Interactions between the *Escherichia coli* MelR transcription activator protein and operator sequences at the *melAB* promoter

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The *Escherichia coli* MelR protein binds to two sites at the *melAB* promoter and activates transcription in response to melibiose. The binding of purified MelR to the *melAB* promoter and to a number of derivatives carrying point mutations has been studied. A *melAB* promoter fragment that is very weakly activated by the *melR* gene product has been made by creating multiple symmetrical changes in both MelR-binding sites. A library of random substitutions in MelR was screened to find

mutants that were able to activate this mutant promoter. One of these mutants contained a substitution in a proposed helix-turn-helix region that most likely relaxes the sequence-specificity of MelR binding. The other mutants contain substitutions throughout the central part of MelR and appear to alter the conformation of MelR, such that activity is triggered at lower melibiose concentrations and by arabinose.

INTRODUCTION

The *Escherichia coli* MelR protein is a transcription activator essential for expression of the *melAB* operon, that encodes the proteins for melibiose metabolism and transport (Webster et al., 1987). MelR activity can be triggered by the inclusion of melibiose in the growth medium; the role of MelR is to activate transcription initiation at the *melAB* promoter (*pmelAB*) in response to melibiose or, possibly, some other metabolite. We previously described the overexpression and purification of MelR, and showed that MelR binds independently to two apparently identical 18 bp sites located at *pmelAB*, upstream of the transcription start site (Caswell et al., 1992a,b). Mutational analysis at the upstream site (Site 1) suggested that a guanine at position –104 with respect to the transcription start might be crucial for MelR binding.

In this work we have investigated MelR binding to both target sites at *pmelAB*, and examined the effects of point mutations at and around both position –104 and the corresponding position in the second MelR-binding site. Our aim was to find point mutations in the MelR-binding sites that stopped MelR binding and prevented MelR-dependent transcription activation, with the goal of exploiting these mutations to set up a selection for substitutions in MelR that compensated for the changes in the binding site sequence. Our hope was that these substitutions would identify segments of MelR directly involved in operator recognition. Here we report that most of the changes we studied had but small effects on MelR-dependent activation of *pmelAB*. However, it did prove possible to find a weakly activated *pmelAB* derivative, by combining mutations at both MelR-binding sites, and we were able to screen for *melR* alleles that were active at the defective *pmelAB*. We describe the unexpectedly diverse properties of the different *melR* mutations identified by our screen.

recombinant DNA were used as previously (Caswell et al., 1992a,b).

The starting *pmelAB* material was the KK33 *EcoRI*–*HindIII* fragment carrying *pmelAB* sequence from –136 to +21 with respect to the *melAB* transcript start (we used the convention of denoting upstream locations with a ‘–’ or ‘p’ and downstream locations with a ‘+’ prefix). In this study we used the KK43 fragment derived from KK33 by a GC-to-AT transversion at –73 (the p73A mutation) that creates a unique *BglII* site between MelR-binding Sites 1 and 2. This transversion was made by oligo-directed site-directed mutagenesis using the Amersham mutagenesis kit (cat. no. RPN1523). The KK43 promoter fragment was cloned into the vector pAA121 (Table 1) and the resulting plasmid was used as starting material for further mutagenesis. Point mutations in MelR-binding Site 2 were created by PCR mutagenesis exploiting the *BglII* site just upstream of Site 2. Point mutations in MelR-binding Site 1 were made by site-directed mutagenesis using the Amersham kit. Mutations in Site 1 and Site 2 were combined by exploiting the unique *BglII* cleavage locus between the two MelR-binding sites.

The starting plasmid carrying *melR* was pJW15. The starting point for this plasmid was the KK20D59 *BamHI*–*HindIII* fragment carrying *melR*, the *melR* promoter and flanking sequences, described in Figure 5 of Webster et al. (1988). An *EcoRI* linker was added at the *BamHI* end of this fragment and the resulting *EcoRI*–*HindIII* fragment was cloned into pAA121 to give pJW15. A library of random mutations in *melR* (cloned in pJW15) was created, using the PCR method described by Zhou et al. (1991) that exploits the high error rate of *Taq* DNA polymerase. pJW15 derivatives carrying mutations in *melR* were characterized by sequencing using a set of primers to cover the entire gene.

