DNA Bending by Cro Protein in Specific and Nonspecific Complexes: Implications for Protein Site Recognition and Specificity

Dorothy A. Erie, Guoliang Yang, Holly C. Schultz, Carlos Bustamante*

Scanning force microscopy was used to resolve λ Cro protein when bound as a single dimer or multiple dimers to its three operator (O_R) sites. The bend angles induced by binding of Cro to specific and nonspecific sites were determined and are 69° ± 11° for specific and 62° ± 23° for nonspecific complexes. Bending of the nonspecific sites is advantageous for a protein such as Cro that bends its specific site, because it increases the binding specificity of the protein and it can be used by the protein to sample contacts required for the recognition of its target sequence. It is proposed here that bending of nonspecific DNA may be a general property among DNA binding proteins that bend their specific sites.

The intracellular control and regulation of gene expression is mediated through complex interactions between specific DNA binding proteins and DNA control elements. DNA binding proteins can achieve rapid target localization by initially binding to a nonspecific site on the DNA and then finding the specific site by one-dimensional diffusion along the DNA, by intersegment transfer, or by both (*1*). Moreover, to overcome binding to the vast excess of nonspecific DNA and to ensure occupancy at the target sequence, specific DNA binding proteins must also discriminate between specific and nonspecific DNA. The greater affinity of these proteins for specific sites provides the discrimination energy and thus determines their binding specificity.

Recognition of the cognate binding sequence requires the formation of specific contacts between the protein and the DNA and is often accompanied by conformational changes in the protein, the DNA, or both (*2*). Many DNA binding proteins, especially transcription regulatory proteins, induce DNA bending upon specific binding (*2*, *3*); however, the importance of this bending is not fully understood. The role of DNA bending in the binding specificity and mechanism of specific site recognition of these proteins remains unclear.

To help elucidate the role of DNA bending in the mechanism and energetics of specific site recognition, we investigated the conformation of the DNA in specific and nonspecific complexes of λ Cro protein and a 1-kb DNA fragment (Fig. 1). Cro, a transcription regulatory protein from bacteriophage λ, binds as a dimer (molecular weight, 14.7 kD) to three operator sites, O_R1, O_R2, and O_R3, in the O_R region in order to repress transcription from the divergent promoters λP_R and λP_{RM} (*4*). We have investigated Cro because it (i) binds to all three O_R binding sites with very high affinities (10^{10} to 10^{12} M^{-1}) and to nonspecific DNA strongly (10^7 M^{-1}) (*5*); (ii) bends DNA upon specific complexation (*6*–*8*); and (iii) appears to undergo facilitated target location by one-dimensional diffusion along the DNA (*5*, *9*). Cro-DNA interactions, therefore, provide a system for investigating the effect of protein-induced DNA bending on the dynamics of target localization, the binding specificity of the protein, and the mechanism of specific site recognition.

To visualize a protein of such small dimensions (14.7 kD), we used scanning force microscopy, which makes it possible to image the protein both free and bound under controlled humidity conditions and without external means of contrast (*10*). The 1-kb DNA fragment (Fig. 1) is incubated with Cro at room temperature to form Cro-DNA complexes (*11*). The complexes are deposited onto freshly cleaved mica and imaged in air with a Nanoscope II scanning force microscope (Digital Instruments) with tips that were modified with an electron beam (*12*).

DNA deposited in the absence of Cro is essentially free of sharp bends and high features (Fig. 2A). In contrast, DNA deposited in the presence of Cro consistently reveals sharp bends with a discrete high feature at the locus of each bend (Figs. 2B and 3). In addition, at stoichiometric Cro concentrations, more than 80% of the high features on the DNA are located at positions consistent with those of the O_R binding sites (two-fifths from one end) and are identified as specific Cro-DNA complexes. The remaining structures are identified as nonspecific Cro-DNA complexes. Invariably, complexes in both groups appear bent (*13*).

A small fraction (<5%) of the observed specific complexes showed elongated structures with two partially resolved high features consistent with the size of two Cro dimers (Fig. 4, A and B). During the course of this study, we observed a few complexes showing three high features at the position of the operator site, which are identified here as triply bound Cro dimers. The ability to observe adjacent dimers separated by ~20 base pairs (bp) (*14*), as in this case, indicates a spatial resolution of about 50 Å. The conformations of the multiply bound complexes do not appear to be related in a simple manner to those observed in singly bound complexes. A more extensive body of data than that available at present on multiply bound complexes will be necessary to investigate the conformational effects associated with the relative phasing between Cro dimers occupying adjacent sites (*15*).

We have obtained distributions of bend angles for both specifically (Fig. 5A) and nonspecifically (Fig. 5B) bound Cro dimers (*16*). The distribution of bend angles for specifically bound Cro is very narrow (Fig. 5A), with an average angle of 69° ± 11°.

