetate.* Dissolve 57.8 g ammonium acetate in a total volume of 100 mL of water. Store at 4°C.
7. *80% ethanol.* Mix 80 mL ethanol with 20 mL water. Store at 4°C.
8. *Oligonucleotide annealing buffer.* Mix 1 mL of 1 M Tris-HCl of pH 7.5, 200 μ L of 500 mM EDTA of pH 8 and 10 mL of 1 M NaCl in a total volume of 100 mL.
9. *Ligation buffer, 5 times concentrated.* 500 mM Tris-HCl of pH 7.6, 100 mM MgCl₂, 100 mM DTT. Store at -20°C.
10. *5 mM ATP.* Dissolve 3 mg of ATP (disodium salt) in 1 mL of TE.
11. T₄ polynucleotide kinase: 10 U/ μ L.
12. T₄ DNA ligase: 1 U/ μ L.
13. Competent *E. coli* cells: e.g., strain DH5 α .
14. *LB-amp plates.* Suspend 10 g of LB powder and 7.5 g of Bacto-agar in 500 mL of water; autoclave and dissolve agar by swirling, cool solution to 55°C in a water bath, add 50 mg of ampicillin (for final concentration of 100 μ g/mL), dissolve by swirling and pour plates, store plates in a plastic bag at 4°C.
15. *LB-media.* Suspend 10 g of LB powder in 500 mL water and autoclave. Store at room temperature. For the ampicillin containing LB, add and dissolve the specified amount to the media at room temperature.
16. *Tris-sucrose.* Dissolve 10 g of sucrose and 5 mL of 1 M Tris-HCl of pH 7.8 in a total of 100 mL water (prepare fresh).
17. *Lysozyme solution.* 10 mg/mL in 250 mM Tris-HCl of pH 8, keep frozen aliquots at -20°C, thaw once immediately before use, discard unused portions.
18. *200 mM EDTA, pH 8.* Prepare by dilution of 500 mM EDTA (see earlier).
19. *TLM.* Mix 3 mL of 10% Triton X100, 75 mL of 250 mM EDTA of pH 8, 15 mL of 1 M Tris-HCl of pH 8, and 7 mL water 20. Phenol: add 62.5 mL water to bottle with 250 g phenol, mix, warm as little as possible, add 300 mg 8-hydroxyquinoline, fill 20 mL aliquots in 30-mL Falcon tubes. Upon use, thaw and add 1 mL of 1 M Tris-base to one aliquot. Keep in fridge no longer than one month.
20. *R Nase A.* 250 μ g/mL in 10 mM Tris-HCl, pH 7.5.

21. *Tris–acetate, 20 times concentrated.* Dissolve 96.8 g of Tris–base, 22.84 mL glacial acetic acid, 40 mL of EDTA (500 mM, pH 8), add water to a final volume of 1 L.
22. *Agarose gel, 2%, about 5-mm thick, 7-cm long, and 10-cm wide.* Mix 0.6 g agarose (electrophoresis grade), 1.5 mL of 20 times concentrated Tris–acetate, and 30 mL of water in a 100-mL reagent bottle. Be sure that the cap of the bottle is loose before the agarose in the mixture is melted in a microwave oven. Swirl the mixture occasionally and dissolve the agarose completely. Adjust the volume with water to 30 mL and pour the gel. Insert a comb for about 3-mm wide slots and let the agarose solidify. Cover the gel with Tris–acetate electrophoresis buffer containing 1 µg/mL ethidium bromide (CAUTION, ethidium bromide is mutagenic).
23. *Ethidium bromide solution.* 10 mg/mL; dissolve 1 g of ethidium bromide in 100 mL of water by stirring for several hours. Store in the dark at 4°C.
24. *Agarose-loading buffer.* Mix one volume of 50% glycerol, 1 volume of Tris–acetate electrophoresis buffer, and 1/10 volume of a 2.5% (w/v) bromophenol blue solution in TE.
25. DNA molecular weight markers in the range of 100–1,000 base pairs, e.g., *HaeIII*-digest of FX174.
26. UV transilluminator.
27. Horizontal electrophoresis apparatus for agarose gel, approximate dimensions of 7 × 10 cm.

2.2. Detection of Plasmid Clones by Blue/White Color

LB-amp-XG plates: LB-amp plates (see Subheading 2.1, **item 14**) containing 0.05 mg/mL of X-gal and 5×10^{-5} M of IPTG. Stocks of 2% X-gal in dimethylsulfoxide and 0.1 M IPTG in water can be made and appropriate amounts can be spread onto LB-amp plates the day before use.

