

Ethylation interference and X-ray crystallography identify similar interactions between 434 repressor and operator

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In the crystal structure of a repressor-operator complex (the 434 repressor DNA-binding domain and its 14-base pair (bp) operator), Anderson *et al.*¹ elsewhere in this issue identify six positions of likely contact between repressor protein and phosphates of the DNA backbone. At each of these positions, electron densities of protein and DNA merge. Experiments presented here indicate that intact 434 repressor approaches these phosphates very closely when it is bound to DNA in solution. Specifically, when any one of these phosphates is ethylated², repressor cannot bind to the modified operator. We also identify another position where ethylation has a significant but less dramatic effect on repressor binding, and note that in the structure, repressor closely approaches this phosphate. Our results strongly support the idea that the interactions between protein and the DNA phosphate backbone in the crystallized complex¹ are the same as those made by intact repressor to operator DNA in solution. In addition, our results suggest that DNA is slightly bent by repressor binding.

Phosphates of the 434 operator which, when ethylated, interfere with 434 repressor binding were identified by the method of Siebenlist and Gilbert² (modified as described by Hendrickson and Schleif³, see Fig. 1 legend). Briefly, the 2-fold-symmetric consensus 434 operator used by Anderson *et al.*¹ was cloned on a plasmid, end-labelled and ethylated such that each DNA molecule had an average of no more than one ethylphosphate. Purified ethylated fragment was incubated with excess 434 repressor, then operators bound to repressor were separated from the unbound operators by electrophoresis³⁻⁵. The unbound DNA molecules contain ethyl groups that prevent binding. Bound and unbound DNA fragments were isolated, cleaved at sites of ethylation and examined by electrophoresis on a polyacrylamide sequencing gel. Each band on the gel is generated by cleavage at a specific ethylphosphate. Bands enriched in the unbound fraction and depleted in the bound fraction indicate phosphates at which ethylation inhibits repressor binding.

Ethylation at any of three phosphates found 5' to bases -1, 9, and 10 on each strand prevents binding at the repressor concentration used here (compare lanes 1 and 2, Fig. 1a, b). Anderson *et al.*¹ show that these phosphates lie near residues Asn 16 and Gln 17 at the N-terminus of helix $\alpha 2$ (phosphate -1); and Lys 40, Arg 41 and Arg 43 (phosphates 9 and 10). Ethylation of a fourth phosphate, 5' to base 11, has a significant but weaker effect on binding (hatched triangles in Fig. 1). In our interpretation of the structure, this phosphate is positioned within reasonable hydrogen-bonding distance of the γ -O of Ser 30. Thus, there is excellent agreement between the results of these chemical probe experiments and the co-crystal structure. The four phosphate contacts are symmetrically disposed about the centre of the operator (Fig. 1c) on one face of the B-DNA helix (Fig. 2).

Ethylation of a DNA phosphate removes the negative charge and adds a group with a van der Waals radius of ~ 4.5 Å. Both steric and charged-group effects can therefore contribute to its influence on binding. Model building with straight DNA shows that with the relationship of DNA and protein seen in the crystal, the side chains of Asn 16 and Gln 17, the only residues near the -1 phosphate, are too far from the DNA backbone for hydrogen-bond formation (Fig. 3a). If the DNA is bent to a radius of curvature of ~ 100 Å, then a hydrogen bond to the -1

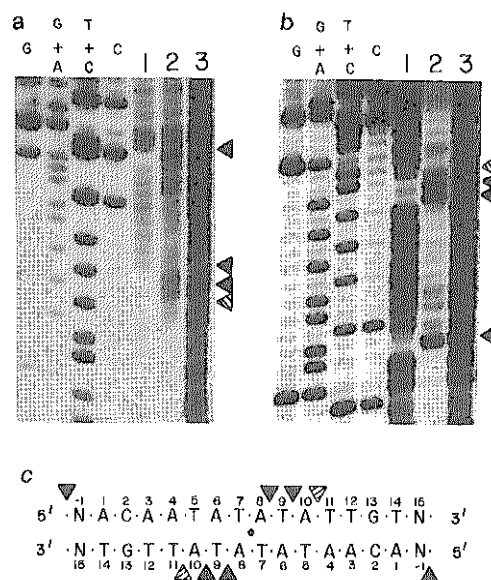


Fig. 1 Ethylation interference analysis of 434 repressor-operator interactions. *a*, Results for top strand; *b*, results for bottom strand. Lane 1, DNA bound by repressor; 2, DNA not bound by repressor; 3, ethylated unseparated control. In *a* and *b*, bands depleted in lane 1 and enriched in lane 2 relative to the control in lane 3 indicate ethylphosphates that interfere with repressor binding. *c*, Ethylphosphates that interfere with repressor binding aligned with the 434 operator sequence. Filled triangles, strong interference; hatched triangles, weaker interference.