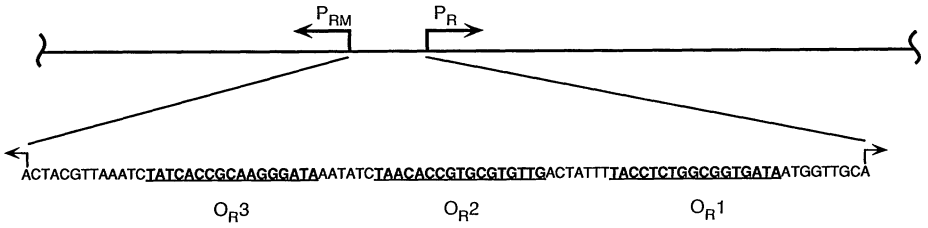

Fig. 1. Schematic diagram of the 1.0-kb Sph I–Hind III DNA fragment of pDE13, showing the positions and sequence of the operator sites (*39*). The start sites for transcription from bacteriophage λ promoters P_{RM} and P_R are shown as leftward and rightward arrows, respectively. The region between these sites, the O_R region, contains three Cro binding sites (O_R1, O_R2, and O_R3) whose centers are separated by 23 bp. The 17-bp recognition sequences are underlined and boldfaced. The three binding sites span approximately 70 bp and are located 370 to 440 bp (two-fifths) from the Sph I–cut end of the template. Cro binds to O_R3 with a higher affinity (10^{12} M^{-1}) than to O_R1 and O_R2 (10^{11} M^{-1} and 10^{10} M^{-1}, respectively) and to nonspecific DNA with a binding affinity of approximately 10^7 M^{-1} (*40*).

D. A. Erie, G. Yang, H. C. Schultz, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA. C. Bustamante, Howard Hughes Medical Institute, University of Oregon, Eugene, OR 97403, USA.

*To whom correspondence should be addressed.

This average bend angle (*17*) is larger than those determined from crystallography (*7*), gel mobility assays (6), and oligonucleotide cyclization experiments (*8*). This larger bend angle is not an artifact of the experimental method. With the same deposition procedure, average bend angles of 88° ± 10°, 48° ± 12°, 50° ± 15°, and 20° ± 15° have been measured for specifically bound yeast TATA binding protein (TBP), Eco RV endonuclease, Eco RI methylase, and the mutant His^{235} → Asn (H235N) of Eco RI methylase, respectively (*18*). These angles are consistent with those determined from the x-ray crystal structures or by gel mobility assays (*19*). The angles measured previously (6–8) either are determined from Cro binding to very short (17 to 21 bp) oligonucleotide recognition sequences or are sensitive to experimental conditions. The methods used are likely to yield only a minimum estimate of the bend angle and underestimate the extent of DNA bending (*20*).

The average nonspecific bend angle is very similar to the specific one, but the distribution of nonspecific bend angles is substantially broader than that of the specific angles (62° ± 23° versus 69° ± 11°) (Fig. 5, A and B). Presumably, this broader distribution reflects the multiplicity of microstates associated with the binding of the protein to many different positions along the DNA (*16*, *21*), a larger flexibility of the nonspecific complexes over the specific ones (*22*), or both.

The observation that Cro bends DNA at nonspecific sites has important implications about (1) its dynamics of one-dimensional diffusion along the DNA, (2) its binding specificity, and (3) its mechanism of specific site recognition.

1) The nonspecific complexes observed in these studies depict Cro molecules trapped, by deposition on the mica surface, before they arrive at the specific binding site. These observations indicate that Cro induces DNA bending at all positions (Fig. 5, A and B). If Cro diffuses along the DNA in search of its operator (*5*, 9), the observed bending of the nonspecific DNA would result in the propagation of a "bending wave" along the DNA, with Cro riding at its vertex. It can be shown that propagation of such a bending wave along the DNA should not present a significant energy barrier to protein diffusion along the DNA (*23*), although it will reduce the rate of diffusion (*24*).

2) The binding specificity of a protein is determined by the difference in binding affinities of the protein for the specific versus the nonspecific sites. Any factor that can alter this difference will necessarily affect the binding specificity of the protein. Protein-induced DNA bending can contribute to binding specificity through the energy cost associated with the DNA distortion (*25*). If the protein does not bend

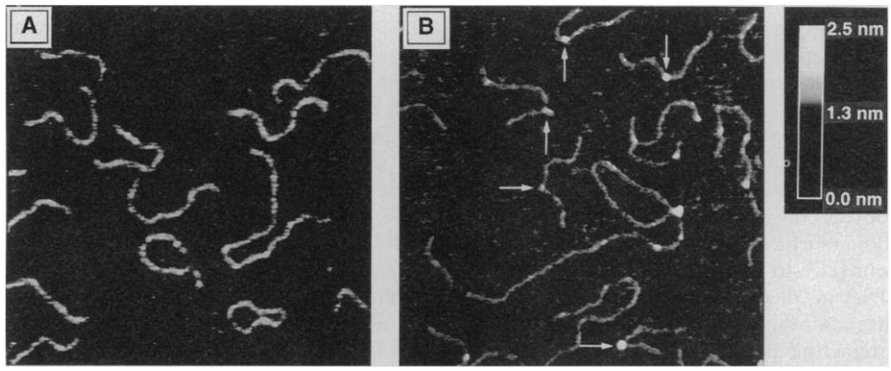

Fig. 2. Scanning force microscope images of (**A**) free 1-kb DNA fragment and (**B**) Cro-DNA complexes formed on the 1-kb fragment (*11*, *41*). The scan sizes are 1000 nm. In (B), the high features observed on the DNA, some of which are marked with arrows, are bound Cro molecules. Such DNA fragments are scanned at a closer range (Figs. 3 and 4), and the position and size of the features are measured. Only those complexes in which the size of the feature is consistent with of one, two, or three bound Cro molecules are analyzed.