Assays of *pmelAB* activity in vivo

To assay the activity of *pmelAB* and derivatives, *EcoRI*–*HindIII* KK43 fragments were cloned into the broad-host-range low-copy-number *lac* expression vector pRW50, such that the *lac* genes were placed under the control of *pmelAB*. Resulting

EXPERIMENTAL

Strains, plasmids and gene manipulation

The bacterial strains, plasmids and DNA fragments used in this work are listed in Table 1. Standard methods for manipulating

Abbreviation used: *pmelAB*, *Escherichia coli* *melAB* promoter.

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Table 1 Bacterial strains, plasmids and promoter fragments used

Name	Brief description	Origin and references
Bacterial strains		
M182	$\Delta lac mel$ <i>E. coli</i> K12 strain	Casadaban and Cohen (1980)
pop2094	$\Delta lac mel^+$ <i>E. coli</i> K12 strain	Gaston et al. (1988)
Bacterial plasmids		
pAA121	pBR322-derived vector for cloning <i>EcoRI</i> – <i>HindIII</i> fragments	Kelsall et al. (1985)
pRW50	Broad-host-range <i>lac</i> expression vector for cloning <i>EcoRI</i> – <i>HindIII</i> fragments	Lodge et al. (1992)
pJW15	<i>EcoRI</i> – <i>HindIII</i> fragment carrying <i>melR</i> and <i>melR</i> promoter cloned in pAA121	This work
<i>pmelAB</i> promoter fragments (all <i>EcoRI</i> – <i>HindIII</i> fragments)		
KK33	Fragment carrying <i>pmelAB</i> sequences from –136 to +21 with an <i>EcoRI</i> linker upstream and a <i>HindIII</i> linker downstream	Caswell et al. (1992b)
KK43	Derivative of KK33 carrying GC-to-AT transversion at –73 creating a unique <i>BglII</i> site between Sites 1 and 2	This work
KK431	Derivative of KK43 carrying four mutations in Site 1 (p104A, p105T, p106G, p107C)	This work
KK432	Derivative of KK43 carrying four mutations in Site 2 (p56G, p57C, p58A, p59T)	This work
KK433	Derivative of KK43 carrying four mutations in Site 1 (p104A, p105T, p106G, p107C) and four mutations in Site 2 (p56G, p57C, p58A, p59T)	This work

plasmids were transformed into the *mel lac* strain M182. To supply wild-type or mutant MelR, the transformants were further transformed with pJW15 or derivatives (note that pJW15 and pRW50 are compatible since they carry different replication origins and encode resistance to different antibiotics). Melibiose-induced β -galactosidase expression in cells carrying KK43 cloned in pRW50 and pJW15 was measured exactly as described by Caswell et al. (1992a).

To assay *pmelAB* activity in the presence of a single copy of the *melR* gene, pRW50 derivatives carrying *EcoRI*–*HindIII* *pmelAB* fragments were transformed into the *mel⁺* strain, pop2094, and melibiose-induced β -galactosidase expression was measured. β -Galactosidase activities were measured in Miller units (Miller, 1972) and were taken to be proportional to *pmelAB* activity.

Gel retardation and footprinting protocols

In vitro studies of MelR binding to *EcoRI*–*HindIII* KK43 fragments were performed using MelR purified as described by Caswell et al. (1992a). Gel binding assays were performed using fragments end-labelled with [γ -³²P]ATP, exactly as described in Caswell et al. (1992b). Protein concentrations were estimated by the Bradford (1976) assay to determine MelR concentrations. In order to determine the percentage of active MelR in our preparations, the KK43 *EcoRI*–*HindIII* fragment was cleaved with *BglII* to generate fragments carrying just one MelR-binding site. Gel-binding assays were used to determine the amount of MelR required to band-shift 2 pmol of fragment containing binding Site 1 in 10 μ l of buffer (i.e. at 200 nM, a concentration greater than 10 times the estimated association constant). According to these assays, our preparations of MelR were 70–80% active in binding to the operator, and this was taken into account in calculating binding strengths for the different sites.