Methods. A double-stranded consensus 2-fold-symmetric 434 operator of identical sequence to that used by Anderson *et al.*¹ with single-stranded *Sal*I ends was generated by self-annealing of an oligonucleotide of sequence 5' TCGAACAATATATTGT 3' (made on an Applied Biosystems Model 380A DNA synthesizer). This fragment was treated with T4 kinase (BRL) and ATP, then cloned using standard methods⁷ into the unique *Sal*I site within the *lacZ* gene of pEMBL8- (ref. 8) to generate pFB37. Because insertion of the oligonucleotide destroys the *Sal*I site, vectors without the insert in the ligation product could be linearized by digestion with *Sal*I and hence eliminated during transformation. The 18-bp insertion preserves the *lacZ* translational reading frame by inserting 6 codons, none of which are translational stops. Plasmids with cloned operators could therefore be identified by transforming *Escherichia coli* strain JM101 (ref. 9) (phenotypically LacZ⁻), plating on X-Gal¹⁰ and picking blue Lac Z⁺ colonies. Plasmids containing single operators were identified by DNA sequencing¹¹ and cleaved at the unique *Eco*RI site. Cleaved plasmids were 3'-labelled with DNA polymerase I large fragment (NEB) and [α -³²P]dATP (NEN), or 5'-labelled (bottom strand) by successive treatments with bacterial alkaline phosphatase (Boehringer) and T4 kinase and [γ -³²P]ATP (ICN; reactions as in ref. 7). After cleavage with *Hind*III, the labelled operator-containing fragment was isolated by electrophoresis on a 5% polyacrylamide gel, excision of the desired band and electroelution in 0.2× TAE buffer⁷. After filtration (Millex 0.45- μ m HA filter), phenol extraction, ether extraction and ethanol precipitation, DNA was ethylated with ethylnitrosourea (Sigma) as described². Ethylnitrosourea-treated DNA ($\sim 5 \times 10^{-8}$ M) was incubated at 4°C with 2×10^{-7} M 434 repressor (isolated as described previously¹²) in 50 mM KCl, 2 mM MgCl₂, 10 mM Tris pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, 50 μ g ml⁻¹ bovine serum albumin and 5% glycerol. Repressor-operator complexes were separated from unbound DNA that contained ethylations interfering with repressor binding by polyacrylamide gel electrophoresis³⁻⁵. Bound and unbound fractions were excised from the gel and purified as described above. Ethylated DNA fragments were cleaved at phosphotriesters by heating in alkali², prepared for electrophoresis as described³ and analysed on a 10% sequencing gel. Ethylation cleavage products were aligned with the sequencing ladder by correcting down to the nearest base (see ref. 2 for discussion). Alignment of a 5'-labelled sequencing band with a 5'-labelled ethylation cleavage product identifies the phosphate 5' to that base; alignment of a 3'-labelled sequencing band with a 3'-labelled ethylation cleavage product identifies the phosphate 3' to that base.

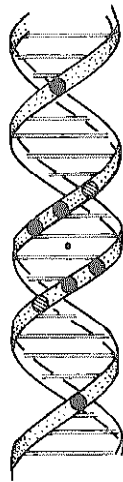


Fig. 2 Phosphates of the 434 operator at which ethylation reduces affinity for 434 repressor. Positions of strong ethylation interference are symbolized by filled circles and weaker interferences by hatched circles on a B-DNA helix. The operator centre of symmetry is indicated with a small filled circle.

