The Mnt Repressor of Bacteriophage P22: Role of C-Terminal Residues in Operator Binding and Tetramer Formation†

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ABSTRACT: A set of C-terminal deletion mutants of the Mnt repressor of bacteriophage P22 has been constructed, and the corresponding truncated proteins have been purified. A truncated protein lacking the three C-terminal residues, Lys^{80}-Thr^{81}-Thr^{82}, binds operator DNA with an affinity near wild type and has a normal tetrameric structure. Loss of the next residue, Lys^{79}, causes a 600-fold decrease in operator affinity, but the truncated protein is still tetrameric. Further sequential deletions of Tyr^{78} and Leu^{77} cause modest decreases in operator affinity, but the truncated proteins are now dimeric. These results indicate that Lys^{79} is an important determinant of the high affinity of Mnt repressor for operator DNA and that Tyr^{78} is an important determinant of tetramer formation by Mnt repressor.

Mnt repressor is a sequence-specific DNA binding protein encoded by the *Salmonella* phage P22 (Botstein et al., 1975; Levine et al., 1975; Vershon et al., 1987a). In conjunction with the closely related P22 Arc repressor, Mnt regulates expression of the phage *ant* gene [for review, see Susskind and Youderian (1983)]. Mnt represses transcription from the *ant* promoter during lysogenic growth, serving to maintain the lysogenic state and to protect against superinfection, while Arc represses transcription from the *ant* promoter during lytic infection. Mnt and Arc bind to different operator sites. Each operator contains 21 base pairs, but the operator sequences are not homologous (Vershon et al., 1987a,b).

Analyses of Mnt and Arc mutants have suggested that these proteins do not use the helix–turn–helix mechanism of DNA binding (Youderian et al., 1983; Vershon et al., 1985a, 1987c). Moreover, these studies have identified residues near the N-termini of both proteins as important determinants of operator recognition. The first 50 residues of Mnt (82 residues) show greater than 40% sequence homology with Arc (53 residues), suggesting that the proteins share structural homology (Sauer et al., 1983; Vershon et al., 1985b). Despite these similarities, Mnt and Arc display several notable differences. First, the oligomeric structures of the proteins differ; Mnt is a tetramer in solution, whereas Arc is a dimer (Vershon et al., 1985b). Second, the patterns of operator contacts are somewhat different for the two proteins. Mnt makes sequence-specific contacts on both the "front" and "back" side of the operator helix, whereas Arc makes contacts on the front side only (Vershon et al., 1987a,b).

It seemed plausible that residues at the C-terminus of Mnt, which are not present in the shorter Arc protein, might account for some of the differences between Mnt and Arc. To test this possibility, we have initiated site-directed mutagenic studies of the C-terminal region of Mnt. In this paper we report on the properties of a series of Mnt mutants bearing sequential deletions of the C-terminal residues, Leu^{77}-Tyr^{78}-Lys^{79}-Lys^{80}-Thr^{81}-Thr^{82}. Our results show that Tyr^{78} is involved in stabilization of the Mnt tetramer and that Lys^{79} is an important determinant of operator binding affinity. However, our results also indicate that the C-terminal residues of Mnt are not responsible for contacts with the back side of the *mnt* operator.

Materials and Methods

Synthesis of Mutagenic Oligonucleotides. Mutagenic oligonucleotides were synthesized with a Systec Microsyn 1450A DNA synthesizer. Following synthesis oligonucleotides were cleaved from the solid support by overnight incubation in concentrated NH_4OH at 55 °C. The supernatant was recovered and dried in a Savant Speed Vac concentrator. Dried oligonucleotides were dissolved in 250 μL of TE buffer [10 mM Tris[1] (pH 7.5), 0.1 mM EDTA] and loaded onto a preparative 20% acrylamide gel containing 50% urea (w/v). Following electrophoresis in TBE buffer [90 mM Tris–borate (pH 7.8), 2 mM EDTA] gel pieces containing oligonucleotide were cut out of the gel, minced, and incubated overnight at 37 °C in TE buffer. The supernatant was loaded onto a Waters C-18 Sep-pak cartridge column and washed with 5 mL of TE buffer. Oligonucleotide was eluted with 70% acetonitrile and dried. Purified oligonucleotides were dissolved in 100 μL of TE buffer and stored at −20 °C.

Oligonucleotide-Directed Mutagenesis. The *mnt* gene, under transcriptional control of the *tac* promoter, is carried on a pBR322-derived plasmid, pTM201 (Vershon, 1986), which contains an M13 origin of replication. Termination codons were introduced into the *mnt* gene by annealing the mutagenic oligonucleotide to a gapped heteroduplex, followed by extension and ligation (Inouye & Inouye, 1987). All mutagenic oligonucleotides used were 17 bases in length and were synthesized such that the single base change required to make each of the termination codons was the central base in the sequence. Gapped heteroduplex was prepared by annealing single-stranded circular pTM201 to a 4350 base pair *Cla*I fragment from pTM201, which lacks the P_{tac}-*mnt* sequences. Single-stranded circular pTM201 was prepared as described (Zagursky & Berman, 1984) following a 50-fold dilution of an overnight culture of *Escherichia coli* strain XTM201 (Vershon, 1986) into 2× YT media and infection with 2 × 10^7 pfu of the M13 derivative RV-1 (Levinson et al., 1984).

