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Proceedings of the National Academy of Sciences of the United States of America,

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DNA twisting and the affinity of bacteriophage 434 operator for bacteriophage 434 repressor

(DNA structure/DNA bending/flexibility/bacteriophage 434 Cro)

Gerald B. Koudelka*, Pehr Harbury*, Stephen C. Harrison†, and Mark Ptashne*

*Department of Biochemistry and Molecular Biology, Harvard University, and †Howard Hughes Medical Institute, Cambridge, MA 02138

Contributed by Mark Ptashne, March 4, 1988

ABSTRACT The affinity of the Escherichia coli phage 434 operator for phage 434 repressor is affected by changes in the sequence of the noncontacted base pairs near the operator’s center. The results presented here show that base composition near the center of the operator affects the operator’s affinity for repressor by altering the ease with which the operator can be overtwisted into the proper configuration for complex formation. We show that both DNA flexibility and repressor flexibility influence the strength of the repressor-operator interaction: an operator with a single-strand nick at its center has a higher affinity for repressor than does the intact operator; and a repressor bearing a mutation that results in a relaxed dimer interaction is less sensitive to changes in the flexibility of the operator. We show that the effect of noncontacted base pairs on operator affinity is independent of the slight overall bend in the operator seen in the repressor-operator complex. Central sequence effects on affinity for repressor are independent of the identity of adjacent base pairs, suggesting that the structure of the individual base pairs, not interactions between them, are responsible for the different torsional rigidities of different operators.

Our current picture of how the Escherichia coli phage 434 repressor recognizes its DNA operator is summarized in Fig. 1. In the repressor-operator complex, one a-helix of the repressor dimer lies in each half-site of the operator. Each of these helices is positioned in the major groove so that its side chains can contact the outermost 5 base pairs in one operator half-site (1). Biochemical and x-ray crystallographic studies suggest that neither this “recognition helix” nor any other part of the repressor contacts the innermost 2 base pairs of each half-site (1, 2). Nevertheless, the operator’s affinity for repressor is determined, in part, by the composition of these base pairs: operators bearing A-T or T-A base pairs at these positions (2). We have proposed that the base composition near the center of the operator affects its affinity for repressor by altering the ease with which operator DNA can be deformed into the optimal configuration for complex formation (2). Crystallographic analysis of the 434 repressor-operator complex shows that when 434 operator binds to 434 repressor, the DNA near the center of the operator is slightly overtwisted, so that the minor groove in this region is compressed (Fig. 1) (1). Evidently this DNA deformation is required to align the two operator half-sites so that each monomer of the bound repressor dimer can make optimal contacts with each half-site.

In this paper we explore more directly the role of both DNA flexibility and repressor flexibility in determining the affinity of operator for repressor. We reasoned that introducing a single-strand break at the central phosphodiester bond of the operator would increase the flexibility of the operator, and we show that an operator with this modification has an increased affinity for repressor. We introduce a mutation into the repressor that should relax its dimer interaction and show that the mutant protein is less sensitive than is the wild-type repressor to changes in the flexibility of the operator. We present other data consistent with the idea that the base composition at the center of the operator affects affinity by changing only DNA flexibility and not the structure of the final complex.

The operator DNA in the repressor-operator complex, in
addition to being overwound near its center, is also gently bent around the repressor (1). The base composition at the center of the operator could affect operator affinity for repressor by influencing how easily the DNA can be twisted or bent. We present evidence consistent with the idea that central sequence effects on the operator's affinity for repressor are due to changes in the ease with which the operator DNA can be twisted.

**METHODS**

Reagents, Operators, Plasmids, DNA Fragments, and End-Labeling. DNA manipulations were performed as described (2, 3). The operators used were synthesized on an Applied Biosystems model 380A DNA synthesizer, gel-purified, and ligated into the unique Sal I site in pUC18 (4). 32P-end-labeling and isolation of operator-containing DNA fragments and verification of their sequences were performed as described (2). For the DNA bending experiments, circularly permuted (5) 82-base-pair fragments containing one phage 434 operator, were constructed by digesting an operator-containing pUC18 derivative with EcoRI, followed by repair of the recessed end by using dATP, dTTP, and the Klenow fragment. Subsequently, this DNA was digested with HindIII, and the resulting DNA fragment was gel-isolated and then ligated, together with an EcoRI-Hae III fragment from the same parent plasmid, into pUC18 that had been digested with EcoRI and HindIII. DNA fragments with operators located at the center or end were generated by cleaving these DNAs with EcoRI or Xho I, respectively.

