
Tet repressor binding induced curvature of *tet* operator DNA

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ABSTRACT

Tet repressor dimer binds to two *tet* operator sites spaced by 30 bp in the Tn10 encoded *tet* regulatory DNA. The effect of repressor binding on the gel mobility of circular permuted DNA fragments containing either one or both operator sequences is reported. The *EcoRI* induced bending of DNA is used to compare the results with other protein binding induced structural perturbations of DNA. Tet repressor bends a DNA fragment with a single *tet* operator to an angle of $42^\circ \pm 7^\circ$. The apparent bend angle of DNA fragments containing the tandem *tet* operator arrangement occupied by two Tet repressor dimers turns out to be $52^\circ \pm 9^\circ$. These results are interpreted with respect to the end to end distances of the bent DNA fragments. They indicate that either the intervening *tet* regulatory DNA between the operators or the bound operator sequences themselves contain additional perturbations from the canonical B-DNA structure. This finding is discussed in the light of previously obtained results from CD, neutron scattering, and electrooptical studies.

INTRODUCTION

Tetracycline resistance determinants in Gram negative bacteria belong mostly to one of five classes named A to E (1,2). Most of the determinants found in wild type enteric bacteria belong to class B and constitute a part of transposon Tn10 (1,3). Resistance is achieved by a membrane residing efflux protein for the drug (4) whose expression is strictly regulated at the level of transcription (5,6,7). The mechanism of regulation is quite complex involving a tandem operator arrangement and a bipolar, overlapping promoter system (5,6). Within only 78 bp the control region contains three *in vivo* active promoters (7) and the two operators, which exhibit differential effects upon occupation with the Tet repressor on transcription from these promoters (8).

Many DNA binding proteins acting in DNA recombination and modification or gene regulation have been shown to bend the DNA double helix (9). Furthermore, A-tracts with correct phasing are capable of inducing bends in free DNA (10). It is conceivable that these DNA structures may be important for the mechanisms by which different proteins communicate along the DNA or mediate regulatory effects (11,12). It is, thus, necessary to study the effects of binding of regulatory proteins on the structure of the DNA, in particular for a regulatory system as complex as the one described above for the *tet* determinant. In this article we determine and discuss the structural changes of various *tet* operator containing DNA fragments upon binding of Tet repressor as derived from gel mobility shifts.

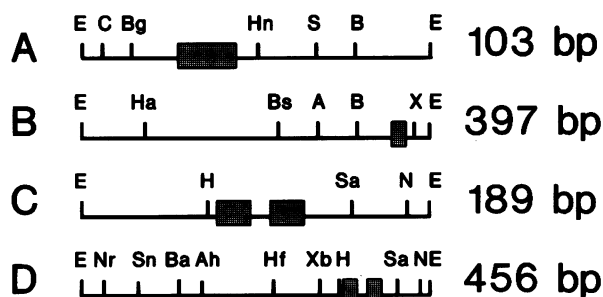


Fig. 1: Overview of the DNA fragments used to produce circular permutations. Four DNA fragments are shown with the positions of relevant restriction sites used to produce the circular permutations. The size of each fragment is given on the right side in base pairs. The positions of one or two *tet* operators are indicated by shaded boxes drawn in the correct scale of the respective fragment. The two DNAs with two *tet* operators contain the wild type *tet* regulatory sequence. Details of the constructions are given in the Materials and Methods section. Abbreviations for the restriction sites are: E=*EcoRI*, C=*ClaI*, Bg=*BglIII*, Hn=*HindIII*, S=*SalI*, B=*BamHI*, Ha=*HaeIII*, Bs=*BstXI*, A=*AvaII*, H=*HincII*, X=*XhoI*, Sa=*Sau3A*, N=*NcoI*, Nr=*NruI*, Sn=*SnaBI*, Ba=*BanI*, Ah=*AhaII*, Hf=*HinfI*, and Xb=*XbaI*.