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; pfu, plaque-forming units; DTT, dithiothreitol; CM, carboxymethyl; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; DMS, dimethyl sulfate; CD, circular dichroism.

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Both the single-stranded pTM201 and the large *Cla*I fragment were gel purified by using 0.7% low-melting agarose (SeaPlaque).

Single-stranded circular pTM201 (0.2 pmol) was mixed with the large *Cla*I fragment of pTM201 (0.1 pmol) in a buffer containing 50 mM Tris (pH 8.0), 1.0 mM EDTA, 0.3 M NaCl, and 50% deionized formamide in a final volume of 40 μL. The gapped heteroduplex was formed by heating the mixture to 70 °C for 15 min and cooling successively at 37 °C and room temperature for 15 min each. DNA was then EtOH precipitated, washed once with 70% EtOH, dried, and redissolved in 10 μL of TE buffer. Phosphorylated mutagenic oligonucleotide (10 pmol) was added and annealed to the gapped heteroduplex by heating to 65 °C for 30 min and cooling to room temperature for 30 min. The mixture was then diluted 2-fold with 2× extension/ligation buffer [100 mM Tris (pH 8.0), 20 mM $MgCl_2$, 100 mM NaCl, 12 mM DTT, 2 mM ATP, and 1.2 mM each of the four dNTPs] and incubated at 16 °C for 60 min with 1.0 unit of the DNA polymerase I large fragment and 80 units of T4 DNA ligase. The mixture was then treated with S1 nuclease in order to remove any free single-stranded circular pTM201 and heteroduplex molecules that had not been completely extended. A 2.5-μL aliquot of 10× S1 buffer [0.5 M sodium acetate (pH 4.5), 2 M NaCl, 10 mM $ZnSO_4$, 5% glycerol] and 200 units of S1 nuclease were added, and the mixture was incubated at room temperature for 20 min. S1 nuclease digestion was stopped by addition of 2 μL of 0.25 M EDTA. Half of the mixture was removed for analysis on a 0.7% agarose gel to test for S1-resistant heteroduplex molecules. The remaining half of the mixture was diluted 5-fold with buffer containing 10 mM Tris (pH 7.5) and 50 mM $CaCl_2$ and transformed into the mismatch repair defective *E. coli* strain MH90 (Hecht et al., 1986). Transformants were selected on LB plates containing 100 μg/mL ampicillin. Plasmids were then transduced into strain X90 (Vershon, 1986) as follows: colonies from a transformation plate were pooled and infected with 2×10^7 pfu of the M13 derivative RV-1 and were grown for 6.5 h at 37 °C. Host cells were removed by centrifugation, and phage stocks were sterilized by heating at 70 °C for 30 min. The stock was diluted 10^6-fold in 2× YT and used to infect late logarithmic phase X90 cells (0.2 mL of cells plus 1.0 mL of diluted phage stock). Transductants were selected on LB-ampicillin plates, and single colonies were restreaked once and used for preparation of single-stranded DNA. Desired mutants were identified by DNA sequence analysis and arose at a frequency of 10–25%.

Purification of Mutant Proteins. The purification described for wild-type Mnt protein (Vershon et al., 1985b) was used for the C-terminal deletion proteins with adjustments made in the salt concentrations used for ion exchange chromatography in order to accommodate the loss of one or two positive charges (Lys^{79} and Lys^{80}). The modifications are described briefly. The buffer used in all purification steps was designated SB and contains 50 mM Tris (pH 7.5), 0.1 mM EDTA, 5% glycerol, and 1.4 mM 2-mercaptoethanol. Following the initial fractionation step using a Sephadex G-75 (fine) column (5.0 × 90 cm), the mutant proteins were dialyzed into SB plus 50 mM KCl and loaded onto a Whatman P-11 phosphocellulose column (2.5 × 8.0 cm) equilibrated in the same buffer. Proteins 1–79 and 1–78 were eluted with a gradient of 50–750 mM KCl, 200 mL each. Proteins 1–77 and 1–76 did not bind tightly to phosphocellulose under these conditions, but their elution during the wash with loading buffer was retarded sufficiently to achieve a significant purification from those proteins in the flow-through. Fractions containing proteins 1–79 and 1–78 were dialyzed into SB plus 50 mM KCl, while proteins 1–77 and 1–76 were dialyzed into SB plus 20 mM KCl. Each protein was loaded onto a CM-Sephadex (C-50) column (2.5 × 5.0 cm) equilibrated in the appropriate buffer. Proteins 1–79 and 1–78 were eluted with a gradient of 50–400 mM KCl, 120 mL each, while proteins 1–77 and 1–76 were eluted with a gradient of 20–300 mM KCl, 120 mL each. The purified mutant proteins were >95% pure as judged by SDS–polyacrylamide gel electrophoresis and were stored in SB plus 100 mM KCl at −70 °C. Protein concentrations were determined by analytical SDS–polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant blue using known amounts of wild-type Mnt as standards or by using the molar extinction coefficient at 280 nm of 4500 previously determined for wild-type Mnt (Vershon et al., 1985b). Unless indicated otherwise all concentrations are given in terms of protein monomer.

