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Bacteriophage P22 Mnt Repressor DNA Binding and Effects on Transcription *in Vitro*

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We have examined the binding of Mnt repressor to operator DNA *in vitro* and have determined how this binding affects the level of transcription from two nearby promoters, P_{ant} and P_{mnt} . Mnt binds to a region of DNA that overlaps the startpoint of transcription of P_{ant} and the –35 region of P_{mnt} . Mnt represses transcription *in vitro* from P_{ant} and enhances transcription from P_{mnt} .

Protection and interference experiments show that Mnt binds to a single, 17 base-pair operator site. The operator sequence and the protein–DNA contacts are symmetric. Mnt makes major groove contacts on both faces of the operator DNA. At pH 7.5, 200 mM-KCl, 22°C, the Mnt tetramer binds operator with high affinity ($K_d = 2.2 \times 10^{-11}$ M) and the protein–DNA complex is quite stable ($t_{\frac{1}{2}} = 48$ min). Operator binding shows large dependencies on pH, salt concentration, and temperature.

1. Introduction

Salmonella phage P22 has two immunity regions, *immC* and *immI*, that are involved in the maintenance of lysogeny or the commitment to lytic growth (for reviews, see Susskind & Botstein, 1978; Susskind & Youderian, 1983). The *immC* region encodes the *c2* and Cro repressors, and is analogous to the immunity region of coliphage λ . The *immI* region encodes the Ant protein and the Arc and Mnt repressors, and has no analog in λ . Ant protein can inactivate the *c2* repressor and thereby induce lytic growth of the prophage. During lysogenic growth, Mnt repressor prevents expression of Ant from the prophage and from any superinfecting phage. This allows the lysogen to be stably maintained. Arc repressor prevents high-level expression of Ant during the late stages of lytic growth.

The organization of genes and regulatory sites in the *immI* region (see Fig. 1) has been determined by genetic studies, by comparison of gene and protein sequences, and by studies of transcription *in vitro* (Botstein *et al.*, 1975; Levine *et al.*, 1975; Susskind, 1980; Sauer *et al.*, 1983; Youderian *et al.*, 1982a,b,

1983; Vershon *et al.*, 1985a; D. Grana *et al.*, unpublished results). Rightward transcription of the *arc* and *ant* genes initiates at P_{ant} and can be negatively regulated by either Mnt or Arc. Transcription of *mnt* initiates at P_{mnt} and proceeds to the left. The P_{ant} and P_{mnt} promoters overlap physically and compete for RNA polymerase.

We have described the purification and properties of the Mnt and Arc repressors (Vershon *et al.*, 1985a). Mnt contains 82 amino acid residues and is a tetramer in solution. Arc contains 53 amino acid residues and is a dimer. The sequences of Arc and Mnt are about 40% homologous and it is likely that they are related structurally. In this paper, we probe interactions between Mnt and its operator by protection and interference experiments. We also present data concerning the kinetics and thermodynamics of Mnt operator binding and examine the effects of Mnt on transcription *in vitro*. In the accompanying paper (Vershon *et al.*, 1987) we study the interaction of Arc repressor with its operator site, and discuss the similarities and differences between the DNA interactions of Mnt and those of Arc.

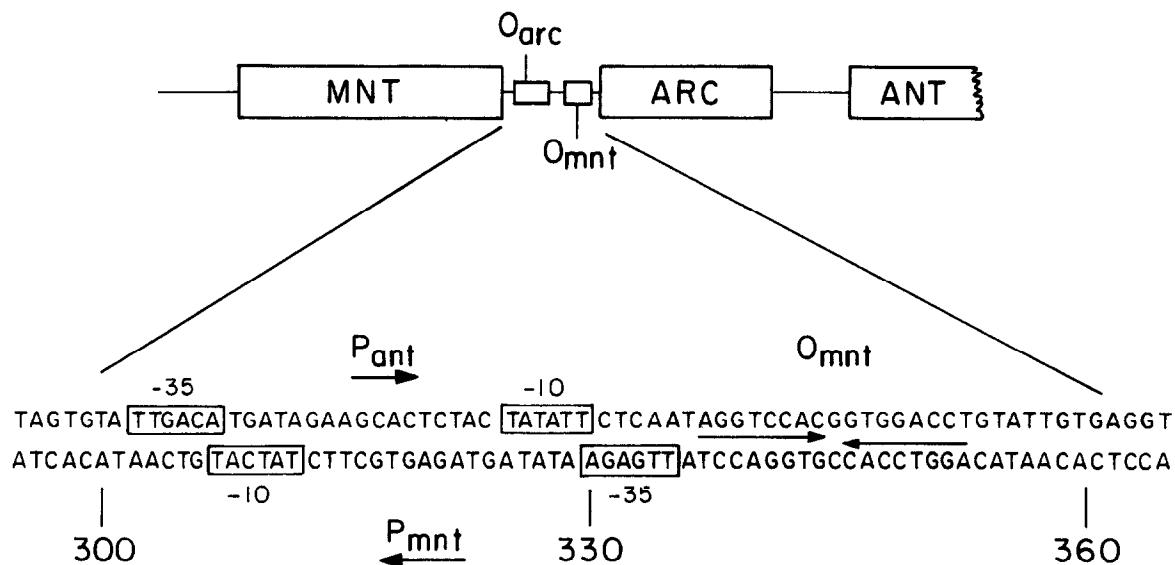


Figure 1. The Immunity I Region of P22. The -10 and -35 regions of P_{ant} and P_{mnt} are boxed. The sequence numbering is from Sauer *et al.* (1983).

2. Materials and Methods

(a) Materials

Materials were obtained from the following sources: restriction enzymes and phage T4 polynucleotide kinase were from New England Biolabs; DNA polymerase I large fragment and calf intestine alkaline phosphatase were from Boehringer-Mannheim; $[\alpha\text{-}^{32}\text{P}]$ dATP and $[\gamma\text{-}^{32}\text{P}]$ ATP were from Amersham; ethylnitrosourea was from Sigma; bis-acrylcystamine was from BioRad; and pancreatic DNase I was from Worthington.

(b) Protein purification

Mnt was purified from *Escherichia coli* strain MS2282 as described (Vershon *et al.*, 1985a) and stored in 50 mM-Tris·HCl (pH 7.5), 0.1 mM-EDTA, 5% (v/v) glycerol, 1.4 mM-2-mercaptoethanol, 100 mM-KCl, at -20°C . A molar extinction coefficient at 280 nm of 18,000/cm was used to calculate the concentration of Mnt tetramer. RNA polymerase holoenzyme was purified by the method of Lowe *et al.* (1979).

(c) Labeled operator fragments

The 240 bp[†] *Eco*RI-*Hind*III fragment from plasmid pMS200 was used as a source for the O_{mnt} operator (Youderian *et al.*, 1982a). The *Eco*RI end was 3'- or 5'-labeled using standard procedures (Maniatis *et al.*, 1982). The 3'-end-labeled fragment was obtained by cleaving pMS200 with *Eco*RI, filling in the *Eco*RI overhang with $[\alpha\text{-}^{32}\text{P}]$ dATP using DNA polymerase I large fragment, and secondary cutting with *Hind*III. The 5'-end-labeled fragment was obtained by removing the 5'-phosphate with calf intestinal alkaline phosphatase, labeling the end with $[\gamma\text{-}^{32}\text{P}]$ ATP using T4 polynucleotide kinase, and secondary cutting with *Hind*III. The end-labeled 240 bp operator fragment was gel-purified on a 5% (w/v) polyacrylamide gel (crosslinked with 0.1% (w/v) bis-acrylcystamine) and located by autoradiography. The appropriate band was excised and solubilized

with 0.9 ml of 0.2 M-NaCl, 20 mM-Tris·HCl (pH 7.5), 0.1 mM-EDTA and 0.1 ml of 2-mercaptoethanol, at room temperature for 1 h. Labeled DNA was recovered from the solubilized gel by chromatography on a Schleicher & Schuell Elutip. The fragment was precipitated in ethanol, dried, and resuspended in 100 μl of 10 mM-Tris·HCl (pH 7.5), 0.1 mM-EDTA and stored at -20°C . The specific activity of either end-labeled fragment was about 100 cts/min per pmol as measured by Čerenkov radiation.

