Construction and characterization of a mercury-independent MerR activator (MerR^AC): transcriptional activation in the absence of Hg(II) is accompanied by DNA distortion

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The MerR regulatory protein of transposon Tn501 controls the expression of the mercury resistance (mer) genes in response to the concentration of mercuric ions. MerR is unique among prokaryotic regulatory proteins so far described in that it acts as a repressor [−Hg(II)] and an activator [+Hg(II)] of transcription of the mer genes, but binds to a single site on the DNA in both cases. This transcriptional activation process has been postulated to involve a protein-induced conformational change in the DNA that allows RNA polymerase more readily to form an open complex at the promoter. It has been shown [Frantz and O'Halloran (1990) Biochemistry, 29, 4747−4751] that activation of transcription by MerR in the presence of mercury is accompanied by hypersensitivity of the operator to chemical nucleases that are sensitive to local distortion in DNA structure. Here we describe specific mutations in MerR that allow the protein to stimulate transcription in the absence of the allosteric activator Hg(II). We demonstrate that the degree of activation caused by these mutants directly correlates with the degree of DNA distortion as measured by the hypersensitivity of MerR−DNA complexes to the nuclease Cu-5-phenyl-o-phenanthroline. These results support the model described above.
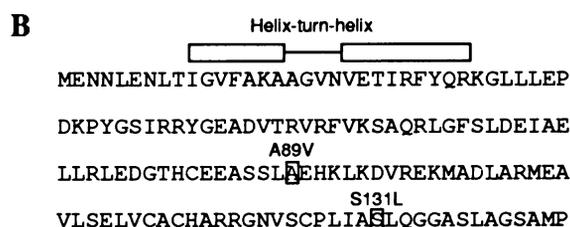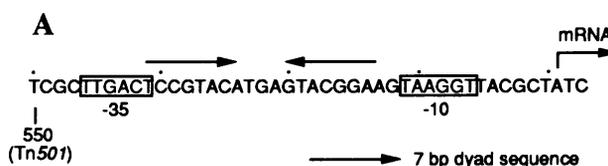Key words: allosteric control/constitutive activation/gene regulation/mercury resistance/positive control

Introduction

Since positively acting transcriptional regulatory factors were first recognized with the description of activation by AraC (Englesberg et al., 1965), many different positive regulators have been identified. Initial hypotheses suggested that activation of transcription could occur by one of two mechanisms: by protein−protein contact, usually between the positive regulator and RNA polymerase, or by distortion of the DNA (Raibaud and Schwartz, 1984). A large number of experiments have subsequently established the role of direct protein−protein contact in many cases of transcriptional activation. Such experiments include the isolation of positive control mutants in λ repressor (Hochschild et al., 1983), the selection of specific second-

site revertants of positive control mutants of CRP (Bell et al., 1990) and the directed mutation of the normally negative-acting λ Cro protein to produce a protein capable of activation (Bushman and Ptashne, 1988). RNA polymerase has also been reported to interact directly with CRP even in the absence of DNA (Pinkney and Hogget, 1988) and regions of RNA polymerase have been identified that are targets for CRP-mediated activation (Igarashi and Ishihama, 1991).

MerR is a regulatory protein that acts both as a repressor and activator of transcription of the mercuric ion resistance (mer) operon of transposon Tn501. Transcription of the mer operon is controlled by MerR in response to the concentration of Hg(II). In the presence of Hg(II), MerR strongly induces transcription of the operon, while in its absence MerR represses transcription. MerR also maintains repression of transcription of its own gene under all conditions (Lund et al., 1986). All of these functions are carried out by MerR bound to a single site within the promoter of the mer operon and activation is achieved by the binding of only a single mercury atom per MerR dimer (O'Halloran et al., 1989). The mer promoter (P_T, Figure 1A) is unusual in that it has a 19 bp spacing between the −10 and −35 elements (Lund et al., 1986) as compared with the 17 ± 1 bp spacing seen in the majority of Escherichia coli promoters (Hawley and McClure, 1983; Harley and Reynolds, 1987). Furthermore there is a close correspondence between the −35 region of P_T (TTGACT) and the canonical −35 sequence (TTGACA; five out of six base pairs), whereas positively controlled promoters in general have poor −35 sequences (Raibaud and Schwarz,

A


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                                                                    mRNA
TCGCTTGACTCCGTACATGAGTACGGAAGTAAGGTTACGCTATC
     -35                              -10
|
550
(Tn501)                        ——————▶ 7 bp dyad sequence
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B


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                    Helix-turn-helix

MENNLENLTIGVFAKAAGVNVETIRFYQRKGLLLEP

DKPYGSIRRYGEADVTRVRFVKSAQRLGFSLDEIAE
                      A89V
LLRLEDGTHCEEASSLAEHKLKDVREKMADLARMEA
                      S131L
VLSELVCACHARRGNVSCPLIASLQGGASLAGSAMP
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Fig. 1. A. Nucleotide sequence of the mer promoter−operator region showing the −10 and −35 promoter elements of P_T (boxed) and the dyad symmetrical sequence at the centre of the MerR binding site. Each dot above the sequence represents 10 bp. B. Amino acid sequence of the MerR gene showing the predicted helix−turn−helix region (DNA binding domain) and the site of the two mutations A89V and S131L.

1984). The P_T promoter also contains a dyad symmetrical sequence within the spacer which is at the centre of the region protected by MerR in footprinting assays (O'Halloran and Walsh, 1987; O'Halloran *et al.*, 1989).

Initial experiments with the P_T promoter *in vivo* demonstrated that the -35 sequence was essential for induction, but not for constitutive activity (Lund and Brown, 1989a), and that selection for up-promoter mutants in the absence of MerR produces mutants with single base pair deletions within the spacer DNA (Lund and Brown, 1989b). *In vitro* experiments showed that both MerR and RNA polymerase were bound to the promoter during repression and activation, and that some MerR contacts with DNA were altered on the addition of Hg(II) (O'Halloran *et al.*, 1989). A model for induction was therefore proposed in which the MerR protein binds to, and represses, the P_T promoter in the absence of Hg(II). On binding Hg(II), MerR causes a conformational change within the DNA that allows the bound but inactive RNA polymerase to extend its contacts to regions downstream of the -10 promoter element and form the melted out and transcriptionally active open complex (Frantz and O'Halloran, 1990).

Further experiments in which the spacing of the promoter elements was altered have provided results that supported this model. They have shown that the 19 bp spacing and the location of the MerR binding site within the spacer are both essential for normal activation: a longer promoter cannot be induced by Hg−MerR, while a shorter promoter is fully active in the absence of MerR, and is repressed by MerR in both the presence and absence of Hg(II) (Parkhill and Brown, 1990).