2.3. Purification of Plasmid DNA

1. *E. coli suspension buffer.* 50 mM Tris–HCl of pH 8, 100 mM NaCl, prepare by mixing 50 mL of 1 M Tris–HCl of pH 8, and 100 mL 1 M NaCl in a total volume of 1 L. Store at 4°C.
2. *Tris–glucose–EDTA.* 25 mM Tris–HCl of pH 8, 50 mM glucose, 10 mM EDTA, prepare freshly by mixing and dissolving 2.5 mL 1 M Tris–HCl of pH 8, 1 g glucose and 2 mL 500 mM EDTA of pH 8, in a total volume of 100 mL of water.
3. *NaOH–SDS.* 200 mM NaOH, 1% SDS, prepare by mixing 10 mL of 10% SDS and 2 mL of 10 N NaOH in a total volume of 100 mL of water.
4. *Sodium acetate.* 3 M, pH 4.8 (adjust pH with glacial acetic acid).

5. *2 M Ammonium acetate*. Dissolve 154.2 g Ammonium acetate in a total volume of 1 L of water. Store at 4°C.
6. Isopropanol.
7. Cheese cloth.
8. Quick-Seal polyallomer centrifuge tubes (e.g., Beckman No. 342413).
9. Cesium chloride.
10. *NaCl, 1 M*. Dissolve 58.44 g of NaCl in 1 L of water, autoclave, and store at room temperature.
11. *CsCl-mix*. Dissolve 122.1 g of cesium chloride (DNA grade) in 128 mL TE, and add 4.13 mL of ethidium bromide (10 mg/mL). The final volume is 165 mL.
12. *n-butanol*. Mix *n*-butanol with an equal volume of water and an amount of cesium chloride which leaves some undissolved. Use only the upper (*n*-butanol) phase.

2.4. Analysis of DNA-Protein Complexes

1. Vertical electrophoresis apparatus for polyacrylamide gel; approximate dimensions are 15 × 15 cm.
2. Acrylamide/Bisacrylamide (30/0.8, w/w) 30% aqueous solution.
3. *TBE (ten times concentrated buffer)*. Dissolve 108 g of Tris-base, 55 g of boric acid, and 9.3 g of EDTA (Ethylenediami-netetraacetic acid, disodium salt) in water. Fill up to a total volume of 1 L.
4. *8% polyacrylamide slab gel (about 15 × 15 cm, 1 mm thick)*. Mix 31 mL of water, 13.3 mL of 30% acrylamide/bisacrylamide, 5 mL often times concentrated TBE, 600 mL of 10% APS (dissolve 1 g of ammonium persulfate in 10 mL of water), and 60 µL of TEMED (*N,N,N',N''*-Tetramethylethylenediamide). Pour solution between the glassplates of the assembled vertical electrophoresis apparatus. Insert a comb for about 1-cm wide slots. Let the gel polymerize for several hours, preferably overnight.
5. *Gal repressor protein-binding buffer (five times concentrated)*. 50 mM KCl, 50 mM Tris-HCl of pH 7.6, 50 mM MgSO₄, 0.5 mM EDTA, 0.5 mM DTT, 250 µg/mL BSA, 50% glycerol.
6. *Ethidium bromide stain*. Add 100 µL of ethidium bromide solution (10 mg/mL) to 1 L of TE.

2.5. E. coli Host Strains

For pBendBlue (8), *Epicurian coli* SURE2 competent cells (Stratagene) are recommended. For the other pBend plasmids, any transformation proficient *E. coli* strain may be used. The Dam methylase present in most laboratory *E. coli* strains recognizes GATC between the *NheI* and *ClaI* sites. If this is of concern, a methylation-defective (dam-) strain should be used.