Labeled Operator Fragments. The *mnt* operator contains a 17 base pair core displaying perfect 2-fold symmetry about an axis passing through the central G·C base pair. We refer to the strand containing the central "C" as the top strand and that containing the central "G" as the bottom strand. The 240 base pair *Eco*RI/*Hin*dIII fragment from plasmid pIO101 was used as one source of operator DNA (Vershon, 1986). The end-labeled top strand was obtained by digestion of pIO101 with *Eco*RI, filling in the 5′-overhang with the DNA polymerase I large fragment in the presence of [α-^{32}P]dATP and [α-^{32}P]dTTP, and subsequent digestion with *Hin*dIII. The end-labeled bottom strand was obtained by digestion of pIO101 with *Hin*dIII, filling in the 5′-overhang with the DNA polymease I large fragment in the presence of [α-^{32}P]dGTP and [α-^{32}P]dATP and nonradioactive dCTP and dTTP, and subsequent digestion with *Eco*RI. End-labeled fragments were gel purified with 4% low-melting agarose (NuSieve). Gel pieces containing the labeled fragments were cut out, mixed with 3 volumes of Elutip-d low-salt buffer, and heated to 65 °C for 15 min. Labeled DNA fragments were then purified on Elutip-d columns as described by the manufacturer. Purified operator fragments were dissolved in approximately 200 μL of TE buffer and stored at −20 °C.

The second source of *mnt* operator DNA was plasmid pMO100 (Vershon, 1986). The 3′-end-labeled bottom strand was prepared by digestion of pMO100 with *Eco*RI and end filling as described above, followed by digestion with *Eco*RV. The 5′-end-labeled top strand was prepared by digestion of pMO100 with *Eco*RI, removal of the 5′-phosphate with calf intestinal phosphatase, labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP, and digestion with *Eco*RV. Labeled fragments were gel purified and stored as described above.

DNA Binding and Protection Assays. The binding of the wild-type or mutant Mnt proteins to operator DNA was assayed by using the gel shift electrophoresis method (Garner & Revzin, 1981; Fried & Crothers, 1981). Details of this assay using wild-type Mnt protein have been described (Vershon et al., 1987a). Reactions were performed at room temperatures in a buffer containing 50 mM Tris (pH 7.3), 10 mM $MgCl_2$, 0.1 mM EDTA, 200 mM KCl, and 100 μg/mL BSA. The amounts of bound and free operator fragment were quantitated by scanning laser densitometry of the autoradiograms using an LKB 2202 Ultroscan laser densitometer with a 2220 recording integrator.

The ability of wild-type or mutant Mnt proteins to protect operator DNA against modification by dimethyl sulfate (DMS) was assayed according to the method of Gilbert et al.

		74	75	76	77	78	79	80	81	82	
Wild-Type	...	Leu	Lys	Asp	Leu	Tyr	Lys	Lys	Thr	Thr	
	...	CTG	AAG	GAT	TTG	TAC	AAA	AAA	ACC	ACC	TGA
1-79	...	Leu	Lys	Asp	Leu	Tyr	Lys				
	...	CTG	AAG	GAT	TTG	TAC	AAA	TAA			
1-78	...	Leu	Lys	Asp	Leu	Tyr					
	...	CTG	AAG	GAT	TTG	TAC	TAA				
1-77	...	Leu	Lys	Asp	Leu						
	...	CTG	AAG	GAT	TTG	TAG					
1-76	...	Leu	Lys	Asp							
	...	CTG	AAG	GAT	TAG						

FIGURE 1: Protein and DNA coding sequences for the C-terminal ends of wild-type Mnt and the truncated mutant proteins. For each mutant, an amber or ochre termination codon was introduced by making a single base change at the appropriate position by oligonucleotide-directed mutagenesis.

(1976) as detailed by Vershon et al. (1987a). Reactions were performed at 4 °C in a buffer containing 50 mM sodium cacodylate (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 200 mM KCl, 1 mM DTT, 1 mM $CaCl_2$, 250 μg/mL BSA, and 5 μg/mL sonicated salmon sperm DNA.

Hydroxy radical footprinting was performed according to the method of Tullius and Dombroski (1986). End-labeled DNA was diluted to 2.5×10^5 Cerenkov cpm/mL in a buffer containing 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 200 mM KCl, 1 mM $CaCl_2$, 2.5 μg/mL sonicated salmon sperm DNA, and 100 μg/mL BSA. Protein–operator complexes were formed by mixing 20 μL of an appropriate dilution of protein with 150 μL of end-labeled DNA and incubating at room temperature for 30 min. DNA cleavage using the Fe(II)EDTA/H_2O_2 cutting reagent and preparation of the resulting DNA fragments were performed exactly as described (Tullius & Dombroski, 1986). Samples were electrophoresed on an 8% polyacrylamide sequencing gel containing 50% urea (w/v) at 1500 V for approximately 2.5 h and autoradiographed for 30 h at −70 °C with Kodak XAR-5 film with an intensifying screen. Band intensities were quantitated by scanning laser densitometry as described above.

Protein Structure and Stability. Circular dichroism (CD) spectra were obtained with an AVIV Model 60DS CD polarimeter. Protein was diluted to 75 μg/mL with a buffer containing 0.1 M sodium phosphate (pH 7.5) and 100 mM KCl. Final spectra were an average of five repetitive scans in the range of 200–270 nm.