Certain chemical nucleases that are sensitive to changes in DNA structure will cleave the centre of the MerR binding site only in the Hg−MerR−DNA complex. In the case of Cu-5-phenyl-*o*-phenanthroline, the cleavage is specific and the DNA site is hypersensitive to this reagent only in the presence of Hg−MerR. That this hypersensitivity is only seen in the activator form of the protein−DNA complex suggests that the DNA distortion is associated with transcriptional activation (Frantz and O'Halloran, 1990). Experiments involving *in vivo* footprinting of the closely related P_T of transposon Tn*21* with $KMnO_4$ have confirmed the presence of this distortion *in vivo* (Heltzel *et al.*, 1990).

Recent evidence indicates that the nature of the Hg−MerR induced distortion is a localized underwinding of the spacer by $\sim 33°$ (Ansari *et al.*, 1992). Such an underwinding of the 19 bp spacer would realign the -10 and -35 elements on the face of the DNA helix to resemble the cylindrical orientation of these elements as they are found in a promoter with a spacer length of 18 bp (i.e. within the bounds of the 17 ± 1 bp of the consensus *E.coli* promoter). This optimization of the promoter configuration by allosteric DNA distortion may be the key step in the mechanism of transcriptional activation by MerR.

In this paper we examine further the role of DNA distortion in the transcriptional activation mechanism by constructing and characterizing allosteric control mutants of MerR that can stimulate transcription in the absence of Hg(II). This class of mutations apparently affects neither DNA-binding nor ligand [Hg(II)] binding directly, but rather partially uncouples ligand binding from the activation event. These mutants are described as 'allosteric control' (MerR^{AC}) mutants since the results described here suggest

that the mutations stabilize a conformational state of the protein that is usually observed only in the activator form of the protein (Hg−MerR). By comparing the phenotype and DNA binding properties of these mutants, which activate transcription from P_T in the absence of Hg(II), we have shown that the degree of hypersensitivity (and therefore distortion) of the DNA at the centre of the operator correlates with an increase in the steady state levels of transcription, irrespective of the presence or absence of Hg(II). These experiments support the hypothesis that DNA distortion is necessary for activation of the P_T promoter by Hg−MerR.
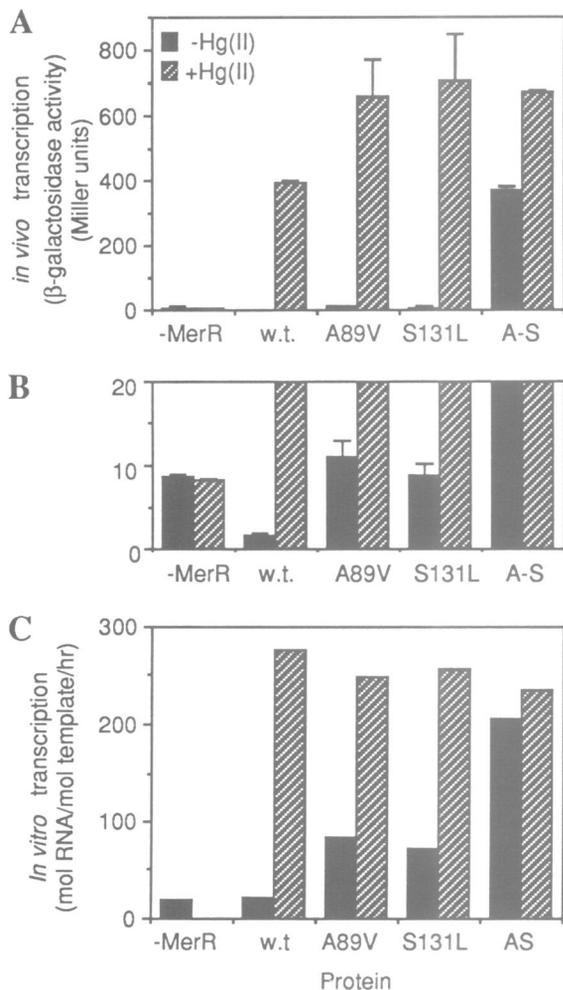
Results

Construction of the constitutively activating MerR mutant

In order to test the model for the induction of the *mer* operon, we produced a constitutively activating MerR protein by site-directed mutagenesis based on data obtained from the mutagenesis of the MerR protein of Tn*21*, which is 94% identical to that of Tn*501* (Ross *et al.*, 1989). Ross *et al.* (1989) generated and analysed a series of random mutations in the Tn*21 merR* gene; two of which, an alanine to valine substitution at amino acid 89 (A89V) and a serine to leucine substitution at amino acid 131 (S131L), were described as repression⁻, activation⁺ and appeared to partially activate transcription in the absence of Hg(II). The equivalent mutants of Tn*501* MerR were generated by site-directed mutagenesis (Figure 1B), and found to activate the P_T promoter to a greater degree than the wild type protein in both the absence and presence of Hg(II) (Figure 2). The double mutant was also constructed in an attempt to produce a stronger Hg-independent activator. This mutant did indeed activate strongly in the absence of Hg(II) (Figure 2).

The site-directed mutagenesis was performed according to the following protocol. The DNA fragment containing *merR* from the MerR-overproducing plasmid pTO90-16 (O'Halloran and Walsh, 1987) was cloned into the mutagenesis vector pMa5-8 (Stanssens *et al.*, 1989) on a 562 bp *Eco*RI fragment to produce the plasmid pMa90-16. The required mutations of pMa90-16 were generated by site-directed mutagenesis with 19 bp synthetic oligonucleotides, in which the mismatched nucleotide was at the centre. The A89V mutation was generated by converting the GCC alanine codon at nucleotide position 283 (nucleotide positions are from the start of Tn*501* and the MerR gene is encoded on the complementary strand) to a GTC valine codon, and in the S131L mutation the TCA serine codon at nucleotide position 157 was converted to a TTA leucine codon. The resultant plasmids were designated pMcA89V and pMcS131L respectively. Mutagenesis of the plasmid pMcA89V with the oligonucleotide determining the S131L mutation produced the double mutant A89V-S131L and the resultant plasmid was designated pMaA-S. The *merR* gene and its flanking DNA in each of the mutant plasmids was completely sequenced to check that the desired mutation had been introduced and that there were no secondary mutations.