3. Method

3.1. Insertion of the Protein-Binding Site into pBend

1. Add 25 μL of pBend DNA (1 mg/mL), 10 μL of ten times concentrated *HpaI* digestion buffer, 55 μL of water, and 10 μL (100 units) of *HpaI* restriction endonuclease to a 1.5-mL Eppendorf tube. Mix and incubate at 37°C for 2 h or overnight.
2. To precipitate the restriction enzyme, place the digest on ice, add 3 μL of 500 mM EDTA of pH 8, 100 μL of ice-cold TE, and 100 μL of ice-cold 7.5 M ammonium acetate. Keep the sample at 4°C for 10 min. Centrifuge in a table-top centrifuge for 10 min. Remove the supernatant (containing the DNA) and add it to an Eppendorf tube filled with 600 μL ice-cold ethanol. Incubate at –70°C for 20 min, and centrifuge in a table-top centrifuge for 10 min. Discard the supernatant, add 500 μL of ice-cold 80% ethanol, centrifuge for 5 min, and discard the supernatant. Dry the DNA pellet in a vacuum centrifuge and dissolve the sample in 250 μL of TE. Store the linearized pBend DNA at –20°C.
3. Synthesize two complementary oligonucleotides which, when annealed to each other, form the protein-binding site. The purification of the oligonucleotides is likely to be unnecessary if they are shorter than 30 nucleotides. Dissolve each oligonucleotide in autoclaved distilled water at a concentration of 200 $\mu\text{g/mL}$.
4. Add 10 μL of each oligonucleotide and 180 μL of oligonucleotide-annealing buffer to a 1.5-mL Eppendorf tube. Incubate the sample for 3 min in a 300-mL beaker with about 150 mL of boiling water. Place the beaker with the tubes in the coldroom at 4°C to allow for annealing of the oligonucleotides over a period of several hours. Store the DNA at –20°C.
5. *Mix in a 1.5-mL Eppendorf tube.* 2 μL of annealed oligonucleotides, 3 μL of five times concentrated ligation buffer, 1 μL of 5 mM ATP, 8.5 μL of water, and 0.5 μL of T₄ polynucleotide kinase. Incubate for 10 min at 37°C. Place the sample on ice and add 3 μL of five times concentrated ligation buffer, 1 μL of linearized vector DNA (from step 2), 1 μL of 5 mM ATP, 9 μL of water, and 1 μL of T₄ DNA ligase. Incubate at 15°C for several hours or overnight. The samples can be stored in refrigerator for several days and aliquots can be used for several transformations.
6. Transform competent *E. coli* cells according to the protocol provided by the vendor and streak on LB-amp plates. Incubate the plates at 37°C overnight or until the colonies appear.
7. For preparation of the plasmid DNA on a small scale, use sterile toothpicks to transfer individual colonies to 15-mL tubes containing 5 mL of LB with 200 $\mu\text{g/mL}$ ampicillin; also, streak cells from each transformant onto an LB-amp plate. Incubate this master plate at 37°C and shake the liquid cultures at 37°C overnight.

8. Pellet the cells by centrifugation for 15 min at about $700 \times g$ at 4°C (e.g., at 3,000 rpm in a Sorvall RT6000B refrigerated centrifuge with an H1000B rotor). Decant the supernatant, add 200 μL of Tris–sucrose, and transfer to 1.5-mL Eppendorf tubes. Add 25 μL of lysozyme solution. Mix and add 130 μL of 200 mM EDTA, pH 8, and 130 μL of TLM. Mix and place at 65°C until lysis occurs (which usually takes a few minutes). Vortex briefly and centrifuge for 15 min in a table-top centrifuge. Remove the pellet with a sterile tooth pick, and add half a volume of the prepared phenol and half a volume of chloroform. Vortex for 10 s, centrifuge for 10 min, and carefully remove about 200 μL of the aqueous (upper) phase while staying clear of the interface. Add 400 μL of ice-cold ethanol, mix and centrifuge for 5 min, decant the supernatant, add 1 mL of 80% ethanol, centrifuge for 2 min, carefully decant the supernatant, and dry the pellet in a vacuum centrifuge. Dissolve the pellet in 30 μL of TE with occasional mixing. Store the samples at -20°C .
9. To verify successful insertion of the protein-binding site, digest an aliquot of the DNA with *EcoRI* and *HindIII*. To a 5- μL aliquot of the plasmid preparation add 2 μL of water, 1 μL of ten times concentrated *EcoRI* digestion buffer, 1 μL of *EcoRI*, 1 μL of *HindIII*, and 1 μL of RNase. As a control, digest 1 μg of pBend DNA. Incubate all samples at 37°C for several hours or overnight. Place digests on ice, add 1 μL of 200 mM EDTA of pH 8, 90 μL of ice-cold TE, and 50 μL of ice-cold 7.5 M ammonium acetate. Keep on ice for 10 min. Centrifuge in table-top centrifuge for 10 min. Collect the supernatant and add it to an Eppendorf tube containing 300 μL ice-cold ethanol. Mix and incubate at -70°C for 20 min. Centrifuge in table-top centrifuge for 10 min. Remove supernatant, add 300 μL ice-cold 80% ethanol to the pellet, centrifuge for 5 min, and discard supernatant. Carefully dry the pelleted DNA in a vacuum centrifuge and dissolve it in 5 μL of TE. Add 5 μL of Tris-acetate-loading buffer and mix briefly.
10. Prepare a 2% agarose gel. Load the samples from **step 9** in parallel with DNA molecular weight markers. Electrophorese at 80 V until the bromophenol blue has migrated about 4 cm. Examine the DNA under a UV transilluminator and take a picture with a Polaroid camera (film type 57 or 55). Successful insertion is indicated by an *EcoRI*–*HindIII* fragment of the expected mobility (242 base pairs plus insert). Electrophoresis can be continued to discover minor mobility differences, but then fresh electrophoresis buffer should be used. Eventually, the nature of the positive clone must be verified by DNA sequencing, which also reveals the orientation of the inserted binding site (see Note 2 for the selection of suitable sequencing primers).