(d) Gel binding assay

Binding of Mnt to labeled-operator fragments was assayed by the gel electrophoresis shift method (Fried & Crothers, 1981; Garner & Revzin, 1981). We performed the assay as follows: labeled fragment was diluted to approximately 5×10^3 cts/min per ml in GAB buffer (10 mM-Tris·HCl (pH 7.5), 10 mM-MgCl₂, 0.1 mM-EDTA, 200 mM-KCl, 100 μg bovine serum albumin/ml). A sample (40 μl) of this diluted operator DNA was mixed with 40 μl of Mnt in the same buffer and the mixture was incubated at room temperature for at least 2 h, unless specified. After this time, 2 μl of loading buffer (10 mM-Tris·HCl (pH 7.5), 50% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol) was added and 20 μl was loaded onto a 5% polyacrylamide gel and electrophoresed at 250 V in TBE buffer (Maniatis *et al.*, 1982). After the bromophenol blue migrated approximately 10 cm, the gel was blotted onto filter paper, dried under vacuum, and exposed to Kodak XR-5 film at -70°C . Band intensities were quantified by scanning laser densitometry using an E-C Apparatus densitometer.

The effects of salt, temperature and pH on the operator DNA binding affinity of Mnt were tested using the gel binding assay described above, with the following modifications. Protein and DNA dilutions in the salt experiments were in 10 mM-Tris·HCl (pH 7.5), 0.1 mM-EDTA, 100 μg of bovine serum albumin/ml with 50, 100, 150, 200, 250 or 300 mM-KCl instead of GAB buffer. Temperature experiments were performed in GAB buffer, except that for some experiments 50 mM-Pipes (pH 8.0) was used instead of 10 mM-Tris·HCl (pH 7.5). Protein dilutions were incubated at each temperature for at least 1 h before adding the labeled DNA and the reactions were

[†] Abbreviation used: bp, base-pair(s).

left to incubate for at least 2 h to ensure that each reaction was at equilibrium. Gels were equilibrated, loaded, and run at temperatures as close as possible to the temperatures of incubation. For studies of the pH dependence of DNA binding, experiments were performed in buffer similar to GAB buffer, except that 10 mM-Tris-HCl (pH 7.5) was replaced by 50 mM-morpholinohethane sulfonic acid for pH 6.5 and 7.0, and 50 mM-Tris-HCl was used for pH 7.5, 8.0, 8.5 and 9.0.

The rate of dissociation of the Mnt-operator complex was measured by mixing protein with DNA at concentrations at which most of the operator was bound and allowing the mixture to equilibrate for several hours. Portions (5 µl) were then removed from the equilibrium mixture, gently diluted into 1.0 ml of GAB buffer, and complexes were allowed to dissociate for a fixed period of time. The concentration of bound complex at each time after dilution was measured by electrophoresis of 20 µl of the dilution mixture in the standard gel assay as described above.

The rate of Mnt-operator association was measured by mixing protein at concentrations of 8.5×10^{-10} M, 1.7×10^{-10} M, or 8.5×10^{-11} M with labeled operator DNA (10^{-12} M) in GAB buffer. At various times after addition of the operator DNA, 20-µl portions of the reaction were removed and gently mixed with a 1000-fold excess of unlabeled operator to block further association of Mnt with the labeled fragment. The samples were electrophoresed as described above and the concentration of bound complex at each time point was quantified.

(e) Protection experiments

DNase I protection experiments were performed as described (Johnson *et al.*, 1979) in gel assay buffer (GAB) supplemented with 2 mM-CaCl₂, 1 mM-dithiothreitol, 250 µg bovine serum albumin/ml, 25 µg sonicated salmon sperm DNA/ml and 50 µg yeast tRNA/ml. A portion (10 µl) of Mnt was mixed with 90 µl of end-labeled fragment (2×10^5 cts/min per ml) and allowed to incubate at room temperature for 30 min. DNase I was added to a concentration of 30 ng/ml and incubated for 15 min at room temperature. Digestion was stopped by the addition of 100 µl of 5 M-ammonium acetate and 600 µl of ethanol, and immersion of the tubes in a solid CO₂/ethanol bath. The DNA was precipitated in ethanol, washed with ethanol, dried under vacuum, and redissolved in sequencing sample buffer (Maxam & Gilbert, 1980). For size standards, Maxam & Gilbert chemical sequencing reactions were performed on the labeled operator fragment. Samples were heated to 90°C, loaded on to an 8% (w/v) polyacrylamide/urea sequencing gel and electrophoresed at 45 mA in TBE buffer. After the xylene cyanol dye marker migrated 34 cm, the gels were blotted on to filter paper, dried under vacuum, and exposed to film at -70°C with a Dupont Cronex intensifying screen.

Dimethylsulfate methylation protection experiments were carried out essentially as described by Gilbert *et al.* (1976). The reaction buffer contained 50 mM-sodium cacodylate (pH 7.5), 10 mM-MgCl₂, 0.1 mM-EDTA, 200 mM-KCl, 1 mM-dithiothreitol, 1 mM-CaCl₂, 250 µg bovine serum albumin/ml, 5 µg sonicated salmon sperm DNA/ml. A sample (10 µl) of protein was mixed with 190 µl of the end-labeled operator fragment (approximately 2×10^4 cts/min), and left to incubate for at least 30 min at room temperature. The mixture was then chilled on ice for 10 min and 1 µl of dimethylsulfate was added. After incubation for 10 min on ice, the reaction was stopped by the addition of 50 µl of G-stop

(1.5 M-sodium acetate (pH 7.0), 1.0 M-2-mercaptoethanol, 100 µg tRNA/ml) and 750 µl of ethanol. The DNA was precipitated in a solid CO₂/ethanol bath, resuspended in 250 µl of 0.3 M-sodium acetate, precipitated in ethanol again, and dried under vacuum. The DNA was then resuspended in 100 µl of 1 M-piperidine, 5 mM-EDTA and heated to 90°C for 30 min. Samples were dried under vacuum overnight, washed and dried twice with distilled water, and resuspended in sequencing sample buffer. Gel electrophoresis was performed as described for the DNase experiments.

(f) Interference experiments

Methylation and ethylation interference experiments were performed using a modification of the procedure described by Siebenlist & Gilbert (1980). Approximately 10⁶ cts/min of labeled operator fragment was methylated by dimethylsulfate as described above but in the absence of Mnt repressor. The DNA was precipitated twice in ethanol, dried, and resuspended in 50 µl of GAB buffer. A portion (10 µl) of the methylated fragment was mixed with 10 µl of Mnt or 10 µl of buffer and left to incubate at room temperature. After 30 min, 2 µl of gel assay loading buffer was added to each mixture, the sample was loaded on to a 6.5% polyacrylamide/TBE gel (crosslinked with 0.1% bis-acrylcystamine), and electrophoresed at 250 V until the bromophenol blue dye had migrated 10 cm. The gel was exposed to film for 1 h at room temperature with an intensifying screen. Labeled fragments migrating at the bound and free positions were excised and purified as described above for the purification of labeled operator fragment. The free and bound DNAs were suspended in 1 M-piperidine and the strand cleavage reaction was carried out as described for the methylation protection experiments. Bound and free samples from the same reaction were electrophoresed on an 8% polyacrylamide/urea sequencing gel together with Maxam & Gilbert sequencing markers.