MATERIALS AND METHODS

General methods

The Tn10 encoded Tet repressor (13) and *EcoRI* (14) were prepared from overproducing *E. coli* strains as described. All other enzymes were purchased from Boehringer (Mannheim), Biolabs (New England), or Pharmacia (Freiburg). Chemicals were purchased from Merck (Darmstadt), Sigma (St. Louis), or Roth (Karlsruhe) at the highest purity available. All cloning procedures were done as published (15). Plasmid DNA was purified on a large scale as described (16). DNA was radioactively labeled using γ - ^{32}P -ATP purchased from Amersham (Braunschweig).

Circular permuted DNA fragments

Three insertions of the 103 bp *EcoRI* DNA from pWH873 (17) into the *EcoRI* site and two insertions of the 397 bp *BamHI* DNA from pWH873 (17) into the *BamHI* site of pWH802 (18) resulted in plasmids pWH533 and pWH949, respectively. The plasmids pWH106 and pWH132 containing multiple insertions of the 189 bp and 456 bp DNAs, respectively, have been described previously (19). These plasmids were digested with the restriction endonucleases indicated in Fig. 1 to yield circular permutations of the inserts.

Native polyacrylamide gel electrophoresis

Protein binding was carried out in total volumes varying from 10 to 40 μl containing 50 mM NaCl, 10 mM MgCl_2 , 10 mM TrisHCl, pH 7.5, 1 mM Dithiothreitol, and either 5% Ficoll 400 and 5% glycerol or 12% glycerol. The amounts of DNA varied between 15 ng and 1.6 μg and are specified in the legends to the respective figures. In *EcoRI* binding experiments the 10 mM MgCl_2 were replaced by 50 mM EDTA. After incubation for 30 min at 37°C the samples were loaded on polyacrylamide gels. Gels (140 \times 280 \times 1 mm) contained between 5 and 10% acrylamide (19:1, bis-acrylamide), 60 mM Tris-borate (pH 8.3), 10 mM EDTA, and 10% glycerol. All gels were prerun for two hours prior to loading. Electrophoresis of the samples was performed at 11 V/cm for 10 to 15 hours. After electrophoresis the gels containing 0.2 to 1.6 μg of DNA per lane were stained with ethidium bromide. Gels containing 15 ng of ^{32}P -labeled DNA per lane were autoradiographed at -70°C .