Activity of the mutant proteins in vivo

The 562 bp *Eco*RI fragments containing *merR* from each of the mutant plasmids and from the wild type pTO90-16 were subcloned into the *Eco*RI site of pACYC184 and the

A

in vivo transcription
(β-galactosidase activity)
(Miller units)

800

600

400

200

0

■ -Hg(II)
▨ +Hg(II)

-MerR   w.t.   A89V   S131L   A-S

B

20

10

0

-MerR   w.t.   A89V   S131L   A-S

C

In vitro transcription
(mol RNA/mol template/hr)

300

200

100

0

-MerR   w.t   A89V   S131L   AS

Protein

Fig. 2. Effect of wild type and mutant MerR proteins on the transcription of the P_T promoter. **A** and **B.** *In vivo* (from β-galactosidase activities of a $P_T::lacZ$ fusion) (panel B is an expansion of the lower portion of panel A); **C.** *In vitro* (from abortive transcription assays). Transcriptional activities are given for assays performed in the presence and absence of $HgCl_2$. Columns labelled '−MerR' represent the activity of the promoter in the absence of the MerR protein.

orientation of the insert was confirmed by *Hind*III digestion. In the correct orientation the *merR* genes are transcribed from the constitutive pACYC184 CAT promoter. These plasmids, designated pAC-A89V, pAC-S131L, pAC-A-S and pAC90-16, as well as the unmodified vector pACYC184, were used to transform an *E.coli* CSH26Δ*recA* strain bearing the resident plasmid pRZH18 (Lund *et al.*, 1986), which carries the P_T promoter from Tn*501* linked to the reporter gene *lacZ*. The activity of the P_T promoter was measured in each case, in both the presence and absence of Hg(II), by determining the level of expression of the *lacZ* gene in β-galactosidase assays.

Figure 2A and B shows that the two single mutations cause the P_T promoter to be transcribed at a level similar to that of the wild type promoter in the absence of MerR. This can be explained as a small degree of Hg(II)-independent activation. In support of this it can be seen that the double mutation causes a high degree of Hg(II)-independent activation of the P_T promoter in the steady state. All three mutant proteins give rise to further activation of transcription on the addition of Hg(II).

## Overexpression and purification of the mutant proteins

Each of the 562 bp *Eco*RI fragments containing a mutant *merR* gene was further subcloned from the pACYC184 vectors into the expression vector pKK223-3 (Amann *et al.*, 1983) and the orientation of the inserted DNA was checked by *Hind*III digestion. This procedure effectively regenerated the MerR overexpression vector pTO90-16, but containing each mutant *merR*. These plasmids were designated pKKA89V, pKKS131L and pKKA-S. Each mutant MerR protein produced by cells containing one of these plasmids was purified as described in Materials and methods.

Activity of the mutant proteins in vitro

The ability of each of the purified mutant MerR proteins to affect transcription from the P_T promoter *in vitro* was examined in an abortive transcription assay (McClure *et al.*, 1978; Ralston and O'Halloran, 1990). Each MerR was incubated with RNA polymerase and a *Bss*HII−*Bst*XI P_T-containing fragment of pTO40 DNA (O'Halloran and Walsh, 1987) for 45 min at 37°C. Addition of the dinucleotide ApU and radioactively labelled [α-^{32}P]CTP allowed RNA polymerase to initiate transcription at the P_T promoter, producing the labelled tetranucleotide ApUpCpC at a rate determined by the transcriptional activity of the promoter−MerR [± Hg(II)] complex. The results of these assays are shown in Figure 2C. It can be seen that the general trend is the same as that *in vivo*. The two single mutant proteins activate transcription slightly in the absence of mercury, whereas the double mutant activates strongly. Again, all the mutant proteins can activate further on the addition of mercuric chloride. A few differences between the *in vivo* and *in vitro* results are also evident; most notable is that *in vitro*, the transcription induced by the two single mutants in the absence of mercury is significantly higher than the transcriptional activity of the promoter alone, whereas the *in vivo* results indicate that the transcription induced by these mutants is the same as that of the promoter alone.