Ethylation interference experiments followed the same general protocol for separation of bound and free operator fragments as the methylation interference experiments. Labeled operator fragment was diluted to 10⁷ cts/min per ml in 100 mM-sodium cacodylate (pH 7.5); 100 µl of the labeled DNA was mixed with an equal volume of freshly prepared ethylnitrosourea saturated in ethanol, and incubated at 51°C for 30 min. The DNA was precipitated in sodium acetate and ethanol in a solid CO₂/ethanol bath, dried under vacuum, and resuspended in 50 µl of GAB buffer. The binding reaction, gel electrophoresis, and purification of the bound and free labeled operator fragment was performed as described above. The DNA was cleaved by the addition of 100 µl of 0.3 M-KOH, 10 mM-EDTA and incubation at 90°C for 30 min. The reaction was neutralized by the addition of 100 µl of 0.3 M-HCl. The DNA was precipitated in sodium acetate and ethanol, dried, and resuspended in sequencing sample buffer. Gel electrophoresis of the samples was performed as described above.

(g) Transcription in vitro

The template used for transcription experiments was the 832 bp fragment from pMS200 containing P_{ant} and P_{mnt}. Template was prepared by cleaving pMS200 with EcoRI, and gel-purifying the 832 bp fragment. To produce smaller run-off P_{ant} transcripts the 832 bp fragment was further cleaved with HaeIII. Transcription assays were performed in buffer containing

40 mM-Tris-HCl (pH 8.0), 100 mM-KCl, 10 mM-MgCl₂, 1 mM-dithiothreitol, 100 µg bovine serum albumin/ml. The DNA template (5.6 nm) was preincubated at 37°C for 10 min with Mnt at concentrations of 0, 2, 4, 8, 14, 20, 40, 100 and 200 nM. RNA polymerase (50 nM) was mixed with each sample of DNA and Mnt, and left to incubate for 10 min. The reactions were initiated by the addition of 200 µM UTP, GTP and CTP, and 3 µM-[γ -³²P]ATP (1.5 × 10⁵ cts/min per pmol). After 10 min, heparin was added to a final concentration of 50 µg/ml and the reaction continued for another 5 min at 37°C. Transcription was stopped by the addition of EDTA to 30 mM. Each sample was mixed with gel loading buffer (7 M-urea, 4% glycerol, 0.02% xylene cyanol and bromophenol blue), and electrophoresed on a 12% polyacrylamide gel in 7 M-urea/TBE buffer. The positions of the RNA products were determined by autoradiography and the appropriate sections of the gel were excised and counted. Calculation of the number of RNA chains employed the specific radioactivity of the [γ -³²P]ATP. The initiation site was established by end-labeling the transcript with [γ -³²P]ATP and limited digestion of the transcript with RNase (D. Hawley *et al.*, unpublished results). The runoff transcripts from P_{mnt} migrated slower than expected on the 12% polyacrylamide gel for unknown reasons. Digestion with RNase T₁ confirmed that this transcript originated from P_{mnt} (data not shown).

3. Results

(a) DNase protection

Mutations in the O_{mnt} operator are located between the *arc* and *mnt* genes (Youderian *et al.*, 1983; D. Granna *et al.*, unpublished results). To determine the precise location of the O_{mnt} operator site, we performed DNase protection experiments using purified Mnt protein. Mnt repressor protects a region from base 330 to 358 on the lower strand of the DNA fragment (Fig. 2) and from base 333 to 363 on the upper strand (data not shown). The protected region overlaps the -35 region of the P_{mnt} promoter and the start site of transcription of the P_{ant} promoter. At the center of this protected region lies a 17 bp inverted repeat that is perfectly symmetric, except for its central base-pair at position 345. Mnt protein does not appear to enhance DNase cleavage of any bonds within the protected region.

(b) Chemical protection and interference experiments

Methylation protection and interference experiments for the upper strand of O_{mnt} are shown in Figure 3. In the latter experiments, labeled operator DNA was partially modified with dimethylsulfate and was then incubated with Mnt repressor. Bound and free DNA fragments were purified by gel electrophoresis and the modified bases were cleaved by treatment with heat and alkali. The cleaved samples were then electrophoresed on a denaturing gel. Modified bases that interfere with binding are those that appear to be overrepresented in the "free" fraction compared to the "bound" fraction.

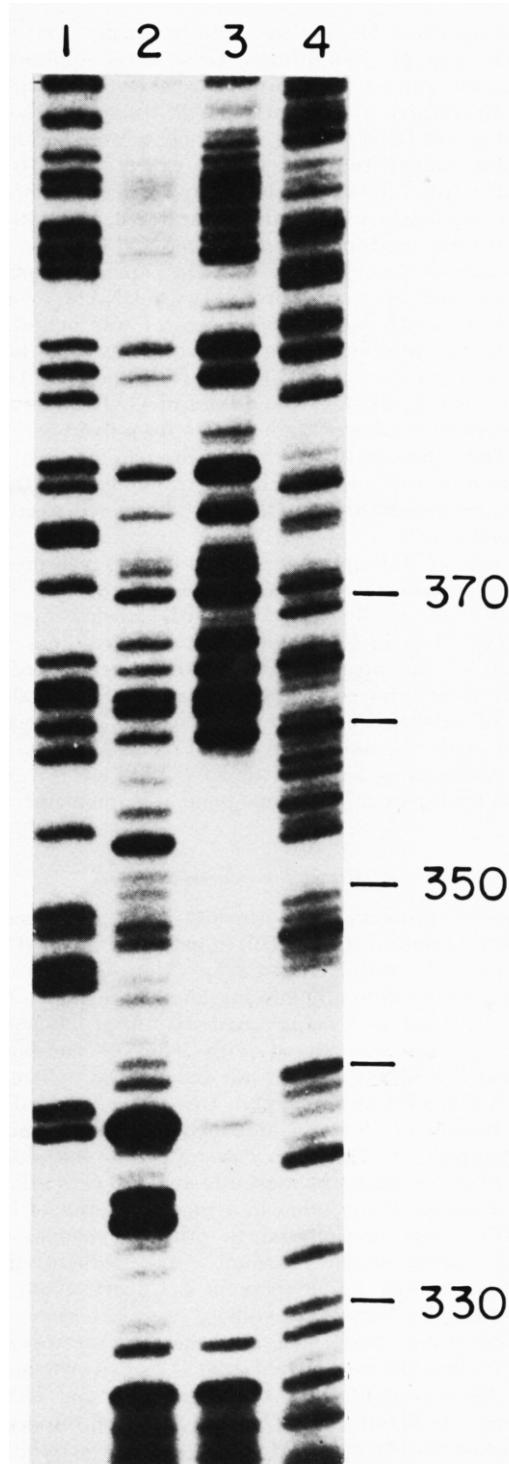


Figure 2. DNase protection of the operator by Mnt. Lanes 1 and 4 are C and A>C Maxam & Gilbert sequencing reactions, respectively. Lane 2 shows DNase cleavage in the absence of Mnt repressor. Lane 3 shows DNase protection in the presence of 1.7×10^{-9} M-Mnt. The DNA was labeled at the 3' end and corresponds to the lower strand shown in Fig. 1.