phosphate can readily be made (Fig. 3*b*). Other small deformations could have a similar effect. An additional contribution to binding might come from favourable interaction between the partial positive charge on the N-terminus of helix $\alpha 2$ and the negative charge on the phosphate⁶. Based on the observed significance of the interaction at the -1 phosphate, we propose an explanation for some aspects of the crystal structure. In the co-crystal, 14-bp operators, each bound by a dimer of repressor DNA-binding domain, pack end-to-end to form rods. Each 14mer is tilted slightly and displaced laterally with respect to the next, so that the 3' terminal base on each strand does not stack on the adjacent operator. A repressor monomer bound to a given 14mer reaches over to an adjacent 14mer to make the -1 phosphate contact, and the DNA structure deviates from regular B-like conformation in this region. We imagine that the tilting and displacement of the 14mers, and possibly the local departure of the DNA backbone from canonical B-form, are consequences of crystallization using discrete fragments rather than continuous DNA. We believe that these effects occur in order to accommodate the -1 phosphate interaction, which in continuous DNA seems to require a gentle bend.

We thank Bill Hendrickson, Scot Munroe, Robin Wharton and members of the Ptashne and Wang laboratories for discussions and materials. J.E.A. is a Burroughs Wellcome Fund Fellow of the Life Sciences Research Foundation. This work was supported by NIH grants GM22526 to M.P. and GM29109 to M.P. and S.H.

Received 19 April; accepted 14 June 1985.

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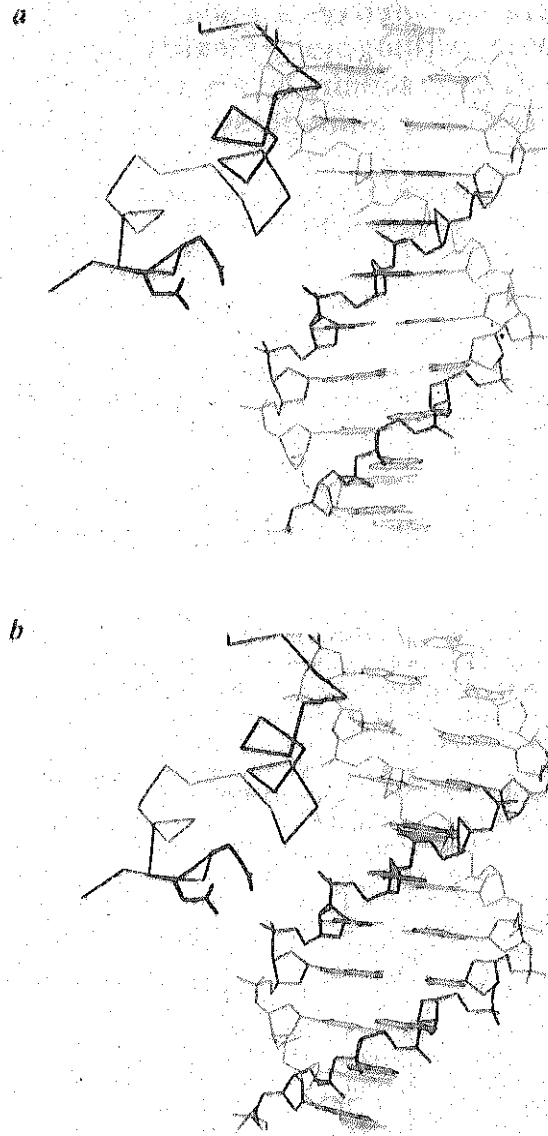


Fig. 3 Interaction of Asn 16 and Gln 17 with straight and bent DNA showing the α -carbon backbone of λ -repressor residues 30-60 (ref. 13), positioned as described by Anderson *et al.*¹, with the side chains of Asn 16 and Gln 17 of 434 repressor extending toward the DNA. In *a*, straight B-DNA was constructed by extending the double helix of the model DNA that best fits the electron density of one 14-bp operator¹. The amino nitrogens of the Asn 16 and Gln 17 side chains are both $>5 \text{ \AA}$ from the oxygens of the -1 phosphate, which is too far for hydrogen-bond formation. In *b*, B-DNA with a slight right-handed supertwist, generated as described by Anderson *et al.*¹, was positioned so that 14 bp of the modelled DNA gave the best fit to the electron density of one 14-bp operator. The side-chain amino groups of Asn 16 and Gln 17 are separated from the phosphate oxygens by $\sim 3 \text{ \AA}$, a typical hydrogen-bond length.

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