Nondenaturing Gel Electrophoresis of Protein–DNA Complexes. Binding reactions were performed in DNase I protection buffer plus 5% (vol/vol) glycerol. After a 30-min incubation at 0°C, the reaction mixtures were fractionated by electrophoresis at 18 V/cm−1 through 4% or 10% polyacrylamide gels (29:1 monomer/methylenebisacrylamide) containing 90 mM Tris-HCl (pH 8.9), 90 mM sodium borate, and 1 mM EDTA at 4°C. The gels were fixed, dried onto Whatman 3MM paper, and autoradiographed by exposure to Kodak XAR-5 film with a DuPont Lightning Plus intensifier at −70°C. The fraction of bound DNA was determined by separation and assay of the radioactive bands and/or densitometric quantitation of the autoradiograph.

Assay for DNA Bending. The DNA fragments used in this study were generated as described above. In this assay, a decreased mobility of the DNA fragment with the operator located near its center, relative to the mobility of the fragment located with the operator near its end, was taken as an indication of DNA bending (5).

DNase I Protection. DNase I protection experiments, used to measure operator affinity for protein, were performed as described (2).

Site-Directed Mutagenesis and Isolation of Mutant Repressor. Site-directed mutagenesis of the 434 repressor was performed as described (6). The mutant protein, bearing the Phe-44 → Ala substitution was purified essentially as described (7).

Generation and Purification of Nicked Operator. The operator bearing a single-strand nick was generated as described (8). Briefly, we designed an operator whose central 6 base pairs comprised the recognition and cleavage site for the restriction endonuclease, EcoRV, and cloned this oligonucleotide as described above. Supercelcided plasmid DNA containing this operator was incubated at 37°C with ethidium bromide and EcoRV. After phenol extraction, butanol extraction, and ethanol precipitation of the reaction mixture, the products of the reaction, consisting of starting material, nicked plasmid, and linearized plasmid, were fractionated by electrophoresis on a 1% agarose gel. After electrophoresis of the nicked circular DNA, we obtained a restriction fragment containing the nicked operator by digesting this DNA with EcoRI and HindIII and labeled it at the 5' end. For generating operator-containing fragments with nicks outside the operator, unphosphorylated operator oligonucleotides were ligated into Sal I-cut pUC18 and treated as described (2). For both fragments, an aliquot of the gel-isolated DNA fragment was electrophoresed on 8 M urea/10% polyacrylamide gel and autoradiographed to verify the integrity of the substrate.

**RESULTS**

Central Operator Sequence and DNA Flexibility. We compared the affinities of two operators for phage 434 repressor; both operators had the sequence A-C-A-G-A-T-A-T-C-T-T-G-T, but one was specifically nicked on one strand at its central phosphodiester bond. Fig. 2A shows that, as assayed by nondenaturing gel electrophoresis, the nicked operator bound phage 434 repressor approximately 5-fold more tightly than did the intact operator. A single-strand nick 4 base pairs outside of the operator did not affect the affinity of the operator for repressor.

Central Operator Sequence and Protein Structure. The x-ray structures of phage 434 repressor and the repressor-operator complex show that Phe-44 is packed into a cleft in the protein. This residue is adjacent to the segment of
repressor that forms the amino-terminal dimer contacts (1, 9) and appears to anchor it, modulating the conformational flexibility of this portion of the peptide chain; thus, Phe-44 influences the rigidity of the dimer interaction. We changed this residue to an alanine by site-directed mutagenesis and studied in vitro the binding of this mutant protein to various synthetic operators. The wild-type repressor and the mutant repressor bearing the Ala-44 substitution had identical affinities for the reference (14-mer) operator (Table 1). In comparison to wild-type repressor, however, the mutant protein discriminated less well between operators having T-A or G-C base pairs at positions 7 and 8. Also, the increase in operator affinity in response to substitution of a homopoly(dA) sequence at positions 6-9 was smaller for the mutant protein than for wild-type (Table 1). We also examined the ability of the Ala-44 repressor to distinguish between the presence and absence of the single-strand nick at the center of the 434 operator. Fig. 2B shows that, in contrast to the wild-type repressor, the repressor with alanine at position 44 bound equally well to the intact and nicked operators. The Phe-44 → Ala change did not alter the repressor’s ability to discriminate between bases at contacted positions in the 434 operator; the protein bearing this change distinguished between A-T and G-C base pairs at operator position 4 as well as wild-type repressor did (data not shown).