Mnt repressor protects each of the guanine residues within the 17 bp inverted repeat. Guanine residues outside this region are not protected. At lower concentrations of Mnt, only partial protection

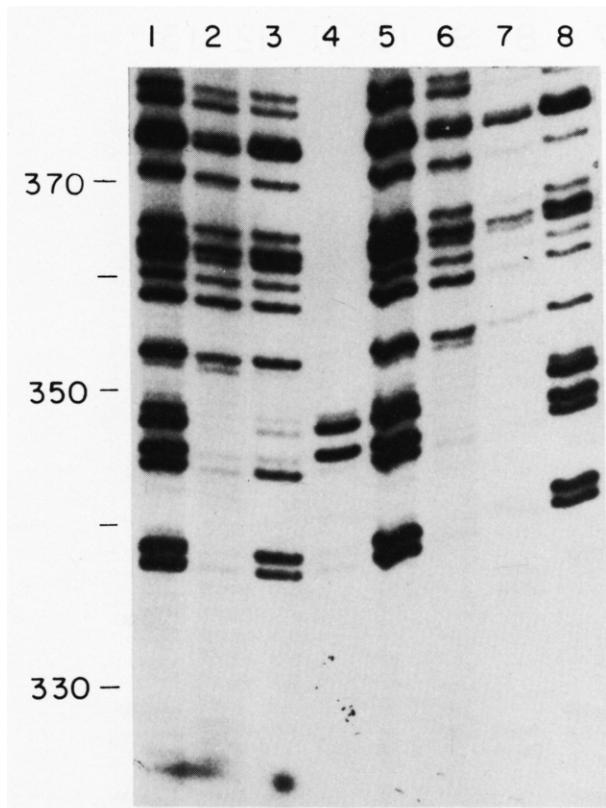


Figure 3. Dimethylsulfate (DMS) protection and interference experiments. Lanes 1 and 5 are DMS reactions in the absence of Mnt. Lanes 2 and 6 are protection reactions in the presence of 8.5×10^{-8} M-Mnt. Lanes 3 and 4 are the bound and free fractions, respectively, from an interference experiment containing 3×10^{-8} M-Mnt. Lanes 7 and 8 are the bound and free fractions from an interference experiment containing 1×10^{-8} M-Mnt. The DNA concentration in all experiments was 2×10^{-8} M. The DNA was 5'-end-labeled and corresponds to the upper strand in Fig. 1.

of the guanine at the central position of the operator, base 345, is observed (data not shown). The results of the interference experiments show that methylation of any of the 11 operator guanine residues weakens binding by the Mnt repressor. Methylation of guanine residues near the center of the operator interferes with binding more than methylation of those near the ends of the operator. Methylation of any of the inner guanine residues, except the central one, reduces Mnt binding affinity to a point that most of the DNA modified at these positions remains unbound (lane 4). Methylation of the outer guanine residues (bases 338, 339, 351 and 352) does not interfere with Mnt binding to the same degree and operator fragments methylated at these positions remain bound at high concentrations of Mnt (lane 3). Methylation of these outer positions causes some binding interference at lower concentrations of Mnt (lanes 7 and 8). Bound Mnt did not appear to protect any adenine residues in the operator from methylation nor did it enhance methylation of any bases.

Ethynitrosourea was used to probe the phosphate contacts made by Mnt. Ethylation of certain phosphate groups interferes with protein binding because salt-bridges or hydrogen bonds to the phosphate are disrupted or because the ethyl group of the modified phosphate sterically hinders protein binding. Ethylation of phosphate groups in two regions of each strand of operator DNA interferes with Mnt binding (Fig. 4). The contacted phosphate groups on either strand are symmetrically related with respect to the center (base 345) of the operator site. Ethylation of some of the phosphate groups appears to interfere with Mnt binding more than others. A summary of the enzymatic and chemical protection and interference data for O_{mnt} is shown in Figure 5.

(c) Transcription in vitro

The Mnt operator site overlaps the -35 region of P_{mnt} and the startpoint of P_{ant} transcription (Fig. 2). Figure 6 shows the effect of Mnt concentration on run-off transcripts initiated at these two promoters. As the Mnt concentration is raised, transcription from P_{ant} is repressed, whereas transcription from P_{mnt} is stimulated. The stimulation of P_{mnt} transcription by Mnt corresponds to an approximate sixfold increase over basal transcription. In the absence of Mnt protein, the P_{ant} promoter is much stronger than the P_{mnt} promoter (Hawley *et al.*, unpublished results; see also lane 1, Fig. 6). It seems likely that Mnt activates P_{mnt} , at least in part, by repressing P_{ant} and relieving promoter competition. However, it is possible also that Mnt plays a more direct role in P_{mnt} activation. Further studies will be required to address this question.

The transcription data also allow us to estimate the activity of the Mnt protein used for these studies, since P_{ant} is completely repressed by addition of 1.2 mol of Mnt tetramer per mol of template DNA. This indicates that the Mnt protein has an activity greater than or equal to 83%.

(d) Equilibrium and kinetic measurements of operator binding

The gel electrophoresis assay has been used to investigate the binding of several proteins to DNA (Garner & Revzin, 1981; Fried & Crothers, 1981, 1984; Hendrickson & Schleif, 1984). This assay relies on the fact that a protein-DNA complex usually migrates at a slower rate than unbound DNA during gel electrophoresis.

A gel binding experiment performed under standard conditions (pH 7.5, 200 mM-KCl, 22°C) and the corresponding Mnt-operator binding curve are shown in Figure 7. The binding data are fit by a simple hyperbolic isotherm. Assuming that Mnt tetramers are the active species, then the apparent equilibrium dissociation constant (K_{app}) for the reaction is 2.2×10^{-11} M. This binding constant corresponds to a standard free energy change of -14.4 kcal/mol (1 cal = 4.184 J) for binding of the