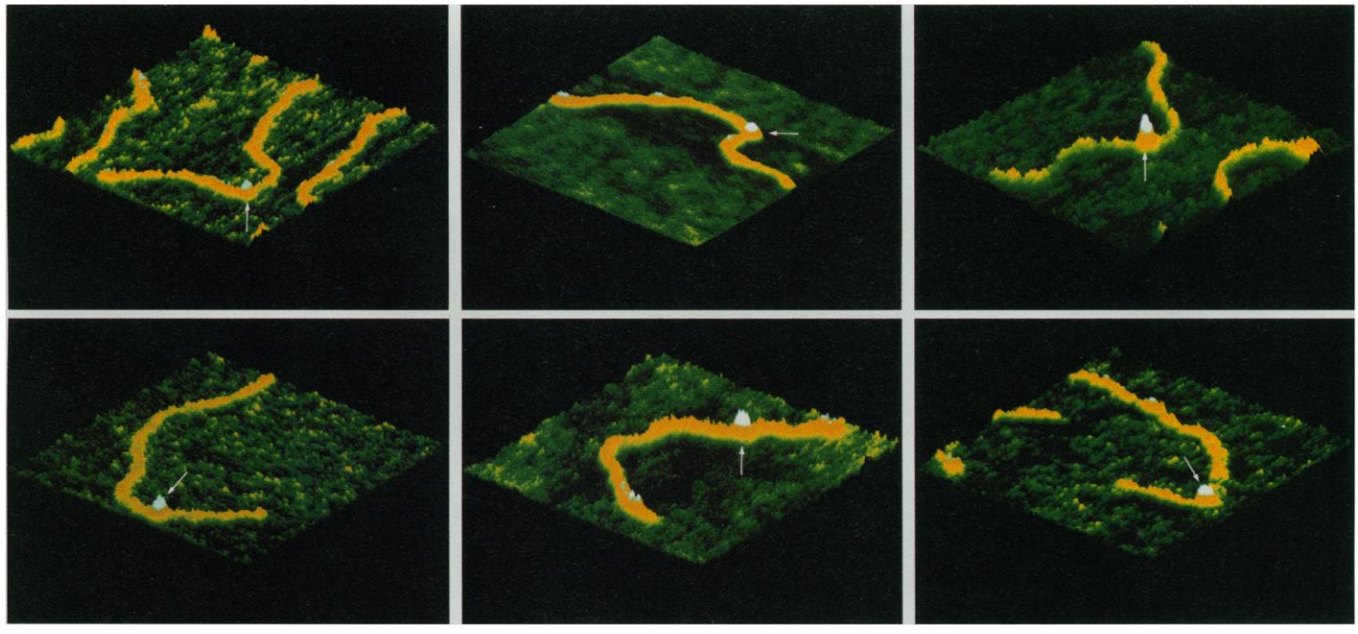

Fig. 3. Surface plots of six individual Cro-DNA complexes. The three images in the top row are specific complexes, and those in the bottom row are nonspecific complexes. The scan sizes are 250 nm. The plane of the mica is displayed with a 30° inclination angle to show the topography of the surface. These images were obtained from several different depositions that were scanned with different tips. Although the absolute heights and widths vary depending on the radius of curvature of the tip and the humidity of the sample chamber (*12*), the observed relative heights do not vary significantly.

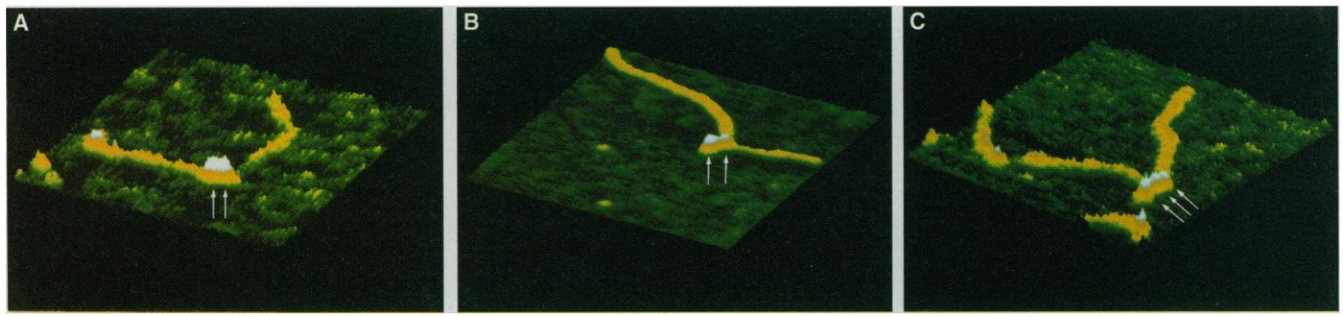

Fig. 4. (**A** and **B**) Surface plots of Cro-DNA complexes in which two Cro dimers are bound specifically. The plane of the mica is inclined 30°. (**C**) Cro-DNA complex with three Cro dimers bound at the O_R3, O_R2, and O_R1 sites (*42*). The scan sizes are 250 nm. These conformations are consistently observed for the multiply occupied species. The structure shown in (B) is the most commonly observed structure for the doubly bound complexes.