The thermal stability of each protein was determined by observing the change in the molar ellipticity at 220 nm as a function of temperature. Initial readings were taken at 25 °C and at 5° intervals up to 85 °C. Following each run samples were equilibrated slowly to 25 °C in order to determine the reversibility of thermal denaturation.

The oligomeric state of each protein was determined by gel filtration chromatography using a Sephadex G-75 (fine) column (1.4 × 46 cm). The buffer for all runs was SB plus 200 mM KCl. The flow rate was maintained at 0.2 mL/min by using a Ranin peristaltic pump. Effluent was monitored continuously at 280 nm. Calibration standards included thyroglobulin, BSA, ovalbumin, myoglobin, and vitamin B_{12}. Apparent molecular weights were calculated as described (Vershon et al., 1985b).

RESULTS

Construction of Mutants. To create a set of Mnt proteins truncated at the C-terminal end, we used oligonucleotide-directed mutagenesis to introduce amber or ochre termination mutations at codons 77, 78, 79, and 80. The resulting mutant proteins consist of residues 1–76, 1–77, 1–78, and 1–79 and

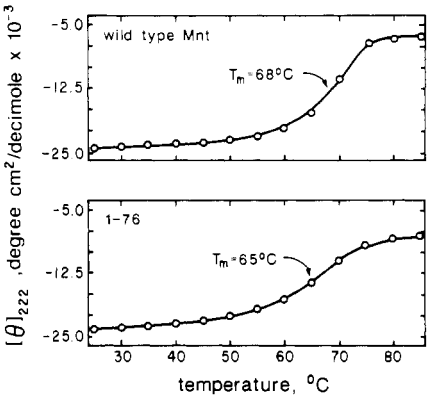

FIGURE 2: Melting curves of wild-type Mnt and the 1–76 truncated mutant protein.

Table I: Properties of Wild-Type Mnt and the C-Terminal Truncated Proteins

protein	DNA binding affinity[a] specific	nonspecific	M_r[e]	T_m (°C)
wild type	1[b]	1[c]	47000	68
1–79	2	1.7	46000	63
1–78	1200	2.3	44000	40, 63[d]
1–77	8000	3.2	30000	65
1–76	12000	2.3	30000	65

[a] Relative values of K_{app} derived from gel binding assays. [b] Value of 1 represents K_{app} of 1.5×10^{-11} M for binding of the wild-type Mnt tetramer to operator DNA. [c] Value of 1 represents K_{app} of 3×10^{-7} M for binding of the wild-type Mnt tetramer to a 750 base pair nonoperator restriction fragment. [d] 1–78 shows a biphasic thermal denaturation profile. We do not yet understand the reason for the 40° thermal transition of this protein. [e] Apparent molecular weights were determined by gel filtration chromatography and have an estimated error of 2000.

are three to six residues shorter than the wild-type Mnt protein (1–82). The protein sequences and the DNA coding sequences for the C-terminal ends of each of these mutants are shown in Figure 1.

Protein Purification and Stability. Upon induction of the *tac* promoter each of the truncated proteins was produced at a high level and could be purified with slight modifications of the procedure described for wild-type Mnt protein (Vershon et al., 1985b; see Materials and Methods). Amino acid analysis of each of the purified mutant proteins gave the expected composition (data not shown).

The circular dichroism spectrum of wild-type Mnt indicates a predominantly α-helical structure (Vershon et al., 1985b). The CD spectrum of each of the mutant proteins was nearly identical with that of wild-type Mnt (data not shown), suggesting that the overall native structure of these proteins is very similar. The thermal denaturation profiles of wild-type Mnt and mutant protein 1–76 are shown in Figure 2. The wild-type Mnt protein undergoes a relatively sharp transition from the native to the denatured form between 60 and 75 °C, with a T_m of approximately 68 °C. The 1–76 mutant protein undergoes a slightly broader transition, with a T_m of approximately 65 °C. Similar values for T_m were observed for the other mutant proteins (see Table I) with the exception of the 1–78 protein, which showed a biphasic transition with T_m values of approximately 40 and 63 °C. Because the purification properties, CD spectra, and thermal stabilities of the truncated proteins are similar to wild type, we conclude that the structural integrity of Mnt is not affected by deletion of up to six C-terminal residues.

Binding of the Mutant Proteins to Operator DNA. Figure 3 shows a set of gel shift operator binding experiments for