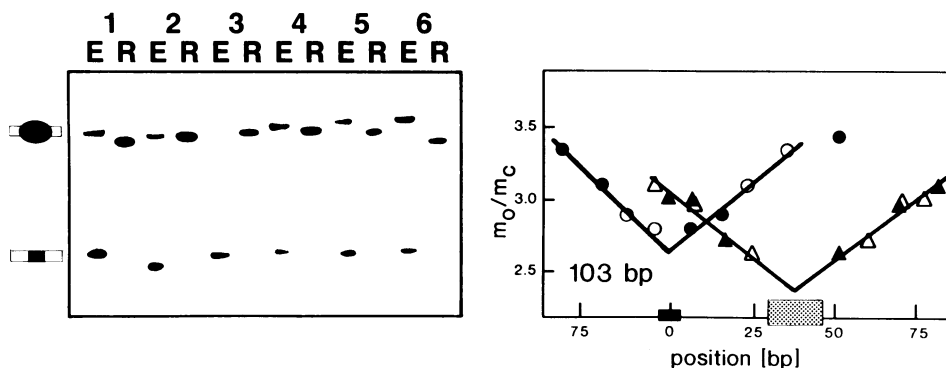


Fig. 2: Gel mobility analysis of circular permutations of the 103 bp DNA complexed with Tet repressor and *EcoRI*. On the left side an autoradiography of a native 10% polyacrylamide gel is shown. The numbers on the top denote the circular permutations. The restriction endonucleases used to produce them were: 1 = *Bgl*II, 2 = *Clai*I, 3 = *Eco*RI, 4 = *Bam*HI, 5 = *Sal*I, and 6 = *Hind*III. In the lanes marked E 15 ng of the respective 5'-end labelled DNA preincubated at 37°C for 30 min with 240 U *Eco*RI in 20 μ l (*tet* operator concentration = 1×10^{-9} M) of 10 mM Tris HCl pH 8.0, 5% Ficoll, 50 mM EDTA, and 10 mM NaCl were loaded. In the lanes marked R the same amounts of DNA preincubated with 1.5×10^{-9} M Tet repressor were loaded. The sketch on the left side indicates the free DNA with the *tet* operator (black box) and the complex of this DNA with one Tet repressor dimer (black ellipsoid). On the right side of the figure the mobility ratios of the free DNAs (m_0) over the complexed DNAs (m_c) are plotted versus the position of the respective restriction site on the DNA fragment. The position of the *tet* operator is marked by a shaded box and the one of the *Eco*RI site by a black box. The filled circles indicate experiments with the *Eco*RI-DNA complex while the triangles indicate experiments with Tet repressor-*tet* operator complexes. Each original data point (filled circles and triangles) was reflected to the respective other side of the fragment (open symbols) to improve the location of the bend in the sequence. The maximal mobility ratio differences are 0.78 for the *Eco*RI-DNA complex and 0.75 for the Tet repressor-*tet* operator complex.

RESULTS

The DNA fragments used for circular permutation and mobility shift determinations are shown in Fig. 1. They contain either the *tet* operator O_1 (17) or the wild type tandem *tet* operator arrangement (5,7). Similar fragments containing the single *tet* operator O_2 (17) were also used and yielded identical results as O_1 . The respective experiments are not shown. Furthermore, for each *tet* operator arrangement a long and a short DNA fragment was studied to address the effect of fragment length on the results. The restriction sites used to generate circular permutations and the operators are also indicated in Fig. 1. Since the restriction enzymes used produce protruding ends of different sizes and the DNA fragments contain various degrees of small internal curvature the mobilities of the free DNAs are not always identical. All of these effects are eliminated by interpreting only the mobility ratio for each restriction fragment (9).

Mobility shift analyses of DNA fragments containing single *tet* operators. Circular permutations of the 103 bp DNA were analyzed on a 10% polyacrylamide gel and the results are shown in Fig. 2. The mobilities of the free DNAs do not indicate any inherent curvature of this sequence, while the mobilities of complexes formed with Tet repressor depend clearly on the position of the *tet* operator in the fragment. This result is also analyzed in Fig. 2. The centre of the bend on the DNA is located exactly at the *tet* operator sequence. This result is anticipated owing to the palindromic symmetry of the *tet* operator and the dimeric structure of the Tet repressor (20). The same mobility shift was also found for