DNA binding by the mutant proteins

The ability of each of the mutant proteins to bind to DNA *in vitro* was examined by a gel retardation assay. Binding curves for each of the wild type and mutant proteins are shown in Figure 3, and the apparent dissociation constants (estimated as the concentration of MerR required for half-maximal binding) are given in Table I. The affinity of the single mutants for the operator does not differ significantly from that of wild type MerR. The double mutant on the other hand has a 20-fold lower affinity than the wild type MerR. In accordance with the prediction, the affinity of all the four proteins is lowered in the presence of Hg(II). We observe that the mutant AS does not bind with the same stability to the operator in the presence of Hg(II). Approximately 30% dissociation of the complex occurs on the time scale of the gel retardation assay (Figure 3D). Hg−AS does saturate the binding site on the time scale of a footprinting assay as shown by a complete protection of the operator from DNase I cleavage (Figure 4, lanes 13 and 17). This apparent difference in the off rate of the double mutant may arise from either the instability of this mutant protein, or from a dynamic rather than static nature of the DNA distortion. Physical studies to differentiate between these two possibilities are under way.
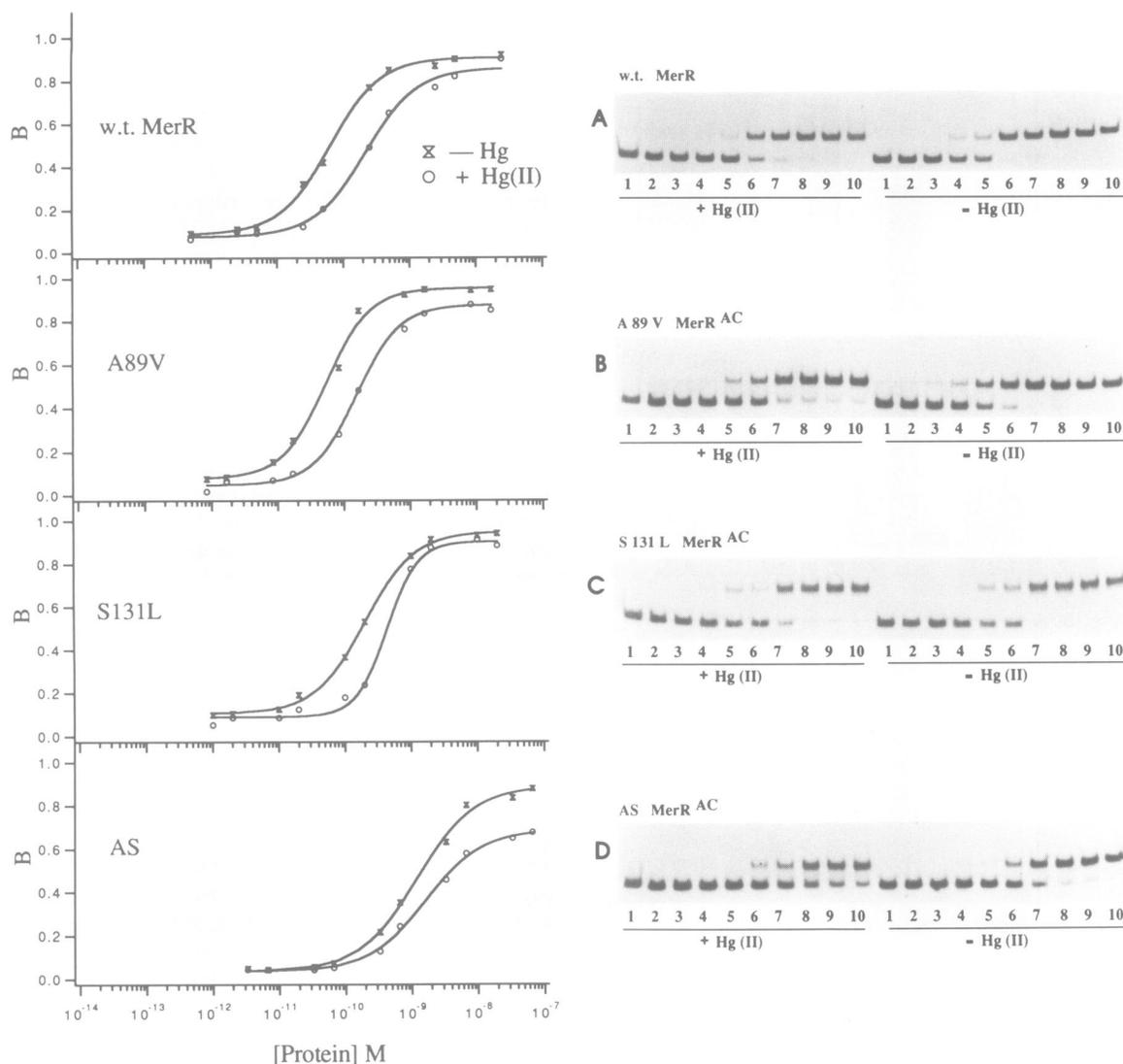
Fig. 3. Determination of the apparent equilibrium dissociation constant (K_D) for respective protein−DNA complexes with or without Hg(II). A representative data set for each of the mutant proteins is shown. Such data sets were used to determine the values for K_D presented in Table I. On the y axes, B represents the fraction of DNA that is bound by the protein. Each reaction was carried out with a constant DNA concentration ($\sim5\times10^{-12}$ M) and the following protein concentrations: **A.** Wild type MerR: lane 1, 5×10^{-13} M; lane 2, 2.5×10^{-12} M; lane 3, 5×10^{-12} M; lane 4, 2.5×10^{-11} M; lane 5, 5×10^{-11} M; lane 6, 2.5×10^{-10} M; lane 7, 5×10^{-10} M; lane 8, 2.5×10^{-9} M; lane 9, 5×10^{-9} M; lane 10, 2.5×10^{-8} M. **B.** A89V MerR: lane 1, 8×10^{-13} M; lane 2, 1.6×10^{-12} M; lane 3, 8×10^{-12} M; lane 4, 1.6×10^{-11} M; lane 5, 8×10^{-11} M; lane 6, 1.6×10^{-10} M; lane 7, 8×10^{-10} M; lane 8, 1.6×10^{-9} M; lane 9, 8×10^{-9} M; lane 10, 1.6×10^{-8} M. **C.** S131L MerR: lane 1, 1×10^{-12} M; lane 2, 2×10^{-12} M; lane 3, 1×10^{-11} M; lane 4, 2×10^{-11} M; lane 5, 1×10^{-10} M; lane 6, 2×10^{-10} M; lane 7, 1×10^{-9} M; lane 8, 2×10^{-9} M; lane 9, 1×10^{-8} M; lane 10, 2×10^{-8} M. **D.** AS MerR: lane 1, 3×10^{-12} M; lane 2, 6×10^{-12} M; lane 3, 3×10^{-11} M; lane 4, 6×10^{-11} M; lane 5, 3×10^{-10} M; lane 6, 6×10^{-10} M; lane 7, 3×10^{-9} M; lane 8, 6×10^{-9} M; lane 9, 3×10^{-8} M; lane 10, 6×10^{-8} M. In each case the first set of samples only was incubated with 0.75 μM HgCl$_2$. The fraction of the DNA in the protein−DNA complex (B) was plotted against log. [MerR] and the apparent K_D was obtained from the concentration of MerR at half-maximal binding.

The similarity in the equilibrium dissociation constants of the single mutants and wild type MerR rules out the possibility that the low levels of transcription observed in the absence of mercury are due to derepression of the promoter.

The position of binding of each of the mutant proteins was examined by DNase I protection assays (footprinting). The protection pattern (Figure 4) shows that the footprint and thus the position and extent of DNA binding, are unchanged between reactions with the wild type and the mutant proteins, in both the presence and absence of mercury. It can also be seen that there is a base at the centre of the operator (marked with an arrow in Figure 4) that is not protected by MerR in the absence of mercury, but is protected by the MerR−Hg(II) complex. This base is protected by

Table I. Equilibrium dissociation constants of the mutant MerR proteins

Protein	K_D (M)	
	−Hg	+Hg
Wild type[a]	$0.5 \pm 0.1\times10^{-10}$	$2.0 \pm 1.4\times10^{-10}$
A89V[b]	$0.5 \pm 0.02\times10^{-10}$	$1.7 \pm 0.1\times10^{-10}$
S131L[b]	$2.1 \pm 0.05\times10^{-10}$	$4.3 \pm 0.05\times10^{-10}$
A-S[a]	$10.0 \pm 4.0\times10^{-10}$	$14.5 \pm 4.0\times10^{-10c}$

[a]K_D determined from five independent data sets
[b]K_D determined from two independent data sets
[c]Apparent half-maximal AS concentration before the onset of non-specific binding. 100% specific binding was never observed in this assay (see Figure 3D).
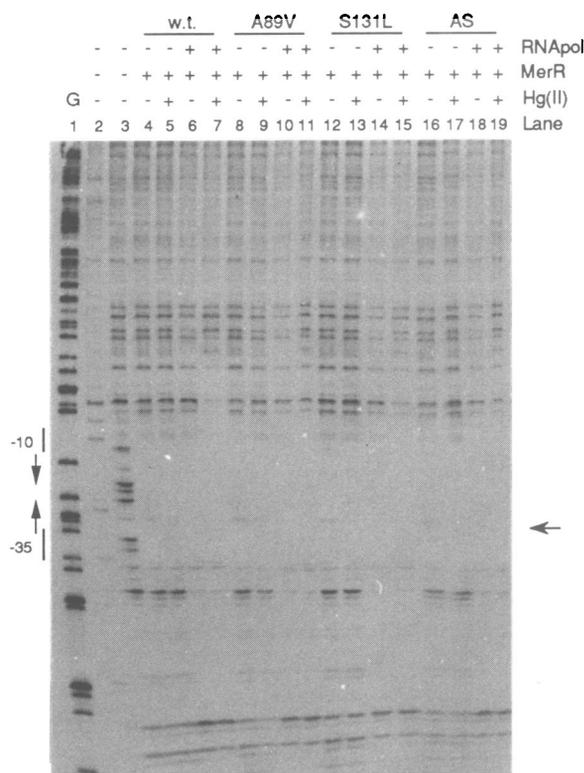
Fig. 4. Autoradiograph showing DNase I protection patterns for the wild type and mutant MerR proteins in the presence and absence of DNA polymerase. Each lane contains ~0.92 nM of the DNA fragment. Lane 1 is a Maxam—Gilbert G-reaction; lane 2 is untreated DNA; lane 3 is free DNA incubated with DNaseI and Lanes 4—19 are DNA incubated with DNase I in the presence of wild type (47 nM), A89V (140 nM), S131L (490 nM) and A-S (331 nM) MerRs, with and without RNA polymerase (15 nM) and HgCl$_2$ (1.6 μM), as shown above each lane. The positions of the -10 and -35 elements of the P$_T$ promoter and the *mer* operator are shown. The base near the centre of the operator that is protected by MerR—Hg(II), but not MerR alone is marked with an arrow.