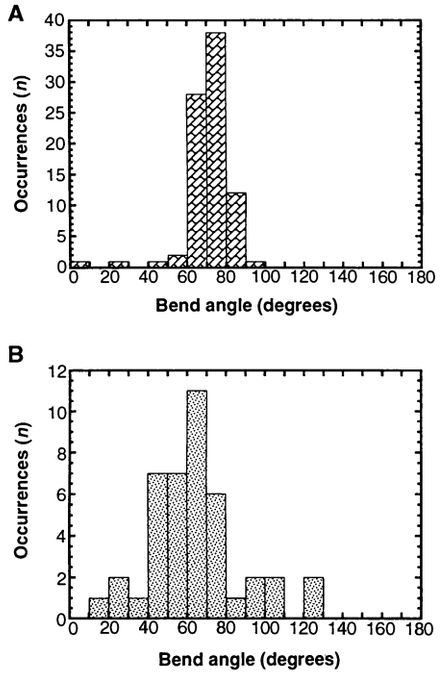

Fig. 5. Histograms of the frequency of occurrence of the bend angles for (**A**) specific and (**B**) nonspecific Cro-DNA complexes (*16*). To determine the bend angles, lines are drawn through the axes of the DNA on both sides of Cro, and the angle of their intersection (Φ) is measured. The apex of the angle is constrained to the center of the Cro molecule. The bend angle (Θ) is the supplement of the measured angle ($\Theta = 180 - \Phi$).

the nonspecific DNA, it must use the energy from specific interactions to induce bending at the specific site. This unfavorable bending energy reduces the difference in energy between the specific and nonspecific complexes—that is, it reduces the binding specificity of the protein. On the other hand, if the bending occurs also at the nonspecific sites, a larger energy differential between the specific and nonspecific complexes can be achieved because the energy to bend the DNA must be expended in order for both these complexes to form. Consequently, to maximize their binding specificity, proteins that bend the DNA at the specific site may bend it also at nonspecific sites, as we observed for Cro (*26*).

3) Bending of the DNA at the nonspecific sites may be an important component of the mechanism of specific site recognition by Cro. Cro may recognize its specific site by sampling the specific contacts at each position on the DNA or by sampling contacts only at certain positions, such as those in which the DNA can be more easily bent (*27*). In both cases, Cro must bend the DNA at all positions, as observed. In the first case, the bending is required to expose interacting groups and to facilitate the formation of the specific contacts; in the second case, the ease of bending could signal the protein of the possible arrival at the specific locus. If the specific site requires less energy to distort, this differential energy might be the signal to "check" for specific contacts (*28*).

The above analyses strongly support the notion that proteins that bend their specific sites may also bend nonspecific locations. In corroboration of this idea, we recently obtained similar results for another transcription factor (*29*). In general, protein-induced DNA bending may modulate specificity at other levels in addition to binding. Many proteins that interact with DNA, such as methylases, recombinases, and restriction endonucleases, also catalyze chemical reactions with the DNA. For such enzymes, DNA bending may play a role in specificity at the level of catalysis as well as at the level of binding. If DNA bending is required for catalysis at the specific binding site (*30*), it may be advantageous for the protein not to bend the nonspecific DNA, because the resulting decrease in binding specificity could be compensated by an equivalent increase in catalytic specificity. Eco RV endonuclease is an example of a DNA enzyme that induces bending of the cognate sequence (*31*) but that may not induce bending of nonspecific DNA (*31*, *32*). As discussed above, specific site recognition by such enzymes may require a preexisting secondary structure of the target sequence. Comparison of the conformations resulting from specific and nonspecific interactions should help elucidate the function of DNA bending in protein-DNA complexes.

Traditional biochemical methods cannot be used to analyze quantitatively bending of nonspecific DNA by proteins, because these methods require a single binding site on a long DNA fragment (*33*). In addition, a bend angle determined by crystallographic methods with short, nonspecific oligonucleotides (*31*) may not represent the average angle of the ensemble of nonspecific complexes, because the conformations of nonspecific complexes depend on the DNA sequence (*21*). Direct visualization methods can be used to characterize the distribution of conformations of specific and nonspecific complexes. Because Cro is one of the smallest proteins to be imaged by any type of microscopy, the scanning force microscope can be used to investigate conformational properties of protein–nucleic acid complexes that have not been previously accessible to direct visualization or to biochemical analysis. Finally, the spatial resolution obtained for the multiply bound Cro molecules shows that scanning force microscopy can be used to study multiprotein assemblies on DNA.