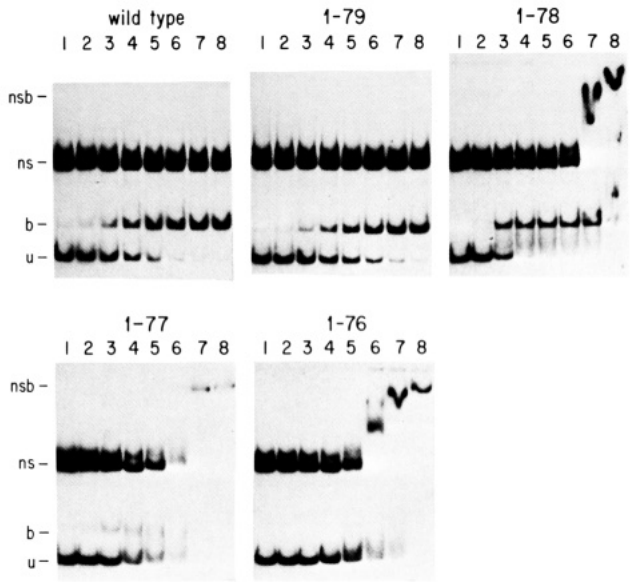

FIGURE 3: Operator and nonoperator DNA binding assays. The unbound operator fragment migrates at position u, the specifically bound operator fragment migrates at position b, the unbound nonoperator fragment migrates at position ns, and the protein-bound nonoperator fragment migrates at position nsb. For wild-type Mnt and each mutant, the total protein concentration is doubled in each successive lane from left to right. For wild-type Mnt and the 1–79 mutant, the lowest protein concentration is 7.4×10^{-12} M (lane 1) and the highest is 9.5×10^{-10} M (lane 8). For mutant 1–78, the lowest protein concentration is 1.5×10^{-8} M and the highest is 1.9×10^{-6} M. For mutants 1–77 and 1–76, the lowest protein concentration is 3.0×10^{-8} M and the highest is 3.8×10^{-6} M.

wild-type Mnt and each of the truncated mutant proteins. The unbound operator fragment migrates at the position marked "u" while the operator fragment complexed with protein migrates at the position marked "b". Under the conditions of these experiments, the protein concentration at which the operator fragment is distributed equally between the bound and unbound forms is a measure of the equilibrium binding constant, K_{app}. A second DNA fragment (marked "ns") was also included in each assay to allow determination of the binding constant for nonoperator sequences. Relative values for the operator and nonoperator binding constants for wild-type Mnt and each of the mutant proteins are listed in Table I.

The 1–79 mutant protein binds operator DNA almost as well as wild-type Mnt (Table I). This indicates that the three C-terminal residues of Mnt, Lys^{80}-Thr^{81}-Thr^{82}, play no significant role in operator binding. By contrast, the 1–78 mutant protein shows a 1200-fold reduction in operator binding affinity relative to wild type. This suggests that Lys^{79} is an important determinant of operator binding. Deletion of Tyr^{78} and Leu^{77} causes additional but less dramatic decreases in operator binding affinity. The affinity of each of the truncated proteins for nonspecific DNA is not significantly different than that of wild-type Mnt (Table I).

DMS Protection. Figure 4 shows the results of DMS protection experiments performed with wild-type Mnt and the 1–76 mutant protein. Considerably higher concentrations of the 1–76 protein are required to achieve protection, and we can estimate from these experiments that the K_{app} for mutant protein 1–76 (5×10^{-8} M) is at least 2500-fold greater than for wild-type Mnt (2×10^{-11} M). These values are slightly different from those obtained from gel shift experiments (see Table I), but conditions of temperature and pH were different for the two assays. Despite the differences in affinity the protection patterns observed with wild-type Mnt and the 1–76

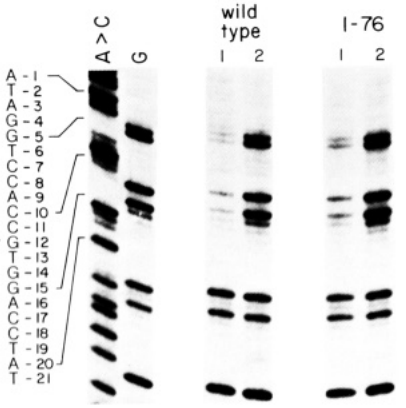

FIGURE 4: Methylation protection experiments using operator DNA that is 3′ end labeled on the top strand (see Figure 6B). Lanes marked A>C and G are Maxam and Gilbert sequencing reactions. Lane 1, [wild-type Mnt] = 5×10^{-11} M; lane 2, [wild-type Mnt] = 1×10^{-11} M; lane 1, [mutant 1–76] = 5×10^{-8} M; lane 2, [mutant 1–76] = 1×10^{-8} M. The N7 group of G-12 lies in the major groove on the back side of the operator helix (see Figure 6A).

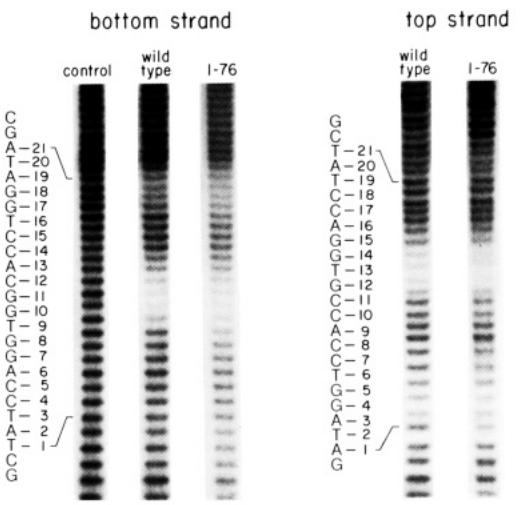

FIGURE 5: Hydroxy radical footprints of Mnt–operator complexes. Reaction mixtures contained operator labeled at the 3′-end (bottom strand) or the 5′-end (top strand). The control lane is the hydroxy radical cleavage pattern obtained in the absence of protein. Reactions contain wild-type Mnt at 3×10^{-10} M or the 1–76 mutant protein at 3×10^{-7} M. Identification of bands was determined by alignment of the bands with Maxam and Gilbert sequencing controls (not shown).

mutant protein are essentially the same. In both cases, guanine residues 4, 5, 12, 14, and 15 are protected by the proteins from methylation. The N7 groups of guanine-4, -5, -14, and -15 lie in the major groove on the front side of the operator helix, while the N7 of guanine-12 lies on the back side of the helix (see Figure 6). Thus, although deletion of the six C-terminal residues of Mnt reduces operator affinity dramatically, it does not alter the ability of the protein to contact guanines on both sides of the operator helix. In the experiments shown in Figure 4, the top strand of the operator was end labeled. In experiments in which the bottom strand of the operator was end labeled, protection of both front- and back-side guanines by wild-type Mnt and the 1–76 mutant protein was also observed (data not shown).