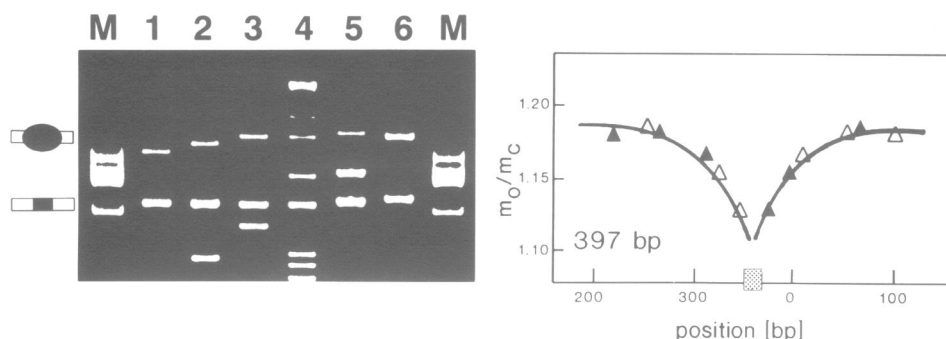


Fig. 3: Gel mobility analysis of circular permutations of the 397 bp DNA complexed with Tet repressor. The photography on the left side shows the mobilities of free DNAs and repressor-operator complexes on a 5% native polyacrylamide gel as indicated on the left side (Sketch as in Fig. 2). The restriction enzymes used to produce the circular permutations were: lane 1 = *Xho*I, lane 2 = *Eco*RI, lane 3 = *Hae*II, lane 4 = *Bst*UI, lane 5 = *Ava*II, and lane 6 = *Bam*HI. In each lane 0.5 μ g DNA incubated for 30 min at 37°C with a half saturating amount of Tet repressor in 20 μ l (concentration of *tet* operator = 4×10^{-8} M) of 50 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris HCl, pH7.5, 1mM DTE, and 12% glycerol was loaded. The lanes M contain molecular weight markers (pWH802/*Eco*RI/*Hae*III, 18). On the right side of the figure the analysis as described in the legend of Fig. 2 is given. The maximal mobility ratio difference is 0.94.

the *tet* operator O_2 -Tet repressor complex in the same sequence context (data not presented).

In order to quantitate the bend a mobility analysis of these permuted fragments with *Eco*RI was also done and the results are included in Fig. 2. In this case the centre of the curvature agrees well with the position of the *Eco*RI site. The curvature of the *Eco*RI-DNA complex has been used to calibrate the mobility scale to results obtained with other protein-DNA complexes (9). The respective extrapolated maximal and minimal mobilities shown in Fig. 2 were converted to bending angles using the data in figure 3 of reference 9. Using this approach the maximal mobility difference for the *Eco*RI-DNA complex yields a curvature of $66^\circ \pm 11^\circ$. This is at the upper limit of the previously reported angles between 52° and 66° (9) and falls within the range suggested by the x-ray structure (21). The curvature introduced in *tet* operator upon binding of Tet repressor is determined to be $72^\circ \pm 11^\circ$.

The *tet* operator contains about 19 nucleotides and represents a large portion of the 103 bp DNA fragment. Therefore, the mobility shifts of a 397 bp DNA fragment containing the *tet* operator O_1 were analyzed on a 5% polyacrylamide gel. The results are shown in Fig. 3. The extrapolation of the relative mobilities locates the Tet repressor induced bend in the centre of the *tet* operator sequence. The angle of the bend is determined to be $48^\circ \pm 7^\circ$.

The result obtained with the long DNA fragment differs from the one obtained with the short DNA. This may be rationalized by the different shapes of the mobility analyses shown in Figs. 2 and 3. The longer DNA contains positions where the location of the restriction site shows no effect on gel mobility. These are at least somewhat more than 100 bp away from the operator. We assume that these positions are more than one persistence length away from the bend (30). Since this situation is not found for the 103 bp DNA we may find a larger total difference in mobility ratios resulting in a larger angle.