Central Operator Sequence and Specific Protein–DNA Contact. Different base pair compositions at the center of the operator might affect its affinity for protein by causing the operator to assume different structures in the final repressor–operator complex, changing the geometry and, thereby, energy of specific protein–DNA contacts. We tested this idea by examining the affinity changes that result when the contacted base pair at position 4 (see Fig. 1) is changed in the presence of three different central sequences. Given in Fig. 3 are sequences of three operators that differ in sequence at the noncontacted base pairs. Despite the fact that these operators have affinities for repressor that differ by >200-fold (see the legend to Fig. 3), changing the base pair at position 4 from A-T to G-C decreased the affinity of each operator for repressor by nearly the same amount (6- to 7-fold, see Fig. 3). This result shows that the central operator sequence did not detectably alter the energy of the interaction of the repressor with base pair 4.

Central Operator Sequence and DNA Bending. Using a gel mobility shift assay (5), we examined the correlation between DNA bending and sensitivity to central operator sequence for four proteins: wild-type repressor, Ala-44 repressor, R1_69 (the proteolytically derived amino-terminal DNA binding domain of repressor), and phage 434 Cro [a single-domain protein, the structure of which is similar to that of R1_69 (9)]. In the absence of protein, the mobilities of the two DNA fragments bearing 434 operators at the end or center were identical (Fig. 4A), indicating that the 434 operator does not contain a static bend (see the legend to Fig. 4).

Table 1. Affinity of phage 434 wild-type (wt) repressor and Ala-44 repressor for synthetic operators

<table>
<thead>
<tr>
<th>Operator</th>
<th>Sequence</th>
<th>Relative K_d</th>
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Binding affinities are expressed as the concentration of repressor monomers needed to occupy half-maximally each operator in a DNase I protection experiment. These values, which have been normalized to the amount of each repressor needed to half-maximally occupy the 14-mer reference operator. This value was determined in an experiment performed in parallel. Under the condition of these experiments 1 = 2 x 10^4 M for both the wild-type and Ala-44 repressors.

FIG. 3. Effect of base composition at operator positions 6-9 on the base-pair contact at position 4. Shown are the sequences of three sets of synthetic operators, I, II, and III, along one strand. The three sets of operators differ by the sequence of the bases at operator positions 6-9 (boxed). The two operators within each set differ only by the identity of the base present at operator position 4. Compared within each set are the resulting changes in operator affinity for phage 434 repressor when the base at position 4, A-T, is replaced by G-C. The relative affinity changes are expressed as described in the legend to Table 1. For set I, 1 = 2 x 10^-8 M. For set II, 1 = 4 x 10^-8 M. For set III, 1 = 1 x 10^-9 M.

Complexed to 434 repressor or the amino-terminal DNA binding domain of repressor, the 434 operator was bent (Fig. 4B and E). A comparison of Fig. 4 B and C shows that, despite their different sensitivities to central operator sequence, wild-type repressor and repressor bearing the Ala-44 substitution bent DNA to the same extent. The operator affinity of 434 Cro, another gene regulatory protein encoded by phage 434 (10), is affected by the central operator sequence, the pattern of the affinity changes being nearly identical to that seen with 434 repressor (2). Nevertheless, in contrast to the results obtained with the 434 repressor proteins, 434 Cro did not bend the operator DNA (Fig. 4D).

Taken together, these results argue that DNA bending is unrelated to the sensitivity of repressor (and Cro) to base composition at the center of the operator.

FIG. 4. DNA bending capabilities of phage 434 proteins. The operator was located at the end (lanes c) or center (lanes c) of the two DNA fragments used in this study. A decrease in the electrophoretic mobility of the fragment having the operator located near the center of the fragment, relative to that of the fragment with the operator near its end, was taken as evidence for DNA bending (5). (A) Mobility of the two uncomplexed DNAs (arrowhead). (B–E) Mobilities of protein–DNA complexes formed with these two fragments and wild-type 434 repressor (B), 434 repressor bearing the Ala-44 substitution (C), 434 Cro (D), and the amino-terminal DNA binding domain of repressor (E).
Operator | Operator Position | Relative $K_s$
---|---|---
I | ACAATATATTG | 1
   | GC | 50
II | ACACTAGATTG | 1
   | GC | 50

**Fig. 5.** Influence of positions 6 and 9 on the affinity changes caused by substituting operator positions 7 and 8. Shown are the sequences of two sets of synthetic operators, I and II, along one strand. The two sets of operators differ by the identity of the bases at operator positions 6 and 9 (boxed). The two operators within each set differ only by the sequence of bases present at operator positions 7 and 8. Compared within each set are the resulting changes in operator affinity for 434 repressor when the bases at positions 7 and 8 TA-A-T to GC-C-G in the presence of two sequence contexts at positions 6 and 9. Fig. 5 shows that changing the T-A-A-T base pairs at positions 7 and 8 to GC-C-G decreased the affinity of the operator for repressor by a factor of 50, independent of whether the base pairs at positions 6 and 9 were TA-A-T or GC-C-G. These results indicate that interactions between adjacent base pairs at the center of the operator do not have a role in determining effects that these bases have on the affinity of the operator for repressor.