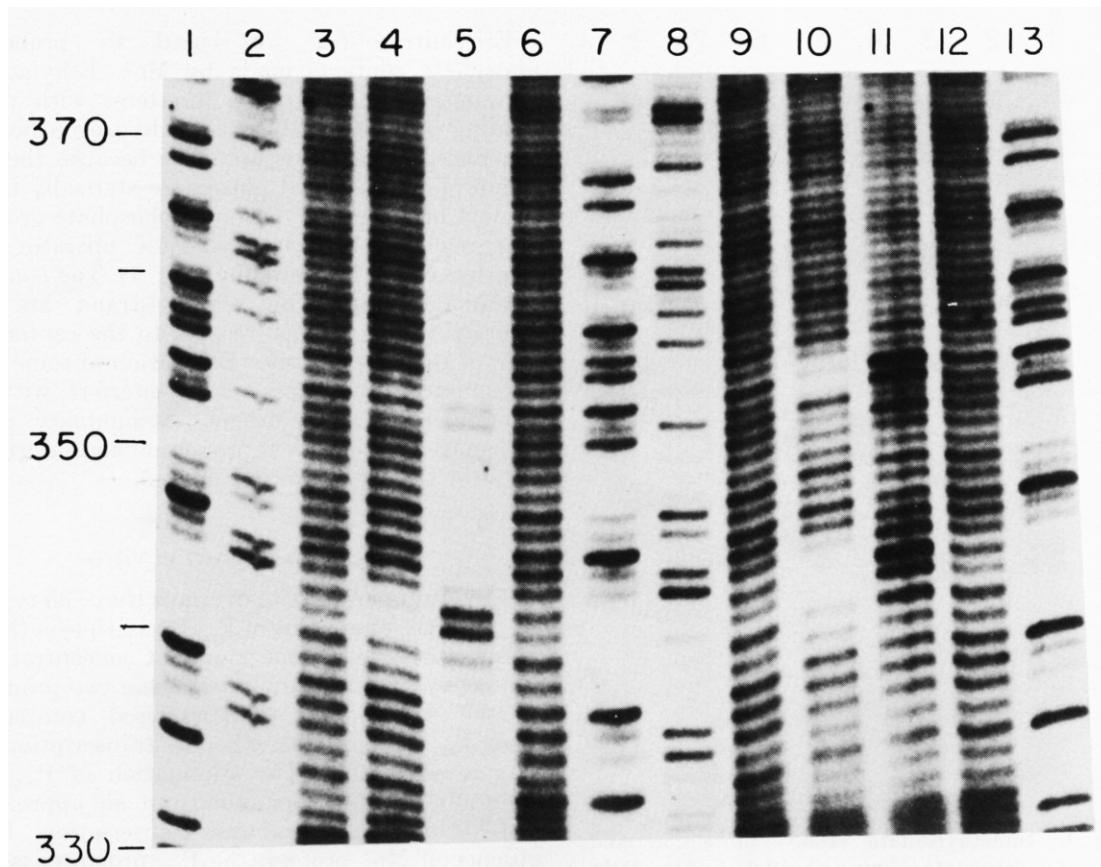


Figure 4. Ethylnitrosourea phosphate interference experiments. Lanes 1, 7 and 13 are A>C sequencing reactions. Lanes 2 and 8 are C+T sequencing reactions. Lanes 3, 6, 9 and 12 are control lanes showing the cleavage pattern of operator DNA after partial modification with ethylnitrosourea. Lanes 4 and 5 are the bound and free fractions, respectively, from an ethylation interference experiment containing 3×10^{-8} M-Mnt. Lanes 10 and 11 are the bound and free fractions from an interference experiment containing 1×10^{-8} M-Mnt. The operator concentration in both experiments was approximately 2.5×10^{-8} M. The DNA was 3'-end-labeled and corresponds to the lower strand in Fig. 1.

Mnt tetramer to the operator at 22°C. Equilibrium experiments similar to those shown in Figure 7 but using lower concentrations of operator DNA gave essentially identical binding curves.

Figure 8 shows a dissociation rate experiment under standard conditions for the Mnt-operator complex. The dissociation reaction appears to be first order with a half-life of approximately

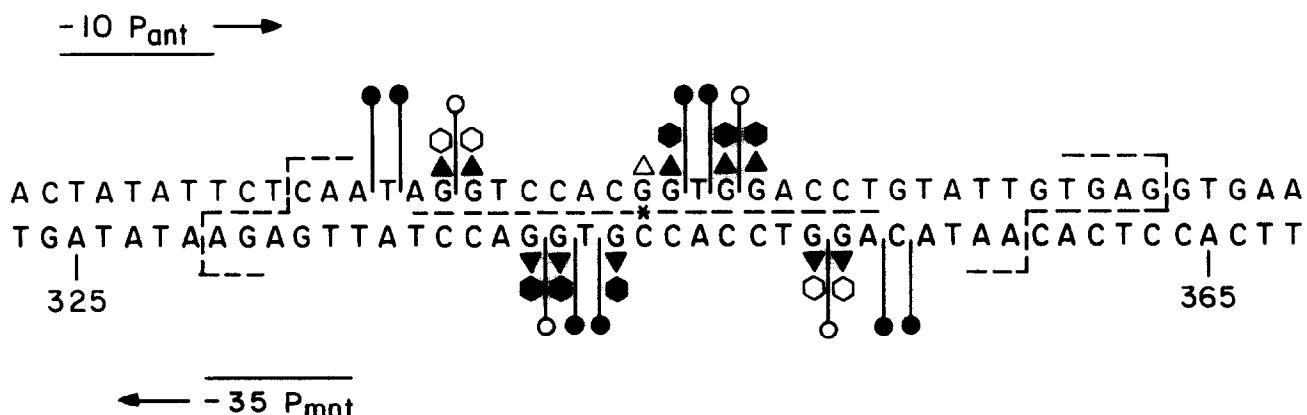


Figure 5. Summary of protection and interference data. The broken line between the DNA strands indicates bases related by the 2-fold axis of symmetry at base-pair 345 (*). Broken lines on either side of the operator show the boundaries of DNase I protection. Triangles represent bases that are protected from modification by dimethylsulfate. Hexagons indicate bases at which modification by dimethylsulfate interferes with binding by Mnt. Circles indicate phosphate positions at which modification by ethylnitrosourea interferes with Mnt bonding. Filled symbols represent positions of strong interference or protection. Open symbols indicate positions of weak interference or partial protection.

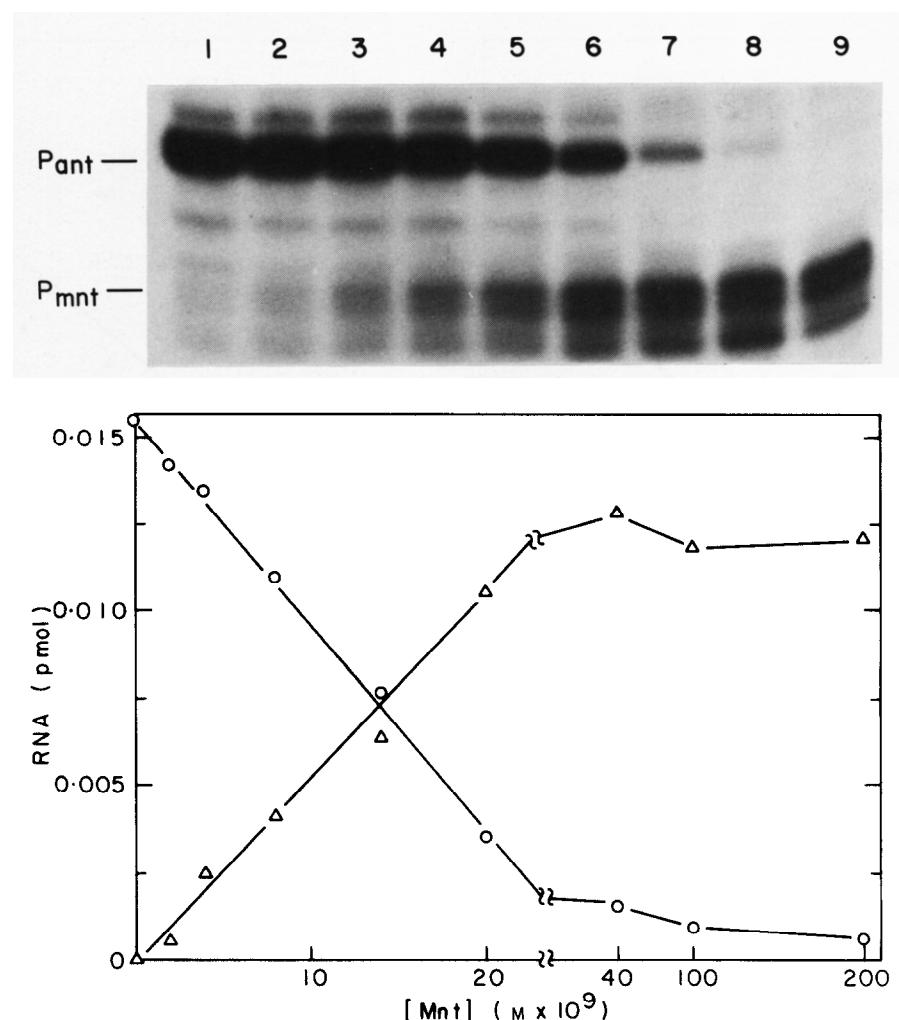


Figure 6. Transcription *in vitro* as a function of Mnt concentration. The upper panel shows an autoradiograph of the run-off transcription products from the P_{ant} and P_{mmt} promoters. The lower panel shows the amount of each run-off transcript produced as a function of different concentrations of Mnt. Circles represent the number of run-off transcripts from P_{ant} . Triangles represent the number of run-off transcripts from P_{mmt} .