DNAase I footprinting was performed exactly as in Caswell et al. (1992b) using the KK43 *EcoRI*–*HindIII* fragment labelled with [γ -³²P]ATP at the *EcoRI* end. The footprinting gels were calibrated with Maxam–Gilbert G reactions of the labelled fragments. Autoradiograms of footprinting and gel binding experiments were scanned using a Pharmacia LKB Ultrascan XL laser densitometer and these scans were used to estimate the concentration of active MelR required for 50% occupation of particular sites.

RESULTS AND DISCUSSION

MelR binds more tightly to Site 1 than to Site 2

In this work we have used the KK43 *melAB* promoter fragment illustrated in Figure 1. The fragment carries two MelR-binding sites upstream of the *melAB* transcription start and –10 hexamer. The KK43 fragment was derived from KK33 by the introduction of a *BglII* site just upstream of Site 2; this has no measurable effect on *pmelAB* activity or MelR binding (results not shown). Gel binding assays and methylation interference experiments performed by Caswell et al. (1992b) suggested that MelR binding to the upstream Site 1 is tighter than to Site 2. To show this directly we have determined the DNAase footprint of the KK43 fragment in the presence of increasing concentrations of purified MelR. Results such as those shown in Figure 2(a) confirm that Site 1 is occupied before Site 2. From scans of our data, we conclude that, under our conditions, the concentration of MelR necessary for 50% protection of bands in Site 1 is 8 ± 3 nM, while 50% protection of Site 2 occurs at 30 ± 10 nM. As a control, the experiment was repeated with the KK431 fragment, a KK43 derivative containing four point mutations in Site 1 (see Table 1 and below). The results in Figure 2(b) show that the point mutations had no effect on MelR binding to Site 2, whereas they weakened binding to Site 1 (50% protection of bands in Site 1 at 150 ± 30 nM MelR).

Effects of point mutations in MelR-binding Site 2

In order to identify important bases for MelR binding, Caswell et al. (1992b) created a set of random point mutations in MelR-binding Site 1. Surprisingly, most of the mutations had very little effect on *pmelAB* activity. However, transversion of the CG base pair at –104 led to a moderate reduction in MelR-dependent activation, suggesting that this base pair might be directly or indirectly involved in MelR binding. Since the nucleotide sequences at Sites 1 and 2 are identical, we created changes at the corresponding position in Site 2, at position –59. The p59T, p59C and p59A mutations were created by site-directed mutagenesis on the KK43 fragment, and the resulting *pmelAB* derivatives were cloned into the *lac* expression vector pRW50. To evaluate the effects of the mutations, pRW50 derivatives were transferred into the *lac mel* strain M182, *melR* was introduced by adding the

Figure 1 Schematic representation of the KK43 *pmelAB* fragment

The figure illustrates the *EcoRI*–*HindIII* fragment used in this work. The location of MelR binding Sites 1 and 2, the –10 hexamer, the transcription start and the *Bgl*II site are highlighted. The base sequence of the 'top' strand around the MelR-binding sites is shown, together with the nature of the substitutions in the KK431, KK432 and KK433 derivatives. The asterisk denotes the substitution that created the *Bgl*II site.

Figure 2 DNAase footprints of MelR binding at *pmelAB*

The figure shows DNAase footprints of MelR binding to end-labelled KK43 (a) or KK431 (b) fragments. Lane 1 in each panel shows a G sequencing reaction of the fragment. Different concentrations of MelR were incubated with 1 nM labelled fragment prior to DNAase treatment, as follows: no MelR, lane 2; 2.5 nM MelR, lane 3; 10 nM MelR, lane 4; 40 nM MelR, lane 5; 160 nM MelR, lane 6; 640 nM MelR, lane 7. The locations of the footprints corresponding to the occupation of Sites 1 and 2 are shown.