3.2. Detection of the Protein-Binding Site Cloned into pBend Blue by Blue/White Color Screening

After ligation of the DNA segment corresponding to the protein-binding site into pBendBlue DNA (8), as described in **steps 1–5** of Subheading 3.1., transform competent *E. coli* cells (*Epicurian* strain) and plate on LB-amp-XG plates. Incubate the plates at 37°C overnight or until the colonies are large enough to distinguish their blue/white color phenotype. By this

procedure, usually 1–10% of the transformed colonies on LB-amp-XG plates are white. Verify the white colonies by purifying on LB-amp-XG plates. Sequence verification shows that almost all the white colonies contain the desired insert. Thus, when the cloning efficiency is poor, the color screening allows successful use of pBendBlue in cloning short DNA sequences for studying DNA binding.

3.3. Purification of pBend DNA

1. To obtain pure DNA of the positive pBend derivative, set up a 5-mL culture of the positive clone in LB with 500 µg/mL of ampicillin starting from an individual colony of the master plate (**step 7** of Subheading 3.1). Shake at 37°C for several hours until the culture becomes turbid. Transfer the cells to a 2-L sterile Erlenmeyer containing 400 mL of LB with ampicillin. Shake overnight at 37°C.
2. Place the culture on ice and transfer the cells into centrifuge bottles. Pellet the cells by centrifugation at 4°C at about $1,600 \times g$ (e.g., 3,500 rpm in a H6000A rotor of a Sorvall RC3C centrifuge). Decant the supernatant, resuspend the pellet in 20 mL *E. coli* suspension buffer, and transfer the cells to a 50-mL centrifuge tube (preferably Nalgene, Cat. No. 3131-0024). Centrifuge at 4°C for 10 min at about $5,000 \times g$ (e.g., in Sorvall SS34-rotor at 10,000 rpm.). Freeze the pellet completely by placing the sample on dry ice or in a –80°C freezer.
3. Thaw the pellet and resuspend the cells in 6 mL of Tris–glucose–EDTA. Add 12 mg of lysozyme powder, mix, and keep on ice for 30 min. Bring to room temperature and add 12 mL of NaOH-SDS. Mix and place on ice for 5 min.
4. Add 9 mL of 3 M sodium-acetate of pH 4.8; shake and leave on ice for 30 min. Centrifuge at about $12,000 \times g$ at 4°C for 15 min (e.g., in an SS34 rotor at 15,000 rpm).
5. Transfer the supernatant to a new centrifuge tube by filtering through a cheesecloth. Add half a volume of isopropanol to the transferred solution, leave 5 min at room temperature, and centrifuge at 4°C for 10 min at $5,000 \times g$ (e.g., in the SS34 rotor at 10,000 rpm).
6. Discard the supernatant and add 6 mL of ice-cold 2 M ammonium-acetate to the pellet. Vortex repeatedly to dissolve the plasmid DNA until only small particles are visible. Centrifuge at 4°C for 10 min at $5,000 \times g$.
7. Transfer the supernatant (containing the plasmid DNA) to a new centrifuge tube, add 4 mL of isopropanol, mix and centrifuge at 4°C for 10 min at $5,000 \times g$.
8. Discard the supernatant and completely dissolve the pellet in 7 mL of TE with occasional shaking. Add 8 g of cesium chloride, 400 µL of 1 M NaCl, 400 µL of 1 M Tris–HCl of pH 8, 160 µL of ethidium bromide (10 mg/mL), and 2.5 mL of CsCl mix.
9. After the CsCl is dissolved, draw the solution into a 20-mL syringe and transfer it into a Quick-Seal centrifuge tube. Fill a second tube with CsClmix and make