REFERENCES AND NOTES

1. P. H. von Hippel and O. G. Berg, *J. Biol. Chem.* **264**, 675 (1989); R. Fickert and B. Müller-Hill, *J. Mol. Biol.* **226**, 59 (1992).
2. T. A. Steitz, *Q. Rev. Biophys.* **23**, 205 (1990); C. O. Pabo and R. T. Sauer, *Annu. Rev. Biochem.* **61**, 1053 (1992); P. H. von Hippel, *Science* **263**, 769 (1994); R. S. Spolar and M. T. Record Jr., *ibid.*, p. 777.
3. A. A. Travers, *Cell* **60**, 177 (1990); P. C. van der Vliet and C. P. Verrijzer, *BioEssays* **15**, 25 (1993).
4. M. Ptashne, *A Genetic Switch, Phage Lambda and Higher Organisms* (Cell Press, Cambridge, MA, ed. 2, 1992).
5. J. G. Kim, Y. Takeda, B. W. Matthews, W. F. Anderson, *J. Mol. Biol.* **196**, 149 (1987).
6. J. Kim, C. Zwieb, C. Wu, S. Adhya, *Gene* **85**, 15 (1989).
7. R. G. Brennan, S. L. Roderick, Y. Takeda, B. W. Matthews, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8165 (1990).
8. Y. Lyubchenko, L. Shlyakhtenko, B. Chernov, R. E.

Harrington, *ibid.* **88**, 5331 (1991).
9. It has been shown that an increase in the length of the DNA fragment from 21 to 800 bp results in a 10-fold increase in the association rate constant for specific Cro complexation. These results have been explained by a two-step mechanism in which Cro first binds the DNA nonspecifically and then finds its target site by facilitated diffusion along the DNA (*5*).
10. C. Bustamante, D. Keller, G. Yang, *Curr. Opin. Struct. Biol.* **3**, 363 (1993).
11. We formed Cro-DNA complexes by incubating the 1-kb DNA fragment (20 nM) with Cro (20 to 250 nM) at 23°C for ~10 min in buffer containing 20 mM tris Cl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, and 75 mM potassium acetate. The higher concentrations of Cro are used to obtain a sufficient population of nonspecific Cro-DNA complexes. The complexes were diluted 10 times in the above buffer without the potassium acetate, deposited immediately onto freshly cleaved mica, rinsed with ~0.5 ml of distilled, deionized water, partially dried with nitrogen, and imaged in air (*12*) [J. Vesenka *et al.*, *Ultramicroscopy* **42–44**, 1243 (1992)]. The free DNA is treated in the same manner as the Cro-DNA complexes.
12. C. Bustamante *et al.*, *Biochemistry* **31**, 22 (1992); D. J. Keller and C. Chihchung, *Surfactant Sci.* **268**, 333 (1992).
13. The bend angle (Θ) is defined as the angle by which the DNA deviates from linearity, with the apex of the angle at the center of the protein.
14. Although the specific site has been determined to be 17 bp (*4*), there may be nonspecific contacts outside this region that could contribute to the DNA bending.
15. Two factors may determine the appearance of the multiply occupied complexes. (i) Cro may induce not only bending but also torsion along the DNA axis. (ii) The centers of the O_R sites are 23 bp apart and therefore out of register with the periodicity of the double helix. In particular, the O_R1 and O_R3 sites are 4 bp, or 137°, out of phase and have opposite polarity. If more than one Cro molecule is bound simultaneously to a single DNA fragment, these effects may produce DNA conformations in solution that do not lie in a plane. Some of the unusual geometries observed may have resulted from the constraints imposed on the solution structures as they lie down on the two-dimensional mica surface. This problem should not arise for DNA fragments with only a single Cro dimer bound.
16. The average length of the DNA fragments is 330 ± 15 nm. Cro is assumed to be specifically bound if it is located between 35% and 45% from one end. Because one end of the DNA cannot be distinguished from the other, some nonspecific complexes may be included in the specific distribution, thus potentially broadening the distribution. However, no bend angles from specific complexes are included with the nonspecific ones. We have analyzed 84 specific complexes and 42 nonspecific complexes. Nonspecifically bound Cro is found at many different sites on the DNA fragment, and no single nonspecific site is overrepresented. At stoichiometric Cro-DNA concentrations, more than 80% of the Cro molecules are found in locations consistent with the position of the O_R region.
17. The angles determined here should be a good representation of those that exist in solution as discussed [(*34*); M. Joanicot and B. Revet, *Biopolymers* **26**, 315 (1987); E. Le Cam *et al.*, *J. Mol. Biol.* **235**, 1062 (1994)]. Furthermore, the angles determined for RNA polymerase complexes with the same deposition procedure (*34*) have been confirmed by electric dichroism measurements [F.-J. Meyer-Almes, H. Heumann, D. Proschke, *J. Mol. Biol.* **236**, 1 (1994)].
18. S. Herman *et al.*, unpublished results.
19. For TBP, the x-ray structure shows a bending angle of ~100° [Y. Kim, J. H. Geiger, S. Hahn, P. B. Sigler, *Nature* **365**, 512 (1993); J. L. Kim, D. B. Nikolov, S. K. Burley, *ibid.*, p. 520]; gel mobility assays yield a bending angle of 93° (D. Starr, B. Hoopes, D. K. Hawley, in preparation). For Eco RV, the x-ray structure gives a value of ~50° (*31*); gel mobility assays indicate a value of 44° [T. Stöver *et al.*, *J. Biol. Chem.* **268**, 8645 (1993)]. Gel mobility assays with Eco RI methylases indicate a value of 55°, whereas the mutant protein indicated no substantial bending (R. García, unpublished results).
20. A bend angle of 30° was determined by gel electrophoresis of Cro bound to ~200-bp DNA fragments (*6*). This angle may be underestimated as a result of the (i) flexibility of the DNA fragment (*35*), (ii) nonspecific binding and sliding of Cro along the DNA during electrophoresis [P. Prentki, M. Chandler, D. J. Galas, *EMBO J.* **6**, 2479 (1987)], or (iii) the empirical calibration of bandshift assays [J. F. Thompson and A. Landy, *Nucleic Acids Res.* **16**, 9687 (1988); S. S. Zinkel and D. M. Crothers, *Biopolymers* **29**, 29 (1990)] (*35*). In a single gel mobility assay on phage 434 Cro and 434 repressor, protein-induced DNA bending was observed for repressor protein but not for Cro [G. B. Koudelka, P. Harbury, S. C. Harrison, M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4633 (1988)]. However, it was later demonstrated by both crystallography (*36*) and gel mobility assays [G. B. Koudelka, *Nucleic Acids Res.* **19**, 4115 (1991)] that both of these proteins bend DNA to approximately the same extent. This result indicates that a single gel mobility assay may yield an accurate estimate of DNA bending for one protein-DNA complex and not for another, even if the extents of bending are very similar. The angle determined from crystallography (*7*) and oligomer cyclization experiments (*8*) was 45°. The DNA in the crystal structure is only 17 bp long, and therefore some of the Cro-DNA interactions may not be present (*13*). A recent crystal structure of Cro from phage 434 bound to a 20-bp DNA fragment (*36*) reveals more bending than had been previously determined with a 14-bp fragment [C. Wolberger, Y. Dong, M. Ptashne, S. C. Harrison, *Nature* **335**, 789 (1988)]. Finally, in the cyclization experiments (*8*), ligation of 21-bp oligomers was performed in the presence of Cro, and the bend angle was defined as 360/(number of oligomers in the resulting closed circles). This method assumes that every site has a Cro dimer bound at the time of ligation; however, the actual site occupancy at the time of ligation is not known. As a result of steric hindrances, it may not be possible for Cro to bind the two sites adjacent to the ligation site when DNA ligase is present. Accordingly, the minimum bend angle determined from these cyclization experiments may be closer to 60° (360°/6), and not 45° (360/8) as concluded (*8*).
21. Y. Takeda, P. D. Ross, C. P. Mudd, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8180 (1992).
22. The standard deviation of the angular distributions (s) can be shown to be