MerR $-$ A $-$ S in both the presence and absence of mercury. Figure 4 also shows the DNase I protection pattern of RNA polymerase in the presence of each of the MerR proteins. It can be seen that there is a region downstream of the -10 element that is protected by RNA polymerase only in the presence of wild type MerR and mercury; this can be ascribed to the open complex. This area is protected by RNA polymerase and MerR $-$ A $-$ S in both the presence and absence of mercury.

Hg(II) binding by the mutant proteins

The affinity of the mutant MerR proteins for Hg(II) was measured using a competitive mercuric binding assay developed for the MerR system (J.G.Wright and T.V.O'Halloran, manuscript in preparation). The ability of MerR to compete with buffering thiols, in this case L-cysteine, was measured by monitoring the change in electronic absorption spectrum observed for MerR upon the addition of mercuric ion (Watton *et al.*, 1990; Wright, 1991). Hg(II) $-$ MerR exhibits an intense λ_{max} at 242 nm ($\Delta\epsilon$ = 19 800 M^{-1}cm^{-1}), which is not observed for Hg(II) $-$ L-cysteine under the same conditions of pH and concentration. By measuring the absorption change (ΔA) accompanying the titration of a thiol-buffered MerR solution with Hg(II), the

Table II. Hg(II) binding of the mutant MerR proteins

Protein	K_{app} (M)	σ(M)[a]
Wild type MerR[b]	585	73
Wild type MerR−operator[c]	338	52
A89V	411	63
S131L	473	82
A-S	423	84

[a]For wild type protein, σ is the standard deviation of three determinations. For the mutant proteins σ is calculated for the 95% confidence level of Student's factor, with K_{app} being obtained from two measurements for each mutant.
[b]J.G.Wright and T.V.O'Halloran (manuscript in preparation).
[c]Binding to a MerR complex with a 30mer synthetic operator [J.G.Wright and T.V.O'Halloran (manuscript in preparation)].

Table III. Plasmids used in this study

Plasmid	Brief description	Reference
pACYC184	Cm^r, Tet^r cloning vector	Chang and Cohen (1978)
pMa5-8	Amp^r, Cm^s mutagenesis vector	Stannsens *et al.* (1989)
pMc5-8	Amp^s, Cm^r mutagenesis vector	Stannsens *et al.* (1989)
pRZ5255	Promoter probe vector containing promoterless *lacZ* gene, Kn^r	Lambert and Reznikoff (1985)
pRZH18	Derivative of pRZ5255 carrying the P$_T$ promoter (500−701 bp of Tn501) such that *lacZ* expression is driven by P$_T$, Kn^r	Lund *et al.* (1986)
pTO90-16	Contains the *merR* gene under the control of the *lac* promoter: overexpresses MerR in the presence of IPTG; Amp^r	O'Halloran and Walsh (1987)
pKK223-3	Expression vector, Amp^r	Amann *et al.* (1983)

apparent equilibrium constant, K_{app}, can be calculated (J.G.Wright and T.V.O'Halloran, manuscript in preparation). The expression of K_{app} is

$$K_{app} = \frac{[\text{HgMerR}][\text{L-Cys}]^2}{[\text{MerR}][\text{HgCys}_2]}$$

The K_{app} values for the mutants are compared with that for the wild type protein in Table II. The results suggest that the mutant proteins have slightly lower binding affinities for Hg(II) than the wild type protein, but the difference is close to the experimental uncertainty. Since Hg(II) has a lower affinity for the MerR$-$DNA complex than for MerR alone, consistent with the thermodynamic cycle observed for Hg(II)-binding (J.G.Wright and T.V.O'Halloran, manuscript in preparation), the lower binding constant of the mutant proteins for Hg(II) could be due to the mutant MerR adopting a conformation similar to that of the operator-bound protein. Alternatively, the low K_{app} values could reflect incomplete renaturation or the possibility that the mutant proteins are less stable than the wild type
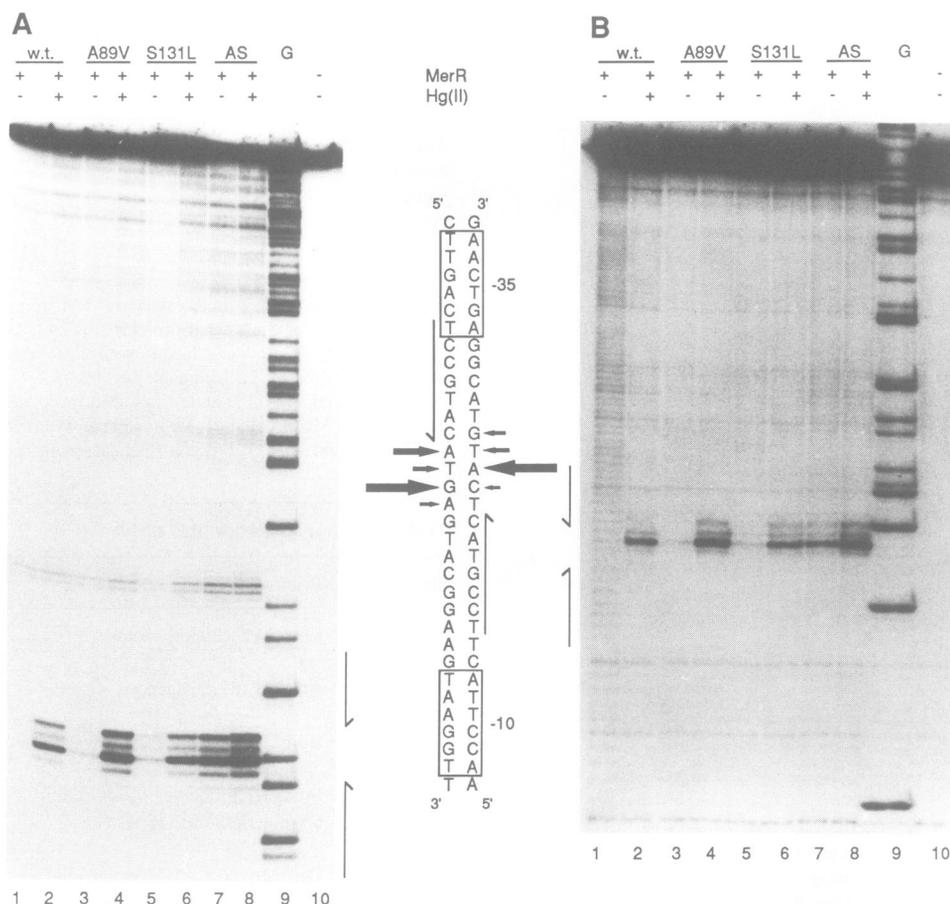
Fig. 5. Cu-5-phenyl-o-phenanthroline nuclease reactivity of the wild type MerR−DNA and mutant MerR^{AC}−DNA complexes. (A) 3' labelled P$_T$ non-template strand (B) 5' labelled P$_T$ template strand. In either A or B each lane contains ~3 nM of the DNA fragment; lane 1, 100 nM wild type MerR; lane 2, 100 nM MerR + 1.6 μM HgCl$_2$; lane 3, 70 nM A89V MerR; lane 4, 70 nM A89V MerR + 1.6 μM HgCl$_2$; lane 5, 116 nM S131L MerR; lane 6, 116 nM S131L MerR + 1.6 μM HgCl$_2$; lane 7, 110 nM A-S−MerR; lane 8, 110 nM A-S MerR + 1.6 μM HgCl$_2$; lane 9, Maxam−Gilbert 'G' sequencing reaction of the footprinting fragment; lane 10, unreacted initial fragment. The sequence of the mer promoter−operator region is given down the centre of the figure. The −10 and −35 regions are boxed and the operator is marked on both the sequence and the autoradiographs with thin convergent arrows. The filled arrows beside the sequence represent the sites and relative intensities of Cu-5-phenyl-o-phenanthroline cleavage.