multi-copy plasmid pJW15, and melibiose-induced *lac* expression was measured. To measure the effects of the Site 2 mutations in a strain with a single chromosomal copy of *melR*, we used the *mel*⁺ strain pop2094, and again measured melibiose-induced *lac* expression. The results in Table 2 show that, with multi-copy

melR, the p59T mutation reduced expression to 60 % of the level found with the wild-type *melAB* promoter. With single-copy *melR*, melibiose-induced expression was reduced further, to 15 % of the level with the wild-type promoter. The p59C and p59A mutations led to minimal reductions in *pmelAB* activity with multi-copy *melR*, but caused some reductions with single-copy *melR* in pop2094. Since mutations at the same position in Site 1 (–104) caused similar small effects, and the combination of the p59T and p104A changes failed to suppress MelR-dependent expression from *pmelAB* (Table 2), we conclude that the GC base pairs at positions –59 and –104 are not essential. Presumably the small effects of mutations at –59 and –104 are indirect. For this reason, we created a number of different changes at the neighbouring positions –58, –57 and –56, and measured their effects on *pmelAB* expression in the presence of multi-copy or single-copy *melR*. The results in Table 2 show that, again, these changes led to but moderate reductions in *pmelAB* activity, but that the effects were greater in pop2094 with single-copy *melR*.

Effects of multiple mutations in Site 1 and Site 2

During the mutagenesis of the KK43 fragment to make point mutations in Site 2, we isolated by chance a derivative carrying four changes, p56G p57C p58A p59T, at consecutive bases. MelR-dependent expression from this *pmelAB* derivative (which we denote as KK432) cloned in pRW50 was reduced to 25 % of the wild-type level with multi-copy *melR* and to 8 % with single-copy *melR*, suggesting that the multiple mutations had reduced MelR binding to a greater extent than any previously characterized mutation. The same mutations were then created in Site 1 (Figure 1) to give the KK431 derivative, and the two sets of four mutations were combined to give the KK433 derivative. The effects of the four mutations in Site 1 (KK431; Table 2) were significantly less than their effects in Site 2, although, in both cases, the reduction in activity was greater in pop2094 with single-copy *MelR*. When combined (in KK433) the mutations resulted in very low activity with both single- and multi-copy *melR*.

To quantify the combined effects of the four base changes on MelR binding, the KK43, KK431, KK432 and KK433 *EcoRI*–*HindIII* fragments were purified and labelled and the binding of

Table 2 MelR-dependent activation of *pmelAB* carrying different point mutations in MelR-binding Sites 1 and 2

The table lists the β -galactosidase levels in cells containing the KK43 *pmelAB* promoter fragment or different derivatives cloned in pRW50. Assays were performed in strain M182 carrying pJW15 as a source of *melR* or in strain pop2094 carrying chromosomal *melR*. Cells were grown in minimal medium containing fructose as a carbon source plus 1 mM melibiose for activation of MelR (Caswell et al., 1992b). β -Galactosidase levels were assayed by the standard Miller (1972) protocol and are expressed as percentages of the activity found with full melibiose/MelR-dependent induction of the wild-type (wt) *pmelAB* sequence on the KK43 fragment; 100% activity corresponds to 1500 Miller units in M182 (pJW15) and 800 Miller units in pop2094. Expression levels in the absence of melibiose were 2–3% of the full melibiose-induced activity. Each assay was performed independently three or more times; the values shown are the averages of these determinations, which vary by less than 10%.

Promoter/mutant cloned in pRW50	Melibiose-dependent β -galactosidase expression in:	
	M182 carrying pJW15 (multi-copy <i>melR</i>)	pop2094 (single-copy <i>melR</i>)
No promoter	2	2
KK43 (wt <i>pmelAB</i>)	100	100
p59T	60	15
p59C	90	35
p59A	95	70
p104T	100	90
p104A	90	40
p59T, p104A	55	10
p58T	80	30
p58C	70	30
p58A	90	75
p56C	95	60
p56T, p57T	75	40
p104A, p105T, p106G, p107C (KK431)	90	25
p56G, p57C, p58A, p59T (KK432)	25	8
p104A, p105T, p106G, p107C, p56G, p57C, p58A, p59T (KK433)	8	5

Figure 3 Band shift assays to monitor MelR binding

The figure shows band shifts due to MelR binding to ^{32} P-labelled *EcoRI*–*HindIII* KK43 (a), KK432 (b), KK433 (c) and KK431 (d) fragments. The MelR concentrations were as follows: no MelR, lane 1; 1 nM, lane 2; 10 nM, lane 3; 120 nM, lane 4. The positions of migration of bands due to the complex with MelR at Site 1 (1) and the unbound fragment (f) are indicated.