**DISCUSSION**

Our results indicate that the composition of the noncontacted central base pairs in the phage 434 operator affects its affinity for phage 434 repressor by altering the relative ease with which the DNA at the center of the operator may be overwisted. We show that a DNA modification (a nick) that should facilitate the alignment of two operator half-sites with the repressor increases the affinity of the operator for repressor. A mutation near the repressor's dimer interface, which presumably decreases the rigidity of the dimer interaction, causes the protein to become less sensitive both to changes in the sequence of the noncontacted bases and to the artificial (by nicking) increase in operator flexibility. We also show that the free energy of a specific protein--base pair interaction is independent of the sequence of the noncontacted bases, suggesting that the sequence of these base pairs affect only DNA flexibility. Thus, both the protein and DNA in the repressor--operator complex are strained, and added flexibility in either cases the formation of optimal protein--DNA contacts.

The Ala-44 mutant and wild-type repressor bind with the same affinity to the 14-mer reference operator, despite their different sensitivities to changes in the base composition at the center of the operator (Table 1). We suggest that at the 14-mer operator, a presumed negative effect of the Ala-44 substitution on overwisting is compensated by a positive effect on operator affinity. The affinity of the repressor for an operator is affected by the equilibrium constants of two reactions: formation of repressor dimers and binding of the dimer to the operator. Strong interactions between the two monomers in the dimer, while favoring dimerization, apparently require that repressor overwind the DNA when it binds, causing the protein to be sensitive to the different "twistabilities" of the central sequences. Substitution of phenylalanine with the less bulky amino acid side chain of alanine at position 44 in the repressor apparently decreases the ability of the repressor to dimerize, a matter we have not tested, and certainly increases the accessible geometries of the dimer interface rendering it more flexible. Thus, the Phe-44 → Ala mutation allows the repressor to make its optimal contacts without overwisting the DNA. A different mutation in a repressor dimer contact also decreased its ability to discriminate between base substitutions at the center of the operator; this protein, however, had a 10-fold lower affinity for the reference operator, compared with the wild-type protein (not shown).

Two results argue that the effects of noncontacted bases on the affinity of operator for repressor are independent of DNA bending. First, the Ala-44 repressor binds DNA to the same extent as does the wild-type repressor, despite the fact that these two proteins have different sensitivities to the base sequence in the center of the operator. Second, 434 Cro does not appreciably bend DNA, although its operator affinity, like that of the repressor, is strongly affected by the central operator sequence. We suggest, therefore, that the central operator sequences affect affinity solely by altering the free energy of overwisting (rather than bending) the operator. Since DNA bends on binding to the amino-terminal domain of the repressor, the absence of bending in the complex with Cro is not a function of Cro's small size, relative to intact repressor, or of its lack of a carboxyl-terminal domain.

The measured flexibility of DNA is dependent on its base composition (11–13). Others have suggested that sequence-dependent variation of DNA deformability depends on interactions between neighboring base pairs (14). We show, however, that the effect of substituting base pairs 7 and 8 in the operator is independent of the identities of the bases present at positions 6 and 9. We suggest, therefore, that the differences in torsional rigidity of the operator, and hence affinity for repressor, of G+C-rich and A+T-rich operators derives from an intrinsic difference between AT and GC base pairs—specifically, the additional base-pair hydrogen bond in the GC base pair. The overwinding near the center of the operator causes the central base pairs to assume a nonplanar conformation—e.g., highly propeller twisted or slightly buckled. The extra torsional rigidity imparted by the G+C-rich sequences as a result of the additional base-pair hydrogen bond can account for the observed decrease in affinity.

We thank the members of the Ptashne and Harrison laboratories, especially Cynthia Wolberger, Ansel Aggarwal, and Alfonso Mondragon for communication of results in advance of publication and for helpful discussions. We also wish to thank Grace Gill and Anna Astromoff for critical reading of manuscript and Betsy Burkhardt for artwork. G.B.K. was supported by a Senior Fellowship of the Charles E. King Trust, Medical Foundation of Boston. The work was supported by National Institutes of Health Grant GM29109 awarded to M.P. and S.H.