sure that the two tubes are balanced. Seal the tubes and centrifuge overnight at about $200,000 \times g$ at 20°C (e.g., at 50,000 rpm in a Beckman NTV65 rotor).

10. Remove the tubes from the rotor, puncture the top of the tube, then collect the lower of the two visible bands with a syringe by puncturing the side of the tube. Transfer the DNA into a 15-mL Corex glass centrifuge tube.
11. Extract the ethidium bromide by adding 1 mL of *n*-butanol which has been saturated with water and cesium chloride. Vortex and remove the upper phase with a glass pipette. Repeat this process beyond the point where the color becomes invisible (usually about six times).
12. Add 2.5 mL of water and 7 mL of ethanol. Mix and incubate at -70°C for 15 min. (Do not leave too long; otherwise, CsCl will precipitate). Centrifuge for 15 min at 4°C at about $7,000 \times g$ preferably in a swinging-bucket rotor (e.g., Sorvall HB4 rotor at 10,000 rpm).
13. Pour off the supernatant, add 5 mL of ice-cold 80% ethanol to the pellet, repeat the centrifugation, discard the supernatant, and evaporate excess ethanol under vacuum.
14. Dissolve the DNA in 500 μL of TE and determine the absorbance at 260 nm. Add the appropriate amount of TE to adjust the concentration of the plasmid DNA to 1 mg/mL (1 A_{260} is equivalent to 50 $\mu\text{g/mL}$). Store the DNA at 4°C .

3.4. Analysis of DNA–Protein Complexes

1. To generate restriction fragments with the protein-binding site located at the end or in the middle, digest the pBend construct separately with *Mlu*I (end) and *Eco*RV (middle). One digestion contains 100 μL (100 μg) of plasmid DNA from **step 14** of Subheading 3.3, 30 μL of ten times concentrated *Mlu*I- (or *Eco*RV) digestion buffer, 160 μL of water, and 10 μL of *Mlu*I or *Eco*RV restriction enzyme (100 U). Incubate the samples at 37°C for several hours or overnight. Place the samples on ice, add 15 μL of 500 mM EDTA of pH 8, 150 μL of ice-cold 7.5 M ammonium acetate and leave at 4°C for 10 min. Centrifuge in a table-top centrifuge for 10 min. Remove and add the supernatant (containing the DNA) to a new Eppendorf tube filled with 900 μL of ice-cold ethanol. Mix and incubate at -70°C for 20 min. Centrifuge in a table-top centrifuge for 10 min. Discard the supernatant, add 500 μL of ice-cold 80% ethanol, centrifuge for 5 min., and decant the supernatant. Carefully dry the pellet in a vacuum centrifuge and dissolve the DNA in 50 μL of water. Verify the success of the digestion by electrophoresis of an aliquot on a 2% agarose gel (described in **item 23** of Subheading 2.1).
2. Pour a vertical 8% polyacrylamide slab gel. Assemble the electrophoresis apparatus and pre-electrophorese at room temperature for 1 h at 100 V with TBE buffer in the reservoirs.
3. Isolate the different DNA fragments containing the protein-binding site after gel electrophoresis for end labeling, if necessary (*see* Note 6). Label the ends of the

DNA fragments by T₄ polynucleotide kinase and [γ -³²P]ATP as recommended by the supplier of the enzyme.