purified MelR was measured by gel binding assays. Figure 3 shows gel binding analyses of the binding of purified MelR to the four labelled fragments. We previously showed that MelR binding to the KK43 fragment first results in a retarded band corresponding to MelR binding to Site 1. Site 2 is filled at higher concentrations of MelR, resulting in a number of more retarded bands which are difficult to resolve (Caswell et al., 1992b; and Figure 3a). With the KK432 fragment, carrying four mutations in Site 2, just the retarded band corresponding to occupation of Site 1 appears, and more retarded bands do not form at the higher MelR concentration (Figure 3b). The simplest explanation for this is that the four point mutations in KK432 suppress MelR binding to Site 2. On this assumption, it is possible to calculate the MelR concentration at which Site 1 is 50% retarded as 8 ± 4 nM, which is in good agreement with data from footprinting and our previous work with DNA fragments carrying just MelR-

binding Site 1 (Caswell et al., 1992b). Figure 3(c) shows data from the experiment with KK433, carrying point mutations in both Sites 1 and 2. In this case, again, only the Site 1 complex is seen, but binding to Site 1 is weakened. Titrations show that the MelR concentration required for 50% binding under our conditions is 180 ± 30 nM. Figure 3(d) shows the experiment with KK431, carrying point mutations in just Site 1. In this case, bands corresponding to multiply bound MelR are observed and the data are consistent with normal MelR binding to Site 2 and reduced binding to Site 1. This corresponds to the situation in the footprint experiment in Figure 1(b), where MelR binds more tightly to Site 2 than to Site 1.

From the band shift and footprint experiments, we conclude that the four base changes in KK431 and KK432 weaken MelR binding to Sites 1 and 2 respectively. A summary of deduced binding affinity data is given in Table 3. The changes in Site 1

Table 3 Binding of MelR to Sites 1 and 2

The table shows the concentration of MelR at which 50% binding takes place to wild-type and mutant Sites 1 and 2, as judged by band shift and footprinting assays as detailed in the footnotes. ND, not detectable.

Site	MelR concn. for 50% binding (nM)	
	Wild-type operator (KK43)	Mutant operator (KK433)
1	8 ± 4*	150 ± 40†
2	30 ± 10‡	ND

* Derived from band shift assays with the KK432 fragment, band shift assays with the KK43 fragment cleaved with *Bgl*II and footprint assays with KK43. Each assay was performed twice using a range of MelR concentrations and the empirical concentration of MelR required for 50% occupancy of the site was deduced. The results were averaged and the range of results is shown. Curve fitting and statistical analysis was not performed.

† Derived from band shift assays with the KK433 fragment, band shift assays with the KK433 fragment cleaved with *Bgl*II and footprint assays with KK431.

‡ Derived from footprint assays with the KK43 and KK431 fragments.

weaken MelR binding by 15–25-fold, but MelR binding to mutant Site 1 can be clearly detected. In the case of Site 2, MelR binding to the wild-type site is weaker than for Site 1; MelR binding to mutant Site 2 cannot be detected and is thus impossible to quantify.

Substitutions in MelR that suppress mutations in *pmelAB*

Current evidence suggests that the DNA-binding determinants of MelR are located in the C-terminal domain (Caswell et al.,

1992a). Although this domain contains two potential helix-turn-helix DNA-binding motifs, there is very little direct evidence that these play a role. Since the combination of point mutations in Sites 1 and 2 in the KK433 *pmelAB* fragment suppressed MelR-dependent transcription activation (Table 2), we reasoned that it might be informative to select substitutions in MelR that could suppress the effects of these mutations. This approach has previously been used in other systems to identify residues that make specific contacts with the mutated bases (Ebright, 1991).

The *pmelAB* *Eco*RI–*Hind*III KK43 and KK433 fragments were cloned into the broad-host-range *lac* expression vector pRW50 and transformed into the *lac mel* host strain, M182, carrying *melR* cloned on pJW15. Cells carrying KK43 score as Lac⁺ on MacConkey indicator plates, due to MelR-dependent activation of *pmelAB*. With KK433, cells score as Lac[–] because MelR binding and *pmelAB* activation are reduced. PCR was used to target random mutations into *melR* cloned in pJW15, and the resulting DNA was transformed into M182 cells carrying KK433 cloned in pRW50. Lac⁺ colonies were selected and checked for pJW15 derivatives carrying mutations that permit MelR-dependent expression from *pmelAB* on the KK433 fragment. After screening 10 000 colonies, we purified six independent pJW15 derivatives carrying simple substitutions in MelR that resulted in detectable activation of mutant *pmelAB*. In each case the sequence of the entire *melR* gene was determined, and this revealed that the mutant MelR carried a single amino acid substitution. Figure 4 shows a schematic representation of MelR with the location of the six substitutions, GS119, KE123, QR128, ST167, KR182 and KN218. We had anticipated that the location of the substitutions might identify the segment of MelR involved in recognition of the bases mutated in KK433. However, disappointingly, the substitutions are located throughout the