Hydroxy Radical Footprinting. Figure 5 shows the results of hydroxy radical protection experiments (Tullius & Dombroski, 1986) performed with wild-type Mnt and the 1–76 mutant protein. Both proteins protect the same set of backbone deoxyribose residues in the central and outer regions of the operator. As shown in Figure 6, these regions of the DNA backbone correspond to those previously defined by ethylation

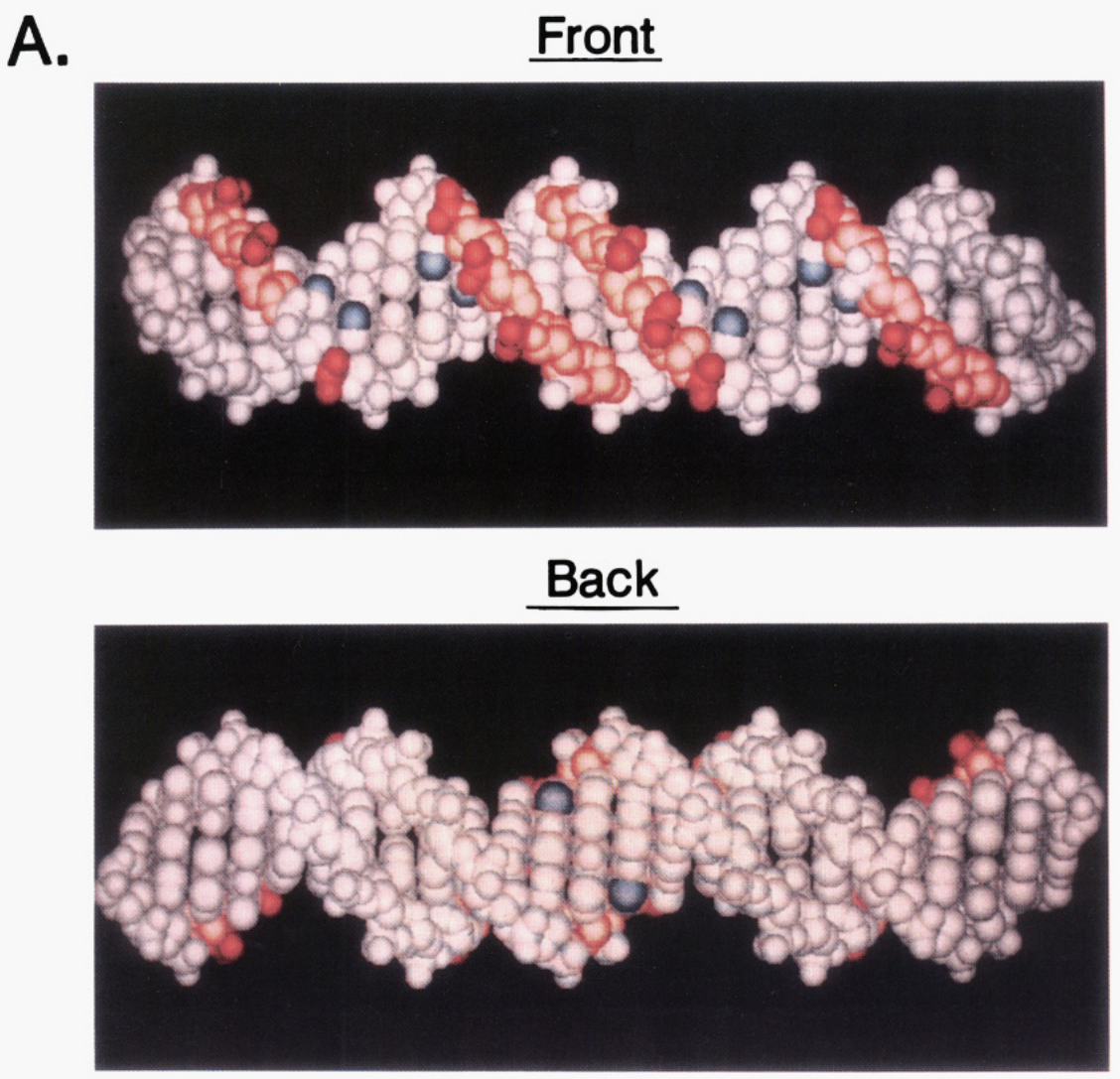

FIGURE 6: Panel A shows B-form DNA models of the front and back surfaces of the *mnt* operator. Blue atoms are the N7 groups of guanine residues that are protected by wild-type Mnt or the truncated mutant proteins from methylation by dimethyl sulfate; orange atoms are the deoxyriboses that are protected by wild-type Mnt or the truncated mutant proteins from attack by hydroxy radicals; red atoms are phosphate groups whose ethylation interferes with wild-type Mnt binding (Vershon et al., 1987a). Panel B shows the DNA sequence of a 21 base pair *mnt* operator, in which position 2 was changed from C·G (wild type) to A·T to facilitate cloning; this change does not affect Mnt binding to the operator. The sequence displays 2-fold rotational symmetry about an axis passing through the central G·C base pair.

interference studies as points of close contact between Mnt and the operator (Vershon et al., 1987a). The 1–77, 1–78, and 1–79 mutant proteins gave hydroxy radical protection patterns similar to those shown in Figure 5 (data not shown).