4. *Mix at room temperature.* 2 μ L of *Mlu*I- or *Eco*RV-digested DNA (from **steps 1** or **3**), 1.6 μ L of five times concentrated Gal repressor protein-binding buffer, 4.4 μ L of water, 2 μ L of diluted protein. Keep the time between diluting the protein and addition to the DNA as short as possible. Do not vortex; mix gently with the tip of the pipette. Prepare a control without added protein. Incubate all samples for 10 min at room temperature.
5. Flush the wells of the polyacrylamide gel with reservoir buffer and load samples without the addition of loading buffer and tracking dyes. The glycerol in the binding buffer gives the sample sufficient density. A long plastic microcapillary tip is helpful to deliver the sample to the bottom of the slot. Glass capillaries should be avoided because proteins tend to stick to glass. In a separate slot, load DNA molecular weight markers with bromophenol blue. Electrophorese at room temperature for 5 h at 200 V. Separate the glass plates and immerse the gel in ethidium bromide stain for visualization of the DNA under UV light. Take a picture and subsequently autoradiograph the gel (consult Notes 3 and 4 if complexes cannot be detected).
6. Measure the distances between the slot and the position of the protein–DNA complex of the *Mlu*I- (μ_E) and the *Eco*RV-digest (μ_M). Also, examine the mobilities of the free DNA (see Fig. 4) to make sure that the DNA fragments contain no intrinsic bending. Calculate the bending angle α using the empirical formula $\mu_M/\mu_E = \cos(\alpha/2)$. See Note 7 for details.

⁶In the initial bending experiment, it is advisable to restrict only with *Mlu*I or *Bam*HI (to place the binding site close to the ends) and *Eco*RV or *Pvu*II (to place the binding site in the middle). Do not select restriction sites which also occur in the protein-binding sequence. When exploiting the 17 circular permuted restriction sites attention must be paid to the property of some of the restriction enzymes as follows: *Cl*aI sites are methylated in most *E. coli* strains; its use is therefore limited to prior growth of the plasmid in a methylation-defective (dam⁻) host. *St*yI will also cut at *N*coI of the repeat; for the purpose of a bending experiment, it can only be used under partial digestion conditions. An additional *Spe*I site is present in the vicinity of the single *Eco*RI site as part of pBluescript SK- (see Fig. 2). *Spe*I digestion generates an additional small fragment, which contains no protein-binding site and does not interfere with the bending assay. Three additional *Dra*I sites are located in the plasmid corresponding to pBluescript coordinates 1,912; 1,931; and 2,623. Depending on their electrophoretic property, some of the vector-derived DNA fragments might comigrate with certain protein–DNA complexes. Make sure to include a control without added protein. Likewise, two additional *Pvu*II sites correspond to pBluescript coordinates 529 and 977. A *Sma*I site is present close to *Eco*RI (see Fig. 2). Two additional *Ssp*I sites correspond to pBluescript coordinates 442 and 2,850; and two additional *Rsa*I sites correspond to pBluescript coordinates 665 and 2,526. *N*coI will also cut at *St*yI of the repeat and can only be used under partial digestion conditions. An additional *Bam*HI site is present close to the *Eco*RI site (see Fig. 2).

³Bending experiments with radioactively labeled DNA are particularly useful if the protein has not been purified or if its availability is limited. Labeling can be accomplished with T₄ polynucleotide kinase and [γ -³²P]ATP. Often, the DNA ends generated by the various restriction enzymes are labeled to different degrees. This problem can be overcome by loading the gel with aliquots of the binding reaction adjusted for the efficiency of fragment labeling. It is best to purify and isolate the protein-binding fragments because the radioactively labeled plasmid DNA might obscure the region where the complexes are located. Another potential problem (which is also the case with unlabeled DNA) might be that bands appear which represent minor digestion products. In order to identify those, make sure to include controls without added protein.

⁴One of the frustrating aspects of conducting a bending experiment can be the inability to detect a complex on the polyacrylamide gel. Even if the binding and electrophoresis conditions are known one should be careful to avoid solutions and equipment which has been in contact with SDS. If possible, dedicate one electrophoresis setup to “gel-shift” experiments. Many DNA-binding proteins are insoluble in the low salt concentration of the electrophoresis buffer and must be stored at high ionic strength. Limit the time between dilution and addition to the DNA. Avoid vortexing during complex formation and do not add tracking dyes because they interact with the complex and might change its mobility. Larger protein–DNA complexes (e.g., Lac repressor, (6)) behave better in low percentage polyacrylamide gels (e.g., 4%) with a high acrylamide/bisacrylamide ratio (80:1). The protein concentration for obtaining about equal amounts of free and complexed DNA should always be determined in a preliminary experiment.