48 minutes. From these data, we calculate a dissociation rate constant of $2.4 \times 10^{-4} \text{ s}^{-1}$. Figure 9 shows the association kinetics for the binding reaction at three different concentrations of Mnt. The association data are fit reasonably well by an association rate constant of $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. The equilibrium dissociation constant calculated from the ratio of the rate constants is virtually the same ($2.0 \times 10^{-11} \text{ M}$) as that calculated from the binding experiment.

(e) Effects of KCl , Mg^{2+} , pH and temperature on operator binding

Figure 10 shows the effect of KCl concentration on K_{app} for binding of Mnt to its operator. As the salt concentration is increased from 150 to 300 mM, the apparent binding affinity is reduced. The slope of the plot of $-\log(K_{app})$ versus $-\log[KCl]$ is between 4 and 6 in this region, suggesting that this number of ions are released when the protein binds to the DNA (Record *et al.*, 1977; Winter & von Hippel, 1981). It should be noted that ethylation of

12 operator phosphate groups interferes with Mnt binding. Thus, there is no simple correlation between the number of ions released and the number of phosphate groups identified in the ethylation experiments. Bound Mnt may be close to many of these phosphate groups, without forming bonds that displace counterions. At low concentrations of salt (50 mM), the apparent binding affinity decreases slightly. We do not know the reason for this effect but similar behavior has been reported for other DNA binding proteins (Winter & von Hippel, 1981; Terry *et al.*, 1983).

In the presence of 200 mM-KCl, the apparent operator affinity of Mnt is reduced almost 25-fold as the $MgCl_2$ concentration is changed from 0 to 10 mM (data not shown). Thus, Mg^{2+} appears to compete with the protein for binding sites on the DNA.

The binding of Mnt to its operator shows a large dependence on the pH of the assay buffer. Figure 11 shows a plot of $\log(K_{app})$ as a function of pH. There is a decrease of at least 1000-fold in binding as the pH is raised from 7 to 9. These data are fit

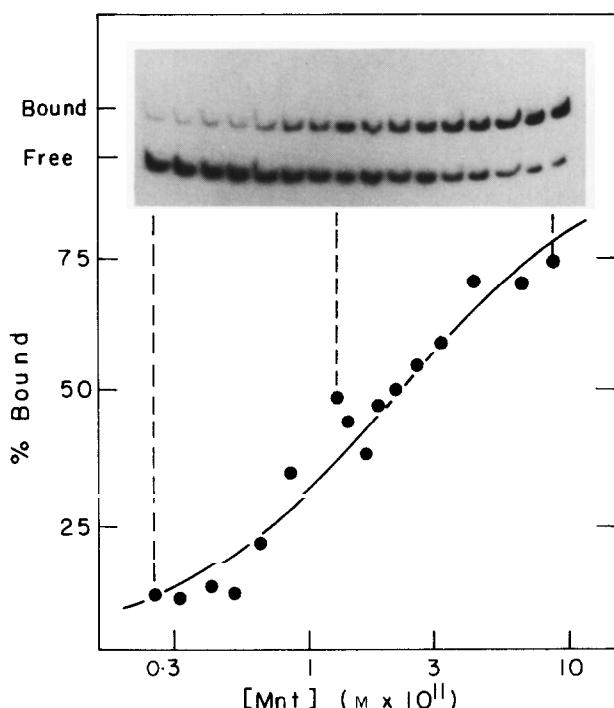


Figure 7. Equilibrium binding of Mnt to O_{mmt} . The gel electrophoresis assay was performed under standard conditions (10 mM-Tris-HCl (pH 7.5), 200 mM-KCl, 10 mM-MgCl₂, 0.1 mM-EDTA, 100 µg bovine serum albumin/ml, at 22°C) using an operator concentration of $1 \times 10^{-12} M$. The data points were obtained by densitometry. The curve is that expected for an equilibrium dissociation constant of $2.2 \times 10^{-11} M$.

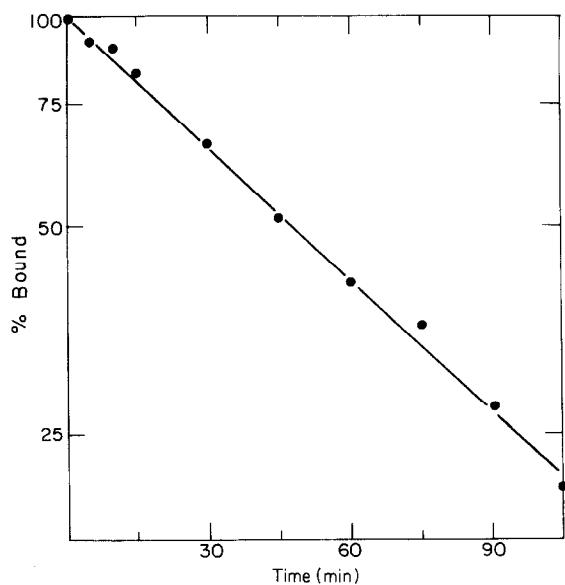


Figure 8. Kinetics of Mnt dissociation from the operator under standard binding conditions. The line is that expected for a dissociation rate of $2.4 \times 10^{-4} s^{-1}$. The half-life of the complex in this experiment is about 48 min.

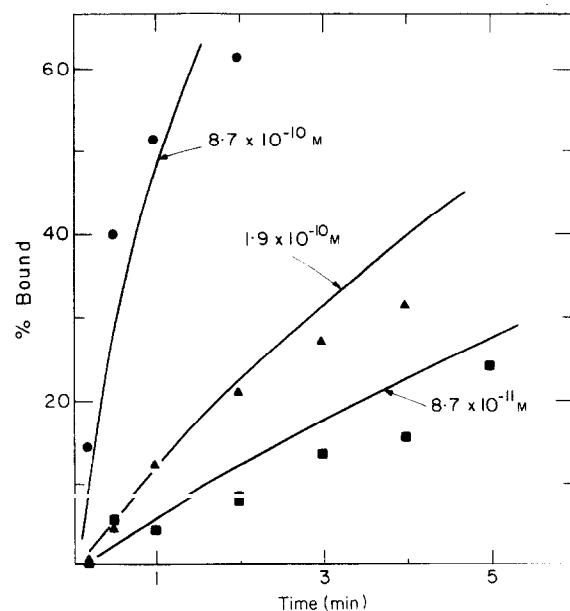


Figure 9. Association kinetics at 3 different concentrations of Mnt. Circles, $8.7 \times 10^{-10} M$; triangles, $1.9 \times 10^{-10} M$; squares, $8.7 \times 10^{-11} M$. The curves are those expected for an association rate constant of $1.2 \times 10^7 M^{-1} s^{-1}$.

moderately well if we assume that two ionizable side-chains (pK_a about 7.5) interact strongly with the operator in their protonated forms but weakly or not at all in their unprotonated forms. Mnt has a single histidine at residue 6 that has been implicated in contacting a G-C base-pair in the major groove of the operator site (Youderian *et al.*, 1983;