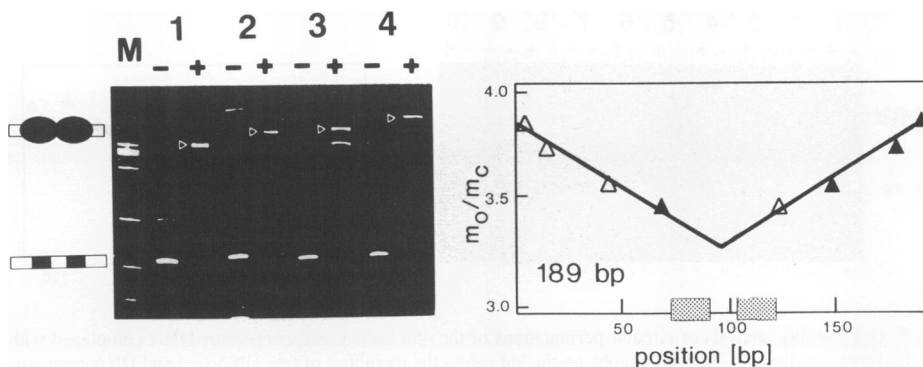


Fig. 4: Gel mobility analysis of circular permutations of the 189 bp tandem *tet* operator DNA complexed with two Tet repressor dimers. The photography on the left shows the mobilities of free DNAs (–) and DNA-repressor complexes (+) on a 10% native polyacrylamide gel. The restriction enzymes used to produce the circular permutations are: lanes 1 = *HincII*, lanes 2 = *Sau3A*, lanes 3 = *NcoI*, and lanes 4 = *EcoRI*. Lane M contains molecular weight markers (see legend to Fig. 3). As indicated by the scetch on the leftside the gel mobilities of free DNA and complexes with two Tet repressor dimers are analyzed. In each lane 0.9 μ g DNA in 20 μ l of the same buffer as described in the legend to Fig. 3 (concentration of operator = 1.6×10^{-7} M) was loaded. In the lanes designated + the samples contained 4×10^{-7} M Tet repressor dimer and were preincubated at 37°C for 30 min. The mobility analysis on the right side was produced as described in the legend to Fig. 2. The maximal mobility ratio difference is 0.86.

These mobility analyses confirm the importance of the end to end distance of DNA for the mobility shifts (27). Thus, we trust that the results obtained with the longer DNAs are more reliable for comparisons. This may also be the reason for the large angle determined here for the *EcoRI* induced bend (9).

Mobility shift analyses of DNA fragments containing the wild type tandem *tet* operator arrangement. The wild type *tet* regulatory region contains two *tet* operator sequences spaced by 30 bp between their palindromic centres (5,20). To probe the overall bending of this regulatory DNA upon binding of two Tet repressor dimers the gel mobility shifts of circular permutations of a 189 bp DNA fragment were analyzed on 10% polyacrylamide gels. The results are displayed in Fig. 4. The centre of the induced curvature is located between the two *tet* operators as would be expected for a combined bending at these two sites. The overall bending angle is analyzed to be $56^\circ \pm 7^\circ$.

The mobility shift experiments of a 456 bp DNA fragment with the wild type *tet* control region were also performed to increase the amount of flanking DNA with respect to the bent sequence. This DNA fragment contains, in addition to the sequence in the 189 bp DNA, 267 bp more of the *tetR* structural gene. The results of this experiment on a 5% polyacrylamide gel are displayed in Fig. 5. The mobility analysis of circular permutations from this DNA fragment reveals a sequence induced curvature which centres around nucleotide 270 of the *tetR* gene (22). As expected, this region contains properly spaced A-tracts (23).

The centre of the Tet repressor induced bend is again located between the two *tet* operators. The apparent bending angle is $52^\circ \pm 9^\circ$, agreeing very well with the respective result obtained with the 189 bp DNA.

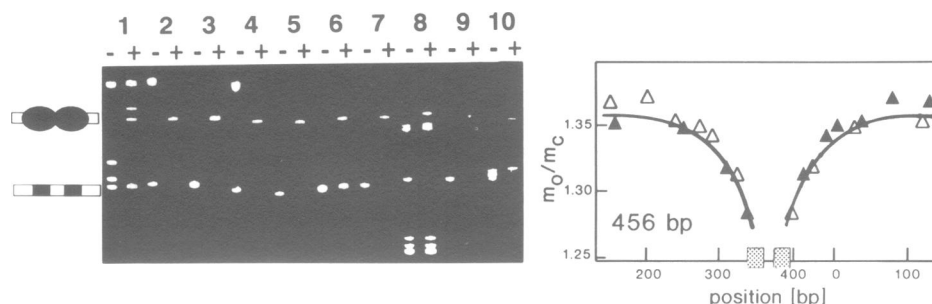


Fig. 5: Gel mobility analysis of circular permutations of the 456 bp tandem *tet* operator DNA complexed with two Tet repressor dimers. The photography on the left shows the mobilities of free DNA (–) and DNA-repressor complexes (+) on a native 5% polyacrylamide gel. The restriction endonucleases used to produce the circular permutations were: lanes 1 = *Sau3A*, lanes 2 = *NcoI*, lanes 3 = *EcoRI*, lanes 4 = *NruI*, lanes 5 = *SnaBI*, lanes 6 = *BanI*, lanes 7 = *AhaII*, lanes 8 = *HinfI*, lanes 9 = *XbaI*, and lanes 10 = *HincII*. In each lane 1.6 μ g DNA in 10 μ l of 60 mM Tris-borate, pH 8.3, 10 mM EDTA, 3% Ficoll, and 5% glycerol was loaded. In lanes marked + the mixtures were preincubated with Tet repressor for 30 min at 37°C. The mobility analysis on the right side was done as described in the legend to Fig. 2. The maximal mobility ratio difference is 0.93.