DNA distortion by the wild type and mutant proteins

The chemical nuclease Cu-5-phenyl-o-phenanthroline is sensitive to DNA structure and rapidly cleaves the DNA backbone at regions where the geometry of the DNA is distorted (Frantz and O'Halloran, 1990; Thederan et al., 1990). DNA, radioactively labelled at either the 3' or 5' terminus, was incubated with each of the mutant and wild type MerR proteins in the presence and absence of RNA polymerase, both with and without Hg(II), and these complexes were then treated with Cu-5-phenyl-o-phenanthroline. With the wild type MerR, a hypersensitive site is generated at the centre of the operator only in the presence of Hg(II) (Figure 5). In sharp contrast to this, the MerR-A-S mutant protein generates a hypersensitive site in the absence of Hg(II) and this hypersensitivity is increased in the presence of mercury. The degree of hypersensitivity is independent of the presence of RNA polymerase (data not shown). As was seen in other experiments, the two single mutants give a pattern that lies between that of the double mutant and the wild type. The single mutants generate a small degree of hypersensitivity in the absence of Hg(II) and this is increased markedly in the presence of Hg(II); this too is independent of the presence of polymerase.

Discussion

The genetic and physical studies described in this paper demonstrate that specific mutations in MerR allow partial decoupling of co-activator binding from both transcriptional activation and DNA distortion, as gauged by hypersensitivity of the MerR−DNA complex to structure-sensitive nucleases. This class of mutant proteins allows a key test of the proposition that a protein-induced DNA distortion at the centre of the P$_T$ promoter is a necessary step in the transcriptional activation mechanism. The strong correlation observed between steady state transcriptional activity and the degree of DNA distortion for this series of MerR mutants, in the presence or absence of Hg(II), supports the distortion model.

We have constructed a pair of mutants in MerR that show a slight Hg(II)-independent activation of transcription from the P$_T$ promoter, and from them we have generated a mutant, MerR-A-S, that shows a strong Hg(II)-independent activation of transcription. In vivo the two single mutants weakly activate the P$_T$ promoter to the level of constitutive transcription and cause a further activation in the presence of Hg(II) to a level above that of the promoter activated by

the wild type Hg—MerR. The double mutant demonstrates an Hg(II)-independent activation of about the same magnitude as the fully activated wild type protein and will further activate the promoter on addition of Hg(II). The abortive initiation assay *in vitro* gives similar results, with the difference that the two single mutants appear to show an Hg(II)-independent activation of transcription to levels above those of the constitutive promoter. This cannot be easily explained, but it must be remembered that the template *in vivo* is negatively supercoiled, while that *in vitro* is linear and this may have an effect at this promoter. A second possible reason for this difference is that the position of the equilibrium between the repressing and inducing conformations of MerR (as discussed below) may be different *in vivo* and *in vitro*. The degree of activation displayed by the Tn21 MerR single mutants (Ross *et al.,* 1989) is broadly consistent with our results for the equivalent single mutants.

The properties of these mutants can best be understood if the MerR protein is envisioned as being in an equilibrium between two stable conformational states, one repressing and one activating. Binding of Hg(II) to the protein causes the equilibrium to shift, increasing the number of molecules in the activating conformation. Each of the single mutations causes a slight shift in the equilibrium towards the activating conformation, while a combination of the two causes a much larger shift. In all of the mutants, the equilibrium can still be shifted further towards the activating conformation by the addition of Hg(II).

The DNA binding affinities of the mutants shed more light on the properties of these proteins. As was first demonstrated for the wild type protein (O'Halloran *et al.,* 1989), all of the mutants show a lower DNA binding affinity in the presence of Hg(II) (Table I). The free energy change involved in DNA distortion was postulated to arise at the expense of the MerR—DNA binding energy. The fact that all of the mutants are able to increase the distortion of the DNA on binding Hg(II) and that all show a lowered binding affinity in the presence of Hg(II), fits with this view. According to this hypothesis, it should also be expected that as the mutants can distort DNA in the absence of Hg(II), they should also show a lowered affinity for DNA than the wild type in the absence of Hg(II). This is what occurs, although the reduction in affinity of the double mutant compared with the wild type is greater than that seen in the wild type on addition of Hg(II), suggesting that the mutations have additional effects on the ability of the mutant proteins to bind to DNA. Using the method described by Fried and Crothers (1981) we have determined that the lower affinity of AS for the operator is not due to a large fraction of inactive protein present in the purified protein sample. It follows then that the lowered affinity must be an inherent property of the double mutant. In fact, in the presence of Hg(II) we observe that AS does not saturate the operator, even at 10^{-7} M, when examined by gel retardation experiments. DNase I footprints on the other hand show a complete protection of the binding site. This situation could arise if AS—Hg dissociated from the DNA complex more rapidly than the wild type and within the time frame of the gel mobility experiment. These off-rates are currently under study.