$$s = \sqrt{kT/(\mu + \gamma + \kappa)}$$

where k is the Boltzmann constant and T is the absolute temperature, μ and γ are the second derivatives with respect to the bending angle of the mica-DNA interaction energy and of the protein-DNA interaction energy, respectively, and κ is the intrinsic bending constant of a DNA fragment of length ℓ and is related to its persistence length in solution, P, by

$$\kappa = PkT/\ell$$

The signs of the second derivatives determine the effect of the surface and of the protein on the spread of the observed angular distributions. Generally, $\mu < 0$; that is, the surface tends to increase the spread of the distributions because the surface binding energy can stabilize otherwise unfavorable departures from the mean bending angle of the complex. Conversely, γ can be both positive or negative. Our results indicate that γ is positive for the specific complexes because the distributions appear tighter (s = 11°) than expected for DNA in solution (s = 20°); for the nonspecific complexes (s = 23°), γ is just positive enough to offset the broadening effect of the surface.
23. The energy required to bend a DNA fragment of length ℓ, modeled as a wormlike chain of persistence length (P), through an arc of radius of curvature R is

$$E_{bend} = PkT\ell/2R^2$$

Because $R = \ell/\Theta$, the bending energy can be written also as

$$E_{bend} = PkT\Theta^2/2\ell$$

[L. D. Landau and E. M. Lifshitz, *Theory of Elasticity*, vol. 7 of *Course of Theoretical Physics* (Pergamon, Oxford, ed. 2, 1970), p. 82; *Statistical Physics, Part I*, vol. 5 of *Course of Theoretical Physics* (Pergamon, Oxford, ed. 3, 1980), p. 396; J. B. Hats, M. E. Mager, B. H. Zimm, *Biopolymers* **8**, 531 (1969); J. A. Schellman, *ibid.* **13**, 217 (1974)]. With P = 550 Å [C. Bustamante, J. F. Marko, E. D. Siggia, S. B. Smith, *Science* **265**, 1599 (1994)], the energy required to bend a 20 Å by 3.4 Å piece of DNA (*37*) by a 65° angle at room temperature (300 K) is ~3.1 kcal/mol. Clearly, this bending energy must be provided by the interactions that result from the formation of the nonspecific complex (*38*). The elastic energy stored in a homogeneously bent molecule distributes evenly along the molecule and therefore is a linear function of its length, as shown above. Accordingly, the energy required to add 1 bp with the same amount of curvature to the bend (that is, the activation energy required to displace the bend laterally by 1 bp) is simply 3.1 kcal/20 mol·bp = 0.15 kcal/mol·bp, substantially smaller than the value for kT.
24. The additional activation energy required to propagate a bend (*23*) will reduce the rate of one-dimensional diffusion of the protein along the DNA by

$$\left\{1 - \exp\left[\frac{-(E_a + 0.15)}{kT} + \frac{E_a}{kT}\right]\right\} \times 100 = 22\%$$