Oligomeric Structure of the Mutant Proteins. Gel filtration studies were performed to characterize the oligomeric state of each of the truncated mutant proteins. The data in Table I indicate that wild-type Mnt and mutant proteins 1–79 and 1–78 elute from a Sephadex G-75 column at a position near that expected for a tetramer (expected value is 38 000 Da; experimental value is ca. 46 000 Da). However, with the loss of Tyr^{78} there is an abrupt shift, and the 1–77 and 1–76 proteins elute at a position that more closely approximates that expected for an Mnt dimer. The apparent molecular weights of the putative dimeric and tetrameric species are probably greater than expected because the oligomers are asymmetric. The possibility that the 1–76 and 1–77 proteins elute at positions different from the other proteins because of sticking to the G-75 resin seems unlikely as identical results were obtained when the experiments were repeated with silica-based gel filtration columns (Bio-Rad TSK-125). The G-75 elution positions of wild-type Mnt and each of the mutant proteins were also found to be independent of loading concentration in the range from 0.26 to 90 μM.

DISCUSSION

In an effort to understand the differences between the closely related Mnt and Arc repressors and to begin a detailed study of the Mnt protein, we have created a set of *mnt* mutations that give rise to a series of stable, truncated Mnt proteins.

Mutations were constructed by introducing termination codons into the *mnt* gene at several positions encoding residues near the C-terminus of the protein. The corresponding mutant proteins were purified and studied in vitro by using several structural and functional assays.

Our results indicate that residues near the C-terminus of Mnt are involved both in operator binding and in stabilization of the Mnt tetramer. This can be most clearly seen by comparing the properties of the 1–76 mutant protein with those of the 1–82 wild-type Mnt protein. The 1–76 protein binds operator DNA with an affinity that is reduced dramatically (2500–12 000-fold depending on conditions) relative to wild type. This indicates that residues near the C-terminus of Mnt mediate energetically important operator interactions. In addition, the apparent molecular weight of the 1–76 mutant Mnt protein is significantly reduced in comparison with that of wild type; the truncated protein appears to be dimeric whereas wild-type Mnt is tetrameric. Arc, which is homologous to the N-terminal 50 residues of Mnt, is also dimeric (Vershon et al., 1985b). It is therefore likely that the Mnt dimer contacts reside in the N-terminal region whereas the tetramer contacts reside predominantly if not exclusively in the C-terminal region of the protein.

Because wild-type Mnt protein makes contacts on the back side of its operator whereas the shorter Arc protein does not, it seemed possible that the C-terminal residues of Mnt might be responsible for these contacts. However, the 1–76 mutant protein protects the same set of front-side and back-side guanines as does wild-type Mnt, indicating that the C-terminal residues of Mnt are not responsible for making back-side major groove contacts. The truncated 1–76 protein also gives essentially the same pattern of hydroxy radical protection as does wild-type Mnt. The models in Figure 6 show the positions where wild-type Mnt or the truncated proteins protect operator DNA against methylation by dimethyl sulfate and attack by hydroxy radicals. It remains to be determined which residues of Mnt are responsible for the back-side contacts and by what mechanism the residues near the C-terminus of Mnt contribute to operator binding.

By comparing the DNA binding and oligomeric properties of wild-type Mnt and the different truncated proteins, we can infer how indiviual C-terminal residues contribute to these functions. The three C-terminal residues of Mnt, Lys^{80}-Thr^{81}-Thr^{82}, are not required for tetramer formation or for strong binding to operator DNA. This conclusion follows from the finding that the mutant 1–79 protein, which lacks these residues, forms stable tetramers in solution and binds operator DNA with an affinity near that of wild type.

Comparison of the properties of the 1–78 and 1–79 mutant proteins suggests that Lys^{79} plays an important role in operator binding, as deletion of this residue causes a 600-fold decrease in operator affinity. The loss of Lys^{79} could affect operator binding indirectly, for example, by causing a global structural change. However, despite the finding that the 1–78 protein is thermally less stable than wild type, it has the same CD spectrum and oligomeric state as wild type under the temperature conditions (22 °C) of the operator binding assay. For these reasons, we favor the idea that the Lys^{79} side chain makes a direct contact with the operator DNA or that the negatively charged α-carboxylate of Tyr^{78} (at the position formerly occupied by the peptide bond linking residues 78 and 79) causes an unfavorable interaction with the operator. Either model implies close approach of residue 79 to the operator.