The similarity of DNase I protection patterns for the wild type, single and double mutants indicate that the general topology of the protein—DNA complex is conserved in all
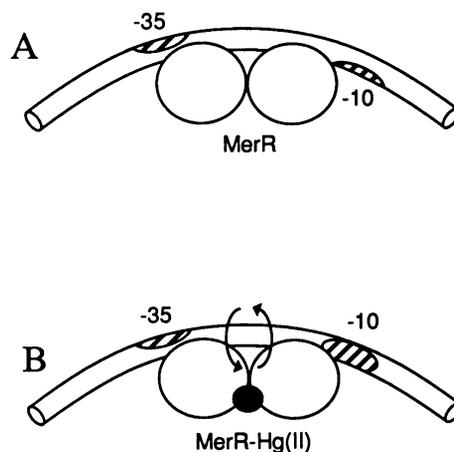
Fig. 6. Diagrammatic representation of the model for induction of the P_T promoter. The RNA polymerase, which is bound to the promoter at all times, is omitted for clarity. A. MerR binds to, and represses, the P_T promoter in which the -10 and -35 elements are sub-optimally aligned due to the 19 bp spacer. MerR induces a bend in the DNA which is unchanged in the presence or absence of mercury. B. MerR—Hg(II) induces a distortion in the spacer DNA which realigns the -35 and -10 elements, thus allowing RNA polymerase to form an open complex at the promoter.

mutants. The data also indicate that a base near the centre of the footprint (indicated by an arrow in Figure 4), protected by the wild type MerR only in the presence of mercury, is protected by the MerR-A-S protein in both the presence and absence of mercury, supporting the hypothesis that in the absence of mercury, the MerR-A-S protein is adopting the conformation of the wild type MerR—Hg(II) complex. Consistent with the transcriptional activation data, the DNase I protection profile of the MerR^{AC} mutants in the presence of RNA polymerase resembles the profile observed for the open complex induced by the Hg(II) bound wild type MerR. This protection profile is most striking in the case of the double mutant (Figure 4, compare lane 7 with lane 18) which in the absence of Hg(II) activates transcription almost to the Hg(II)-induced wild type levels (Figure 2A).

Hypersensitivity to the unmodified Cu-5-phenyl-*o*-phenanthroline nuclease appears to be associated with distortion of the B-DNA structure (Spassky and Sigman, 1985). When a 5-phenyl substituted *o*-phenanthroline complex was used, hypersensitivity was reported to occur at the centre of the *mer* operator on activation of the promoter by MerR (Frantz and O'Halloran, 1990). Cu-5-phenyl-*o*-phenanthroline cleavage was therefore an appropriate method to use in order to test the role of DNA distortion in the mechanism of transcriptional activation by the MerR^{AC} mutants. Complexes of each of the mutants with the *mer* operator exhibited varying degrees of Cu-5-phenyl-*o*-phenanthroline hypersensitivity in the absence of the effector Hg(II). The cleavage intensity increased in the presence of Hg(II) and, as with the wild type, was not dependent on the presence of RNA polymerase (data not shown). Within experimental error, the degree of cleavage (and therefore DNA distortion) effected by each mutant directly correlates with its ability to activate transcription at the P_T promoter. As this distortion is occuring within the spacer DNA of the P_T promoter, the correlation leads us to suggest a direct relationship between the distortion and the activation of the promoter.

These results are consistent with a model for activation in which distortion of the spacer DNA of the promoter alters the relative separation and orientation of the -10 and -35 elements of the promoter and allows them to be more effectively utilized by RNA polymerase, thus potentiating formation of the open complex (Figure 6). This model is also supported by genetic experiments that have shown that the length of the spacer DNA and the positioning of the -35, -10 and dyad symmetrical elements are vital for normal induction of the promoter by wild type MerR (Parkhill and Brown, 1990) and an *in vitro* biochemical analysis, which shows that Hg$-$MerR underwinds the centre of the operator by $\sim 33°$, thus improving the phasing of the -10 and -35 RNA polymerase binding motifs (Ansari *et al.*, 1992).

Although the analogous CRP mutants that activate transcription in the absence of cAMP are designated as CRP* mutants the analogy is limited. Unlike the CRP* mutants, which in the absence of the ligand (cAMP) require the presence of RNA polymerase to bind the lacP$^+$ promoter (Ren *et al.*, 1988), the MerR mutants described here bind DNA independently of the RNA polymerase. The MerR mutants also distort the DNA in the absence of RNA polymerase and Hg(II) and this distortion appears to be at the expense of DNA binding free energy. These results support the hypothesis that the mutant MerR proteins exist in a conformation that is achieved by Hg(II)-mediated allosteric modulation of the wild type protein. In keeping with the CRP nomenclature, we have designated these mutants as MerR^{AC} for allosteric control mutants.

Taken together, our experiments demonstrate that distortion of the spacer DNA is necessary for activation of transcription by MerR.

Materials and methods

Bacterial strains

Site-specific mutagenesis and single-strand DNA sequencing were carried out in the *E.coli* strains WK6 [Δ(*lac proAB*) *galE strA* F' *lacI*q ZΔM15 *proA*$^+$*B*$^+$] and WK6*mutS* (as WK6 but *mutS*::Tn*10*) (Stanssens *et al.*, 1989). β-galactosidase assays were carried out in a *recA* derivative of *E.coli* CSH26 (Miller, 1972; Lund *et al.*, 1986).

DNA manipulations

Restriction endonucleases were purchased from Amersham International plc, Boehringer Mannheim, Bethesda Research Laboratories or Northumbria Biologicals and used according to the manufacturers recommendations. Klenow fragment of DNA polymerase I and T4 polynucleotide kinase were purchased from Amersham International, bacterial alkaline phosphatase from Bethesda Research Laboratories. *Eco*RI was a kind gift of Dr S.E.Halford and T4 DNA ligase was a gift of Dr L.R.Evans. Ligations, transformations and plasmid preparations were according to Maniatis *et al.* (1982). DNA sequence analysis was by the chain termination method (Sanger *et al.*, 1977). Small scale plasmid DNA preparations were performed by a modification of the procedure of Ish-Horowicz and Burke (1981).

Site-directed mutagenesis

Site-directed mutagenesis was carried out according to the gapped-duplex procedure of Stanssens *et al.* (1989) using oligonucleotides manufactured by Alta Bioscience Ltd, University of Birmingham. The sequence of the oligonucleotides used were (where the mutagenic base is shown in lower case): A89V, TTGTGCTCGaCCAGACTGC; S131L, CCCTGTAGTaA-CGCGATCA.

Mutants were selected by direct sequencing of the relevant portion of the *merR* gene using appropriate oligonucleotide primers. The entire sequence of each of the mutant *merR* genes used was checked after the mutagenesis to ensure that there were no additional mutations present.