DISCUSSION

This study indicates clearly that Tet repressor binding to *tet* operator DNA induces curvature at the operator sequence. The mobility shift observed for the circular permuted *tet* operator-Tet repressor complex is much smaller as the one found for binding of CRP to DNA (24). It is in the same range observed for the *EcoRI* and Int complexes with their cognate DNAs (9).

A recent study has used the mobility differences of circular permutations to estimate the bending angles of the bound DNAs for several protein-DNA complexes (9). Following their assumptions we interpret the results presented here with respect to the bending angles induced in *tet* operator upon Tet repressor binding. For this purpose we have made use of the *EcoRI*-103 bp DNA complex to compare the results from our gel system, which is slightly different from the one described in reference 9, with the ones obtained there for *EcoRI* complexes with other DNAs. Our result of a bending angle of $66^\circ \pm 12^\circ$ for this complex falls in the upper part of the range obtained for three other DNA fragments with 52° , 56° , and 66° . This may be explained by a difference in obtaining the mobilities: We have used the extrapolated minimal mobility shift in Fig. 2 while Thompson and Landy used the difference between their most extreme permutations. Thus, the results obtained here should tend to be somewhat larger (9). Furthermore, the 103 bp DNA may tend to yield larger angles owing to its small size (compare above).

Using this estimation, the Tet repressor induced bend at the *tet* operator amounts to $48^\circ \pm 7^\circ$. Previous experiments observing the rotational relaxation rates of Tet repressor-*tet* operator complexes by electric dichroism did not yield a curved DNA structure, however, the resolution of this method may not be sufficient to detect such a slight bend (25). On the other hand, small angle neutron scattering studies revealed structural perturbations of the *tet* regulatory DNA when Tet repressor is bound (26). The distance of two Tet repressor dimers on the wild type *tet* regulatory region derived from these data could be explained by two limit models: One has the repressor dimers on the same side of the DNA which requires a 90° bend at each operator. This would be contradictory to the results

here. The second does not require large bends of the operators but proposes other structural perturbations of the DNA which are discussed below.

The wild type *tet* control sequence contains two operators, each of which should be bent in the same way upon Tet repressor binding. The measured combined curvature, however, is only $52^\circ \pm 9^\circ$, which is nearly the same as that found for a single operator. Clearly, this result is contradictory to a DNA structure with two identical bends in the same plane because this should result in an overall bend of $96^\circ \pm 14^\circ$. It has been suggested that the end to end distance resulting from a bent structure of the DNA determines the gel mobility rather than the bending angle itself (27). Model calculations determining the end to end distances in dependence of a rotation of the two planes defined by the angles at each operator indicate that the measured overall bend would be in agreement with a rotation of these planes by $105^\circ \pm 25^\circ$. The distance between the two operators is 30 bp. Assuming the helical repeat of B-DNA to be about 10.5 bp (29,31) the rotation between identical surfaces of the two operators would be 51.5° . This is clearly distinct from the result obtained here, because the error ranges given represent maximal errors rather than standard deviations. The latter are only about 25% of the ranges indicated. Thus, one may suspect that the end to end distances of tandem and single *tet* operator DNA fragments are roughly the same because they result in the same mobility shifts. If this is the case the rotation between the two operators in the wild type *tet* regulatory sequence should be significantly greater than 50° . This is in agreement with the second model obtained from small angle neutron scattering of the wild type *tet* regulatory region bound by two Tet repressor dimers which assumes that the two repressors are on opposite sides of the DNA (26). This feature could be achieved by two possible structural perturbations of the *tet* regulatory sequence: Either the sequence between the two *tet* operators is of a non-B structure or the binding of Tet repressor induces, in addition to a bend, also a twist in the operator DNA. Evidence indicating perturbations of the helical structure of DNA upon Tet repressor binding has indeed been obtained previously from alterations induced in the CD (28).