The λ Cro repressor has a C-terminal sequence, Lys^{62}-Lys^{63}-Thr^{64}-Thr^{65}-Ala^{66}, that is nearly identical with that of Mnt, Lys^{79}-Lys^{80}-Thr^{81}-Thr^{82}. These residues are partially disordered in the crystal structure of Cro (Anderson et al., 1981), but model building studies have suggested that these residues may form a flexible arm that is involved in sequence-independent minor groove phosphate contacts (Ohlendorf et al., 1982). Studies of truncated Cro proteins suggest that Thr^{64}, Thr^{65}, and Ala^{66} play only minor roles in determining operator affinity, whereas Lys^{63} plays a major role (S. J. Eisenbeis and M. H. Caruthers, personal communication). Although the detailed properties of the truncated Cro and Mnt mutants are somewhat different, it seems possible that the C-terminal residues of Mnt may also form a flexible arm in which Lys^{79} makes operator contacts similar to those proposed for Lys^{63} of Cro.

The operator binding affinities of the Mnt mutant proteins 1–77 and 1–76 are reduced modestly (5–10-fold) relative to the 1–78 mutant protein. However, the apparent molecular weights of the 1–77 and 1–76 proteins are decreased significantly relative to the 1–78 protein or wild type. These data are most simply interpreted by assuming that Tyr^{78} plays a role in stabilizing the Mnt tetramer and that loss of this residue results in a change in the quaternary structure from tetramer to dimer. Since the operator affinity decreases by only about 7-fold in going from the tetrameric mutant 1–78 to the dimeric mutant 1–77, it seems likely that the tetrameric structure of Mnt is not necessary for high-affinity binding to O_{mnt}. It is not clear if the tetrameric structure of Mnt is essential for any of its biological activities, although this question could be addressed by studying mutants that were defective in tetramer formation but had normal operator binding. Our results suggest that residue 78 missense mutants might have such properties.

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Chemical Kinetics of Induced Gene Expression: Activation of Transcription by Noncooperative Binding of Multiple Regulatory Molecules†

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ABSTRACT: A chemical kinetics model is described for the regulation of gene expression by the progressive binding of regulatory molecules to specific binding sites on DNA. Chemical rate equations are formulated and solved for the accumulation of regulatory molecules on DNA, the change in the level of induced mRNA, and the change in the level of the encoded protein in the activated tissue. Some special cases are examined, including that of an activation threshold created by a requirement for the binding of a minimum number of regulatory molecules prior to gene activation. Experimental data for several hormone-activated genetic systems are analyzed in the frame of the proposed model, and kinetic parameters are predicted. The model accounts for a number of experimental characteristics of hormone-inducible genetic systems, including the existence of a lag in the time course of mRNA accumulation, the sigmoidal curve of induced mRNA kinetics, the effect of hormone on mRNA stabilization, and the induction parameters observed when hormone analogues are used. The model also provides an explanation for the phenotypes of genetic variants with altered inducibility as changes in the molecular kinetic parameters of gene activity.

In several disparate biological control systems that involve the binding of regulatory proteins to DNA, the outcome is modulated by the binding of more than one molecule of regulatory protein. Systems as distinct as the initiation of yeast genome replication at ARS sequences (Jaswinski, 1983), integration and excision of prophages (Nash, 1981), and gene activation by steroid–receptor complexes (Payvar et al., 1982; Ringold, 1983; Yamamoto, 1985a) all appear to involve the binding of protein molecules at multiple DNA sequences.

Control systems that operate by multiple binding sites, rather than a single site, offer the possibility of graduated regulation. This property may be especially pertinent in genetic regulatory systems, which have the peculiar characteristic that the properties of the entire cell are decided by the outcome at the one or two gene copies present. This is in contrast to metabolic regulatory systems, where the properties of a single cell derive from the average behavior of many molecules. The potential all-or-none character of the response of a gene will be largely mitigated if levels of gene expression, for example, the rate of transcription, are quantitatively dependent on the number of regulatory molecules bound to the gene. In this case the outcome can be modulated quantitatively rather than by an all-or-none response. Recent direct evidence that quantitative modulation can occur in this way is provided by experiments which vary the number of regulatory elements in synthetic DNA constructions. Searle et al. (1985) have inserted variable numbers of the upstream regulatory element required for metal activation of the mouse metallothionein I gene into the nonresponsive promoter of the herpes simplex virus thymidine kinase gene. Little or no induction by zinc was observed with single insertions of the regulatory element, whereas many constructions with two copies of the regulatory element were inducible, and constructions with additional copies were even more inducible. Similar results were obtained by Toohey et al. (1986), who reported that levels of glucocorticoid activation of the mouse mammary tumor virus promoter were directly proportional to the number of glucocorticoid response elements upstream of the promoter sequence.

To explore the characteristics of multiple-binding systems, we have considered the activation of gene transcription by steroid hormone receptor complexes, a subject that has been much studied experimentally and which has generated a considerable body of quantitative data (Ringold, 1983; Lanne et al., 1976; Janne & Bardin, 1984; Swaneck et al., 1979; McKnight & Palmiter, 1979; Watson et al., 1981, 1985; Perry et al., 1984; Shapiro & Brock, 1985; Karlsen et al., 1986). Using a chemical kinetics approach to analyzing the relationship between regulatory protein binding and gene expression, we have formulated the rate equations and their solutions for the major steps of the gene expression pathway. The equations developed provide a good description of several experimental systems and suggest the physical basis for some

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