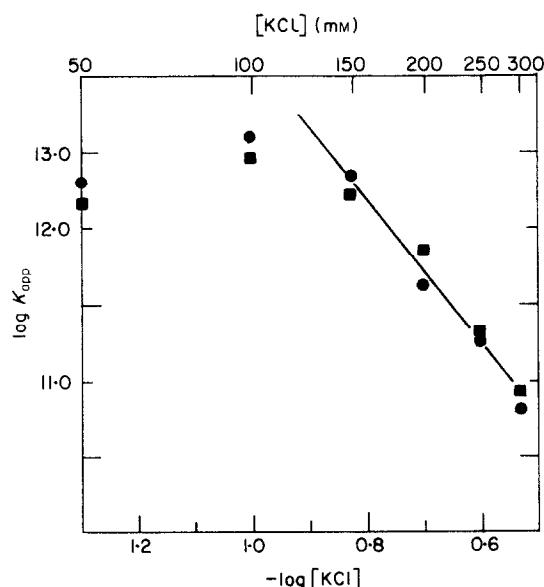


Figure 10. Equilibrium binding as a function of salt concentration. The apparent equilibrium dissociation constants for each salt concentration were calculated from experiments like those shown in Fig. 6. Circles and squares are values obtained from 2 independent experiments. The line is a least-squares fit of the data from 150 mM-KCl to 300 mM-KCl and has a slope of -4.7.

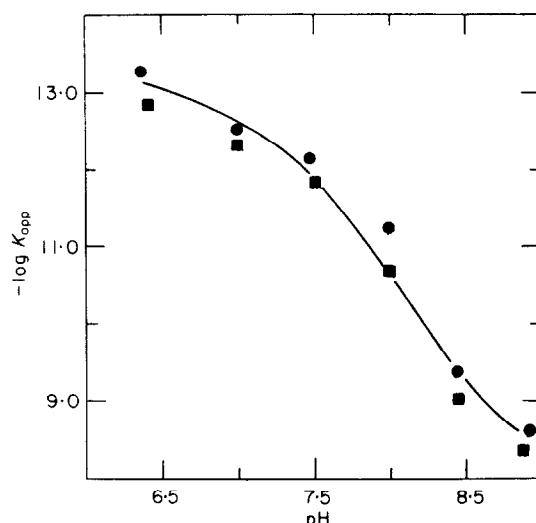


Figure 11. Equilibrium binding as a function of pH. Circles and squares represent data from 2 independent experiments.

Vershon *et al.*, 1985b). We suggest that the protonation of this residue is largely responsible for the observed pH dependence of binding. The protonated form of the α -amino group could also be involved in DNA binding.

The effect of temperature on Mnt binding to its operator was examined using two different buffers (Tris-HCl (pH 7.5) and Pipes (pH 8.0)). As shown in Figure 12, these buffers gave similar overall results. Between 4 and 24°C, there are only small changes in binding affinity but above 30°C the binding affinity decreases dramatically. Since the van't Hoff plots are biphasic, it appears that two or more reactions are involved in the binding of Mnt to its operator. One of these reactions is presumably the binding of the Mnt tetramer to its operator; the other reaction(s) could include a protein or DNA conformational change, Mnt tetramer formation, and possibly protein folding. At low temperatures, the apparent enthalpy change associated with operator binding is modest (0 to +20 kcal/mol). We imagine that this enthalpy change arises from binding of the Mnt tetramer to the operator. At high temperatures, the apparent enthalpy change associated with the overall binding reaction is much larger (approximately -80 kcal/mol). This ΔH is considerably larger than those associated with most protein-DNA interactions and, in fact, is larger than the enthalpy change ($\Delta H = -48$ kcal/mol) that accompanies folding of the Mnt polypeptide chain (Vershon *et al.*, 1985a). This seems suspicious. It is possible that the gel binding assay underestimates the binding affinities at 37 and 42°C, and thus that the apparent enthalpy change is overestimated at these temperatures. However, the temperature dependence of Lac repressor-operator binding measured by filter-binding assays also displays a biphasic van't Hoff plot (Whitson *et al.*, 1986).

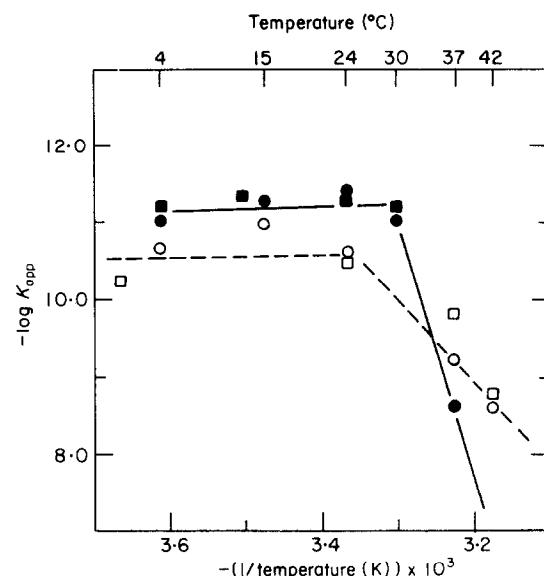


Figure 12. Equilibrium binding as a function of temperature. Open symbols are data from experiments performed in Tris-HCl buffer (adjusted to pH 7.5 at the indicated temperatures). Filled symbols are data from experiments performed in Pipes buffer (pH 8.0). Circles and squares represent data from 2 independent experiments.

4. Discussion

Physiologically, Mnt acts to repress expression of antirepressor from the P_{ant} promoter during lysogenic growth of bacteriophage P22 (Botstein *et al.*, 1975; Levine *et al.*, 1975; Susskind & Youderian, 1982). Mutations in the *mnt* operator are *cis*-acting and prevent stable lysogen formation by an Ant^+ phage. The results presented here show that Mnt binds to a single, 17 bp operator site. The known *mnt* operator mutations fall within this 17 bp region (Sauer *et al.*, 1983; Youderian *et al.*, 1983; D. Granna *et al.*, unpublished results). As shown in Figure 5, the *mnt* operator overlaps the startpoint of transcription from the P_{ant} promoter. When RNA polymerase binds at a promoter, it is also thought to contact the DNA near the startpoint of transcription (Simpson, 1979). So, Mnt apparently prevents transcription from P_{ant} by blocking the binding of RNA polymerase.