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REFERENCES

1. Levy, S.B. (1988) *ASM News* 54, 418-421.
2. Tovar, K., Ernst, A., and Hillen, W. (1988) *Molec. Gen. Genet.* 215, 76-80.
3. Jorgensen, R.A. and Reznikoff, W.S. (1979) *J. Bacteriol.* 138, 705-714.
4. Yang, H., Zubay, G., and Levy, S.B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* 73, 1509-1512.
5. Bertrand, K.P., Postle, K., Wray, L.V., and Reznikoff, W.S. (1983) *Gene* 23, 149-156.
6. Wray, L.V., Jorgensen, R.A., and Reznikoff, W.S. (1981) *J. Bacteriol.* 147, 297-304.
7. Hillen, W., Schollmeier, K., and Gatz, C. (1984) *J. Mol. Biol.* 172, 185-201.
8. Meier, I., Wray, L.V., and Hillen, W. (1988) *EMBO J.* 7, 567-572.
9. Thompson, J.F. and Landy, A. (1988) *Nucl. Acids Res.* 16, 9687-9705.
10. Koo, H.-S., Wu, H.-M., and Crothers, D.M. (1986) *Nature* 320, 501-506.
11. Ptashne, M. (1986) *Nature* 322, 697-701.
12. Schleif, R. (1988) *Science* 240, 127-128.
13. Oehmichen, R., Klock, G., Altschmied, L., and Hillen, W. (1984) *EMBO J.* 3, 539-543.
14. Botterman, J. and Zabeau, M. (1985) *Gene* 37, 229-239.
15. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
16. Hillen, W., Klein, R.D., and Wells, R.D. (1981) *Biochem.* 20, 3748-3756.
17. Wissmann, A., Meier, I., and Hillen, W. (1988) *J. Mol. Biol.* 202, 397-406.

18. Unger, B., Klock, G., and Hillen, W. (1984) *Nucl. Acids Res.* 20, 7693–7703.
19. Hillen, W., Klock, G., Kaffenberger, I., Wray, L.V., and Reznikoff, W.S. (1983) *J. Biol. Chem.* 257, 6605–6613.
20. Hillen, W., Gatz, C., Altschmied, L., Schollmeier, K., and Meier, I. (1983) *J. Mol. Biol.* 169, 707–721.
21. McClarin, J.A., Frederick, C.A., Wang, B.-C., Greene, P., Boyer, H.W., Grable, J., and Rosenberg, J.M. (1986) *Science* 234, 1526–1541.
22. Postle, K., Nguyen, T.T. and Bertrand, K.P. (1984) *Nucl. Acids Res.* 12, 4849–4863.
23. Wu, H.-M. and Crothers, D.M. (1984) *Nature* 308, 509–513.
24. Liu-Johnson, H.-N., Gartenburg, M.R., and Crothers, D.M. (1986) *Cell* 47, 995–1005.
25. Pörschke, D., Tovar, K., and Antosiewicz, J. (1988) *Biochem.* 27, 4674–4679.
26. Lederer, H., Tovar, K., Baer, G., May, R.P., Hillen, W., and Heumann, H. (1989) *EMBO J.* 8, 1257–1263.
27. Lerman, L.S. and Frisch, H.L. (1982) *Biopol.* 21, 995–997.
28. Altschmied, L. and Hillen, W. (1984) *Nucl. Acids. Res.* 12, 2171–2180.
29. Wang, J.C. (1979) *Proc. Natl. Acad. Sci. U.S.A.* 76, 200–203.
30. Diekmann, S., Hillen, W., Morgeneyer, B., Wells, R.D., and Pörschke, D. (1982) *Biophys. Chem.* 15, 263–270.
31. Rhodes, D. and Klug, A. (1981) *Nature* 202, 378–380.

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