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⁷The bending angle α assumes a value of 0° for a straight duplex. Since the mobility of a rigid DNA fragment is related to its end-to-end distance, the latter equals $L \cos(\alpha/2)$, with L being the length of the unbent DNA. The end-to-end distance of a fragment bent at the end will be virtually the same as L . Thus $\mu_M/\mu_E = L \cos(\alpha/2)/L = \cos(\alpha/2)$, where μ_M is the mobility of the complex with the protein bound centrally and μ_E the mobility of the complex with the protein bound at the end of the DNA fragment. The apparent bending angle for two *lac* promoters induced by CRP is 96° (see Fig. 4 of ref. 2). We measure the distance between the top of the gel and the front of the band representing the protein–DNA complex. Whatever method is used, one must be consistent. Possible intrinsic bending in the free DNA must be considered in the calculation of the bending angle. It should be noted that the calculated values may be different from absolute bending angles, since factors other than the end-to-end distance influence the mobility of protein-bound and unbound DNA fragments. The method measures the net bend and cannot distinguish between a single sharp bend at one position and a smooth curving over a larger DNA region. For precise determination of bending angles, at least three independent experiments should be carried out. If possible, control lanes should be added of a similar size complex in which the DNA is bent to a known degree.

Fig. 1.

Schematic representation of the pBend3 insert between the *EcoRI*–*HindIII* sites. pBend3 was constructed by cloning of the 236-base pair *EcoRI*–*HindIII* fragment of pBend2(4) into pBluescript SK (Stratagene). pBluescript is a high copy number plasmid and generates a large amount of DNA upon plasmid extraction. The *EcoRI*–*HindIII* fragment contains 17 duplicated restriction sites to generate DNA fragments of identical length, but in which with the protein-binding sequence (gray rectangle in the upper part of the Figure) is shifted. The sites *XbaI* and *SalI* (in boxes) are unique and suitable for cloning of the protein-binding sequence. Restriction sites are not drawn to scale. The sequence of the insert is shown in the lower part of the figure.

Fig. 2.

Restriction and cloning sites of pBend3, 4, and 5. Portions of pBluescript SK (Stratagene) are shown in gray; the *XbaI* and *SalI* sites of pBluescript are abolished by partial digestion with *XbaI* or *SalI*, filling in with DNA polymerase (Klenow), and ligation with T₄ DNA ligase. Promoters for T₃ and T₇ RNA polymerase are indicated by the arrows. They can be used to study the bending by proteins which bind to double-stranded RNA or to RNA–DNA hybrids. The region between the *EcoRI* and *HindIII* sites (indicated in black) is identical to the one shown in Fig. 1. pBend4 and pBend5 contain additional *SalI* and *HpaI* sites as shown.

Fig. 3.

Restriction and cloning sites of pBendBlue along with the ORF of the *lacZ*(α) gene passing through the entire permuted sequence of pBend3, including the two cloning sites (*XbaI* and *SalI*). The 241-base pair permuted sequence of pBend, including the *XbaI* and *SalI* cloning sites (shown in gray), was inserted between the *SacI* and *ApaI* sites of plasmid pBSK (Stratagene) and is cloned such that the *lacZ* ORF remains uninterrupted. The corresponding amino acid sequence is shown above the DNA sequence. Note that pBSK carries only the α -complementing portion of *LacZ*, necessitating the use of the *lacZ* α host strain (*Epicurian coli*, Stratagene) which is a ω donor. A complementation between α and ω makes a cell β -galactosidase proficient. The insert is bounded by T₃ and T₇ RNA polymerase promoter sequences, both within the ORF.

Fig. 4.

Gel electrophoresis of permuted fragments of *lac* CRP sites. CRP protein was mixed separately with 11 different 5'-end ^{32}P -labeled DNA fragments. In a volume of 20 μL , a sample of each fragment was mixed with 10 mM Tris-HCl of pH 7.5, 1 mM EDTA, 50 mM KCl, 20 μM cAMP, 50 $\mu\text{g/mL}$ BSA, 10% glycerol, and 1 nM CRP. Polyacrylamide concentration was 10%. The protein and DNA concentrations were such that approximately 50% of the DNA was engaged in complexes with CRP. The DNA fragments used were generated by restriction enzymes, which, from left to right, are *Mlu*I, *Bgl*II, *Nhe*I, *Spe*I, *Xho*I, *Eco*RV, *Pvu*II, *Stu*I, *Nru*I, *Kpn*I, and *Bam*HI. As indicated, the fragments near the bottom of the gel are free DNA and those at the upper part are bound to the cAMP-CRP complex.