The *mnt* operator site is also adjacent to the -35 region of the P_{mnt} promoter, and our results show that bound Mnt stimulates transcription from P_{mnt} *in vitro*. The mechanism of this promoter activation has not been studied in detail. However, P_{ant} and P_{mnt} overlap physically and appear to compete for polymerase, since down-mutations in P_{ant} result in increased transcription from P_{mnt} *in vitro* (Hawley *et al.*, unpublished results). Thus, Mnt may activate P_{mnt} by simple repression of P_{ant} . The role of Mnt as a positive regulator *in vivo* has not been investigated. However, the primary prophage repressors of bacteriophage P22, λ and 434, have positive auto-regulatory functions (Johnson *et al.*, 1981; Gussin *et al.*, 1983) and it is not unreasonable

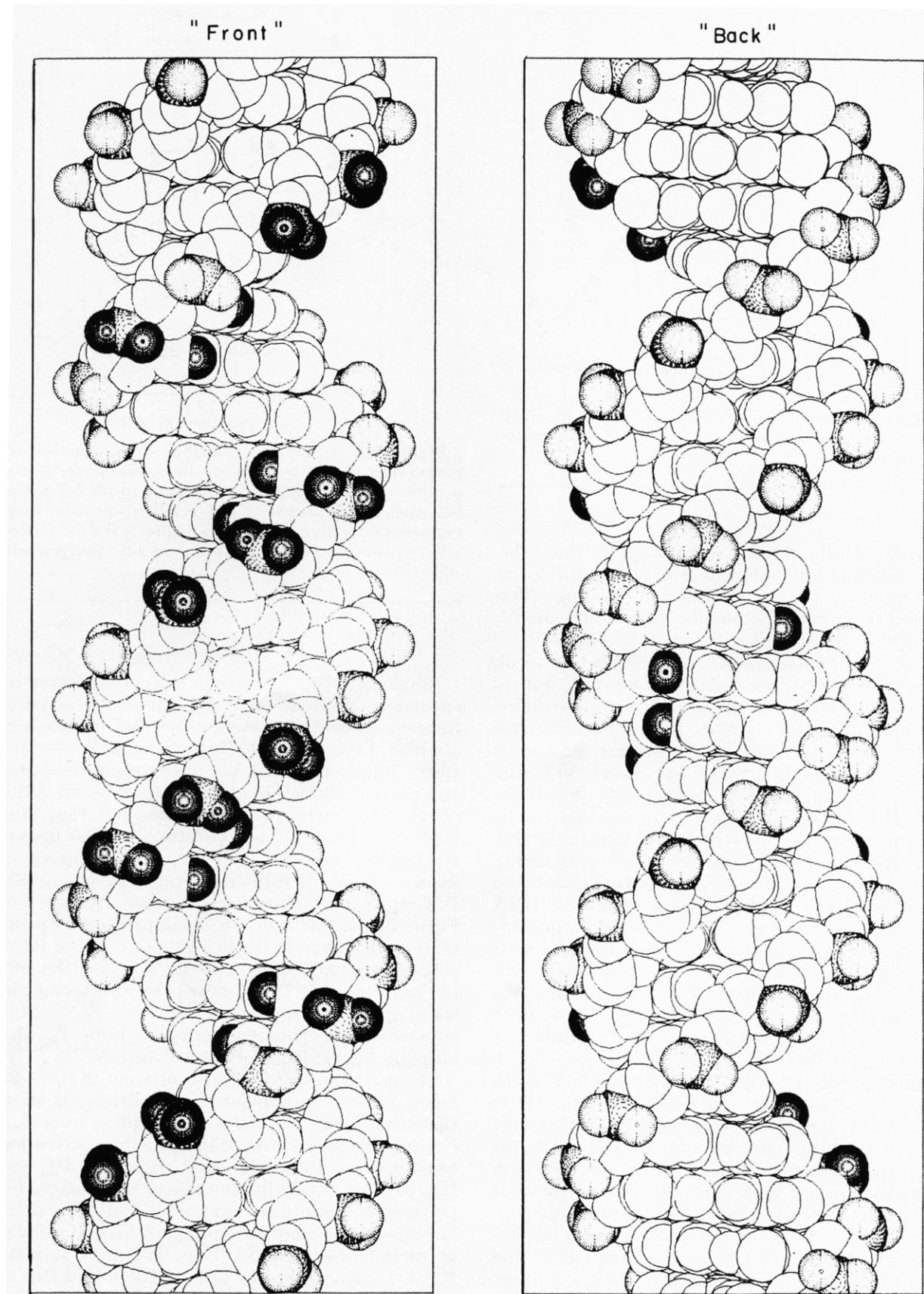


Figure 13. *B*-form DNA model of O_{mnt} showing proposed sites of contact with Mnt. The backbone phosphates are lightly shaded. The darkly shaded atoms are positions at which chemical modification interferes with Mnt binding or Mnt binding prevents modification. Shaded atoms in the major groove are the N-7 group of guanine residues. Darkly shaded atoms on the phosphate backbone are oxygen atoms.

that Mnt would have a similar function. It should be noted, however, that λ repressor is believed to activate transcription *via* a direct contact with RNA polymerase (Hochschild *et al.*, 1983) and the P22 and 434 repressors probably use similar mechanisms.

The concentration of Mnt protein in P22 lysogens has not been measured. However, Mnt shows a high affinity for its operator *in vitro* and relatively low intracellular concentrations of Mnt should be sufficient for efficient repression. For example, even under poor binding conditions (pH 7.5, 200 mM-KCl, 42°C) the apparent affinity constant is 3×10^{-9} M, and 97% repression could be achieved by having approximately 100 Mnt tetramers per cell. At lower temperatures or lower pH, the apparent affinity constant measured *in vitro* is considerably stronger and repression in the cell would be expected to be more efficient. These rough calculations neglect the affinity of Mnt for non-operator DNA. We have not investigated this in detail but preliminary experiments suggest that Mnt binds to non-operator DNA with a site binding constant that is five to six orders of magnitude weaker than for operator binding.

The core *mnt* operator sequence is a 17 bp sequence that has a 2-fold axis of symmetry passing through the central or ninth base-pair. Figure 13 shows that the points of contact between Mnt and the operator share the same 2-fold symmetry axis when the DNA is modeled in a *B*-form conformation. We assume that symmetrically related sub-units of the Mnt tetramer make identical contacts with each half of the operator site. Most of the contacts between Mnt and the operator lie on one side of the DNA, which we will refer to as the "front" side. On this side, Mnt is close to 12 phosphate groups on the DNA backbone and eight guanine N-7 positions in the two successive major grooves. On the "back" side of the operator helix, Mnt contacts are limited to the three guanine N-7 positions in the major groove at the centre of the site.

The three-dimensional structure of Mnt is not known, and there is very little information concerning the mechanism by which it binds to its operator site. Mnt is about 40% α -helical and shares some sequence homology with the λ Cro protein, which is a helix-turn-helix DNA binding protein (Anderson *et al.*, 1981; Sauer *et al.*, 1983; Vershon *et al.*, 1985a). However, altered specificity experiments have shown that the His6 side-chain of Mnt is responsible for the "front side" contacts at the third and 15th base-pairs of the *mnt* operator (Youderian *et al.*, 1983; Vershon *et al.*, 1985b). In the known helix-turn-helix proteins, these base-pairs would be contacted by a side-chain from the second helix or "DNA recognition helix" of the helix-turn-helix structure (for a review, see Pabo & Sauer, 1984). His6 of Mnt could not be part of the DNA recognition helix in a conventional helix-turn-helix, because the preceding residues (1 to 5) are too few to form the first helix and turn of such a

structure. It is possible that Mnt uses a different structural motif for operator recognition.

If the *mnt* operator remains *B*-form when Mnt binds, then a portion of the protein must wrap around the DNA to make the major groove contacts on the "back" side of the operator site (Fig. 13). The λ *cI* repressor also makes major groove contacts on both sides of its operator, apparently by using flexible N-terminal arms to wrap around the DNA (Pabo *et al.*, 1982; Eliason *et al.*, 1985; Weiss *et al.*, 1984; Nelson & Sauer, 1986). Since Mnt uses its N-terminal residues for DNA contacts on the front side of the operator (see above), it seemed likely that its C-terminal residues (Lys-Lys-Thr-Thr) might form a flexible region that contacted the back of the *mnt* operator. However, recent experiments have shown that deletion of these residues does not result in loss of or alteration of the back side *mnt* operator contacts (K. Knight & R. Sauer, unpublished results). It remains to be determined how Mnt makes contacts on both faces of its operator DNA helix.

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