relative to the nonbending case, where E_a is the activation energy for one-dimensional diffusion along the DNA in the absence of bending.
25. D. R. Lesser, M. R. Kurpiewski, L. Jen-Jacobson, *Science* **250**, 776 (1990); D. E. Lesser, M. R. Kurpiewski, T. Waters, B. A. Connolly, L. Jen-Jacobson, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7548 (1993).
26. The suggestion that DNA bending may be an important factor in protein binding specificity is strongly supported by the large amount of energy required to bend DNA. Catabolite activator protein (CAP), which induces a ~90° bend, was found to bind 150-bp minicircles (pre-bent DNA) containing the specific site ~200 times more tightly than if the molecules were linear [J. D. Kahn and D. M. Crothers, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6343 (1992)]. This increase in binding affinity for CAP to pre-bent DNA indicates that at least 3 kcal/mol are required to induce bending at its specific site. In general, the difference in free energies of specific and nonspecific protein-DNA complexes is not greater than ~6 kcal/mol. Because the energy required for DNA bending can have a similar magnitude, differences in bending energies between specific and nonspecific binding sites could potentially contribute a significant amount of favorable free energy to binding specificity. These results suggest, in turn, that bending energy can significantly destabilize a specific protein-DNA complex and therefore reduce the binding specificity, if the nonspecific DNA is not bent. Conversely, if less energy is required to bend the specific site than to bend the nonspecific sites, this differential energy would make a favorable contribution to the binding specificity of the protein.
27. Cro could not recognize its specific site by means of a preexisting secondary structure in the DNA because it alters the conformation of the DNA even upon nonspecific complexation. It may, however, recognize a difference in bending energy associated with this structure.
28. These modes of recognition differ from the possible case in which a protein that bends its target site does not bend the nonspecific DNA. In this case, the protein must recognize either a subset of the specific contacts or a preexisting secondary structure at the specific site, possibly a bend. It is important to note, however, that such a protein could not sample the full complement of specific contacts at every location on the DNA, because a protein that bends its specific site presumably must bend the DNA to make these contacts.
29. The specific and nonspecific bend angles have been measured for yeast TBP, and similar results were obtained (S. Hermann, M. Gulthold, D. Hawley, C. Bustamante, unpublished results). In contrast to Cro, TBP does not appear to use a mechanism of facilitated target location, as its apparent second-order binding rate constant for this site is quite small [B. C. Hoopes, J. F. LeBlanc, D. K. Hawley, *J. Biol. Chem.* **267**, 11539 (1992)]. The molecule in this case

may simply bind, bend, and release the DNA at each location in search of the target site. Nonetheless, our analysis remains valid whether or not the protein diffuses along the DNA.
30. A. J. Zaug, J. R. Kent, T. R. Cech, *Biochemistry* **24**, 6211 (1985); C. K. Surratt, S. C. Milan, M. J. Chamberlin, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7983 (1991).
31. F. K. Winkler *et al.*, *EMBO J.* **12**, 1781 (1993).
32. Crystal structures of Eco RV endonuclease bound to specific and nonspecific DNA fragments recently have been solved (*31*). The DNA in the specific complex has a 50° kink in the central TA step, and the overall conformation deviates significantly from that of B-form DNA; the DNA in the nonspecific complex, on the other hand, has a conformation similar to that of B-DNA. However, two fragments of DNA are bound to Eco RV in the nonspecific complex, with the terminal base pair of each duplex forming what would be the TA step in the specific complex. Because the DNA is discontinuous at the position where the greatest distortion is expected to occur, the conformation of DNA in such a complex might be significantly different from that of Eco RV bound to a continuous, nonspecific site.
33. D. M. Crothers, M. R. Gartenberg, T. E. Shrader, *Methods Enzymol.* **208**, 118 (1991).
34. W. A. Rees, R. W. Keller, J. P. Vesenka, G. Yang, C. Bustamante, *Science* **260**, 1646 (1993).
35. J. D. Kahn, E. Yun, D. M. Crothers, *Nature* **368**, 163 (1994).
36. A. Mondragon and S. C. Harrison, *J. Mol. Biol.* **219**, 321 (1991).
37. In view of the uncertainty about the exact number of base pair contacts involved in the specific and nonspecific Cro-DNA complexes, we have roughly estimated this number to be 20 bp (*14*).
38. It has been suggested that binding of a protein to a single side of DNA, as is the case for Cro, might favor DNA bending [J. B. Matthew and D. H. Ohlendorf, *J. Biol. Chem.* **260**, 5860 (1985)], thereby providing part of the driving force for protein-induced bending of nonspecific DNA.
39. This fragment is generated by first digesting the plasmid pDE13 [D. A. Erie, O. Hajiseyedjavadi, M. C. Young, P. H. von Hippel, *Science* **262**, 867 (1993)] with Sph I and then with Hind III. It is then separated by agarose gel electrophoresis, cut out of the gel, and purified with glass powder [B. Vogelstein and D. Gillespie, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 615 (1979)], followed by ethanol precipitation.
40. Y. Takeda, A. Sarai, V. M. Rivera, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 439 (1989).
41. The concentration of Cro is approximately 25 nM and that of DNA 2 nM for these depositions.
42. Both the position from the ends of the DNA fragment and the distance between the centers of the Cro dimers were measured to determine which sites were occupied.
43. We thank B. W. Matthews, E. Baldwin, W. A. Rees, B. Shoicet, L. Jen-Jacobson, P. H. von Hippel, and many of our laboratory colleagues for helpful discussions and comments on the manuscript. We are grateful to R. Albright and B. Matthews for providing us with purified λ Cro protein. Supported by U.S. Public Health Service (USPHS) research grants GM-32543 (C.B.), GM-15792 and GM-29158 (P. H. von Hippel), GM-20066 (B. W. Matthews), National Science Foundation grants MCB-9118482 (C.B.) and MCB-18945 (C.B. and D.A.E.), USPHS Postdoctoral Fellowship GM-12915 (D.A.E.), and a grant from the Lucille P. Markey Charitable Trust.