β-galactosidase assay

Assays were carried out as described by Miller (1972). *E.coli* CSH26Δ*recA* strains containing the plasmid(s) to be assayed were grown overnight in

M9 medium (Miller, 1972) supplemented with 0.2% glucose, 0.2% casamino acids and 20 μg/ml vitamin B1, in the presence of appropriate selective antibiotics. The cultures were then diluted 1:50 into fresh medium and grown with shaking at 37°C until the OD$_{600}$ was between 0.4 and 0.8. If the strains were to be induced with Hg(II), HgCl$_2$ was added to a final concentration of 1.8 μM 1 h before assaying the culture, this time being sufficient for full induction of the promoter. The copy number of the plasmids in the assayed cultures was checked by preparing DNA from the cultures at the same time as they were harvested for the β-galactosidase assays. The DNA samples were electrophoresed on an agarose gel, the amount being corrected for culture density and checked visually for gross copy number differences. None were apparent. All assays were carried out in triplicate.

Purification of mutant proteins

Cells JM109 containing the plasmids pTO90-16, pKKA89V, pKKS131L and pKKA-S were prepared as previously described (O'Halloran and Walsh, 1987). The cells were lysed by sonication at 4°C and the lysis pellet collected by centrifugation at 25 000 *g*. The overexpressed mutant proteins A89V, S131L and AS formed inclusion bodies, requiring modification of the purification protocol. The pellets were washed with lysis buffer (0.1 M Tris−HCl pH 8.0, 1 mM EDTA, 10 mM PMSF, 10% glycerol and 5 mM DTT) and lysis buffer with 1 M NaCl. The protein was extracted from the pellets with a urea-based buffer (4 M urea, 0.75 M NaCl, 0.1 M Tris−HCl pH 7.5 and 10 mM DTT). The urea was removed by dialysis against the same buffer without urea and the MerR protein was further purified by heparin−agarose column chromatography and FPLC using a mono-S ion exchange column (Wright, 1991).

Competitive Hg(II)-binding

Competitive Hg(II)-binding was measured by a spectroscopic titration method developed for the MerR system which exploits the competition of MerR and buffer thiols for Hg(II) (J.G.Wright and T.V.O'Halloran, manuscript in preparation). The purified protein was adjusted to a concentration of ~5 μM, with the precise concentration determined from the ϵ_{280} (Wright, 1991). Titrations were performed in a stirred, anaerobically sealed cuvette. Conditions for the reaction were 100 mM sodium phosphate, 0.5 M NaCl pH 7.0 25°C and 5 mM L-cysteine. The concentration of the competing L-cysteine was standardized by reaction with DTNB (Riddles *et al.*, 1983). Additions of Hg(II) were made via a gas-tight microsyringe. Details of the method and calculations are presented elsewhere (J.G.Wright and T.V.O'Halloran, manuscript in preparation).

Abortive transcription assay

An abortive transcription assay (McClure *et al.*, 1978) for Hg(II) responsive transcription was adapted from Ralston and O'Halloran (1990). A 0.4 nM solution of the *Bss*HII−*Bst*XI P$_T$ promoter-containing fragment from pTO40 (O'Halloran and Walsh, 1987) was incubated with 50 nM RNA polymerase (Pharmacia), 50 nM MerR and 10 μM HgCl$_2$ where necessary, in 10 mM Tris−Cl pH 8.0, 2 mM MgCl$_2$, 100 mM potassium glutamate, 100 μg/ml BSA, 5% (v/v) glycerol and 1 mM DTT. The reactions were incubated at 37°C for 45 min before adding ApU to 0.5 mM and 0.1 μCi of [α-^{32}P]CTP. The incubation was allowed to continue for 15 min and the products were then separated on a 15% acrylamide−50% (w/v) urea gel. The amount of labelled product, ApUpC*pC* was determined by measuring Cerenkov radiation in a scintillation counter.

DNA preparation and labelling

Non-template strand DNA was obtained by digesting plasmid pTO40 (O'Halloran and Walsh, 1987) with *Bst*BI and 3' end-labelled by filling in the recessed terminus using Klenow DNA polymerase with [α-^{32}P]dCTP and [α-^{32}P]dGTP, then recut with *Bst*NI to give a 256 bp fragment bearing the *mer* operator. Template strand DNA was obtained by digesting plasmid pAA1 with *Sma*I, was 5' labelled using T4 polynucleotide kinase with [γ-^{32}P]ATP, and was redigested with *Eco*RV to give a 134 bp fragment bearing the *mer* operator. These fragments were purified by acrylamide gel electrophoresis and elution; this DNA was used for the Cu-5-phenyl-*o*-phenanthroline reactions. Plasmid pAA1 (Ansari *et al.*, 1992) was digested with *Hind*III and 3'-end labelled with Klenow polymerase, then recut with *Eco*RV to yield a 172 bp fragment that was used for gel retardation experiments. For DNaseI protection assays DNA from the plasmid pRZH18 (Lund *et al.*, 1986) was digested with *Eco*RI, 3' labelled as described above and then redigested with *Hind*III before purification of the 200 bp operator-containing fragment.

Gel retardation assay

Varying concentrations of protein were incubated with the *mer* operator-bearing *Hind*III−*Eco*RV fragment of pAA1; the operator concentration was

held constant at 5×10^{-12} M. Protein−DNA complexes were formed in 20 μl of incubation buffer (10 mM Tris−HCl pH 8, 100 mM potassium glutamate, 0.1 mM EDTA, 1 mM $CaCl_2$, 5% (v/v) glycerol, 1 mM DTT and 100 μg/ml BSA) for 1 h at 10°C. The final concentration of $HgCl_2$ was 0.75 μM in the indicated samples. The equilibrated complexes were resolved on 9% (74:1) native polyacrylamide gels which were prerun at 200 V for at least 45 min in a cold room. The samples were loaded on the gels whilst the gels were electrophoresing at 200 V and run for 45 min. The gels were maintained at 12°C ± 3°C during the run.

The gels were imaged using a Molecular Dynamics phosphorimager and analysed with the Imagequant software. The fraction of radioactivity in the band that represents specific protein−DNA complexes was plotted against concentration of the respective proteins on a semi-logarithmic graph (Figure 3). The apparent K_D in such a plot is the concentration of the protein at half-maximal binding.

In determining the equilibrium binding constants we have used protein concentrations corrected for the fraction of protein that is active. This was determined by the method described by Fried and Crothers (1981). In the protein preparations used, A89V was 95% active, S131L was 30% active, AS was 66% active and the wild type MerR was 95% active.

Nuclease protection assays

The DNase I protection assay was performed as described in O'Halloran et al. (1989). The Cu-5-phenyl-o-phenanthroline protection assay was performed as described in Frantz and O'Halloran (1990).

Acknowledgements

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