24 May 1994; accepted 16 September 1994

Accumulation of HLA-DM, a Regulator of Antigen Presentation, in MHC Class II Compartments

Frances Sanderson, Monique J. Kleijmeer, Adrian Kelly, Desirée Verwoerd, Abraham Tulp, Jacques J. Neefjes, Hans J. Geuze, John Trowsdale*

The *HLA-DM* genes encode an unconventional HLA (human leukocyte antigen) class II molecule that is required for appropriate binding of peptide to classical HLA class II products. In the absence of DM, other class II molecules are unstable upon electrophoresis in sodium dodecyl sulfate and are largely associated with a nested set of peptides derived from the invariant chain called CLIP, for class II–associated invariant chain peptides. DMA and DMB associated and accumulated in multilaminar, intracellular compartments with classical class II molecules, but were found infrequently, if at all, at the cell surface. Thus, DM may facilitate peptide binding to class II molecules within these intracellular compartments.

Major histocompatibility complex (MHC) class II molecules consist of heterodimers of a ~34-kD α and a ~28-kD β chain that interact with a third glycoprotein, the invariant chain (Ii) in the endoplasmic reticulum (ER). This complex is transported out of the ER through the Golgi stacks as a nonamer $(\alpha\beta Ii)_3$ (*1*). Invariant chain is then proteolytically removed and class II molecules accumulate in an acidic, lysosome-like compartment, termed MIIC in B lymphocytes (*2*). MIIC is placed late in the endocytic route and is a potential site for peptides derived from exogenous antigens to associate with class II molecules. Similar compartments have since been characterized in other cell types (*3*–*6*).

Insight into how peptides bind (that is, get loaded onto) class II molecules was provided by a series of cell lines with mutations in genes in the class II region of the MHC (*7*, *8*). These cell lines have three interesting properties: (i) class II molecules on the cell surface are not recognized by certain conformation-specific antibodies, such as the DR3 antibody 16.23; (ii) upon polyacrylamide gel electrophoresis (PAGE) in SDS, class II heterodimers are less stable than those of normal cells and dissociate into their constituent polypeptide chains; and (iii) the mutant cells are defective in the presentation of protein antigens to T cells (*9*). All three phenotypes of the mutant cells are the result of mutations in either of the linked *DMA* and *DMB* genes (*7*, *8*). In addition, another mutant cell line with a large deletion encompassing the *DM* locus (T2-DR3) develops abnormal MIIC and has class II molecules predominantly associated with the class II invariant chain–associated peptides (CLIP) (*10*, *11*).

To investigate further the function of DM, we made rabbit antisera; FS2 recognizes recombinant DM α chain protein and AK3 recognizes the derived COOH-terminal peptide of the DM β chain. To demonstrate the specificities of the antisera, we used glycoprotein preparations from the B lymphoblastoid cell line LCL721, the deletion mutant 721.174, and the cell line 7.9.6, which bears a point mutation in *DMB* (*7*, *9*, *12*). Both antisera recognized specific bands on protein immunoblots of 33 to 35 kD and 30 to 31 kD, for antibody to DMA (anti-DMA) and anti-DMB, respectively (Fig. 1, A and B). These values are consistent with the predicted molecular sizes of 26 kD given that the DMA sequence contains two putative N-linked glycosylation sites and DMB, one (*13*). As additional specificity controls, we demonstrated that anti-DMA did not cross-react with conventional class II molecules expressed in L cell transfectants and identified unique spots on two-dimensional SDS-PAGE (*14*).

The sequence similarities between DM and the classical class II genes, their locations adjacent to one another in the class II region, and the identical phenotypes of *DMA* and *DMB* mutants all suggest that the DM α and β chains associate to form a heterodimer. To confirm this, cells from the Burkitt's lymphoma cell line Raji were metabolically labeled for 20 min and lysed. Extracts were precipitated with the antiserum to DMA, FS2 (Fig. 2A). Immunoprecipitated protein was eluted from protein A–Sepharose and reprecipitated with either anti-DMA or anti-DMB. Reprecipitation with anti-DMA revealed bands at 30 and 33 kD. These were specific for DMA, and both were seen after metabolic labeling with ^{35}S for 5 min (*15*). The lower band is not present in appreciable quantities in the

F. Sanderson, A. Kelly, J. Trowsdale, Human Immunogenetics Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, Holborn, London WC2A 3PX, UK.
M. J. Kleijmeer and H. J. Geuze, Department of Cell Biology, School of Medicine, Utrecht University, The Netherlands.
D. Verwoerd, A. Tulp, J. J. Neefjes, Department of Cellular Biochemistry, Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands.
*To whom correspondence should be addressed.