Figure 4 Location of suppressor mutations in MelR

The figure represents the 302-amino-acid sequence of MelR. The arrows show the positions of the six suppressor substitutions discussed in the text. The different shadings denote the N-terminal and C-terminal domains as defined by Gallegos et al. (1993). The two sequences likely to form helix-turn-helix structures in the C-terminal domain are indicated (hth 1 and 2).

Figure 5 Activation of wild-type and mutant *pmelAB* by wild-type MelR and mutant derivatives

The bars represent measurements of β -galactosidase expression in M182 cells carrying pRW50 containing the mutant KK433 *pmelAB* promoter (a) or the wild-type KK43 promoter (b). The cells also carried pJW15 encoding wild-type MelR (wt) or the different mutants indicated on the x-axis. Cells were grown in the absence (□) or presence (■) of melibiose in the growth media. β -Galactosidase activities were normalized, with 100% taken as the activity of the wild-type KK43 *melAB* promoter with wild-type MelR and melibiose present.

central portion of MelR and do not map to one particular region.

The activity of the different mutant *melR* derivatives at both the mutant and wild-type *pmelAB* fragments, KK433 and KK43, cloned in pRW50, were measured. The results in Figure 5(a) show that the six different substitutions in MelR increased expression from the mutant KK433 promoter 3–4-fold, to give 20–30 % of the expression level found with wild-type MelR at the wild-type *pmelAB* promoter on the KK43 fragment. In each case, MelR-dependent expression was induced by the inclusion of melibiose in the growth medium.

Figure 5(b) shows that all six mutant MelR derivatives could also activate the wild-type *pmelAB* promoter. In each case, activation was triggered by the addition of melibiose to the growth medium, and activation levels were significantly higher than those with wild-type MelR. This shows that we had failed to identify MelR mutants with improved specificity for the changed KK433 MelR-binding sequences; mutants with specificity for the KK433 sequence would have given lower levels of activation with the wild-type *melAB* promoter. Rather, the substitutions that we isolated appeared to result in a general increase in melibiose-dependent activity. To eliminate the possibility that the mutations resulted in increased levels of MelR expression, we assayed the MelR levels in crude extracts from M182 cells carrying pJW15 with the different mutations. To determine the relative levels of MelR protein, we estimated the amount of extract required to totally retard labelled *EcoRI*–*HindIII* KK43 fragment in gel binding assays [in these experiments, we used labelled fragments at concentrations higher than the estimated association constants in order to titrate MelR protein in the extracts independently of small differences in binding; for protocols see Webster et al. (1989) and the Experimental section]. From these experiments we concluded that none of the six substitutions led to changes in MelR levels of greater than 10 % (results not shown), suggesting that none of the changes altered the stability or expression of the protein.

Two new classes of MelR mutants

The six new MelR substitutions shown in Figure 4 pose a problem. We had hoped that their location would identify the region involved in the recognition of base sequence around positions –104 and –59 at *pmelAB*. Clearly, the KN218 substitution might signal a role for helix-turn-helix 1, but the disparate nature of the other substitutions was unexpected. We considered that some of the mutations might be causing a subtle change in MelR that shifts its conformation towards the active form. Since the activity of MelR *in vivo* can be triggered by melibiose, we measured the concentration-dependence of melibiose activation for the different MelR mutants. To do this, M182 cells carrying the KK43 *pmelAB* fragment cloned in pRW50, and pJW15 carrying the different *melR* derivatives, were grown in media containing different concentrations of melibiose. The melibiose concentrations required for 50 % induction were measured and are listed in Table 4. While the activity of wild-type MelR was triggered at 30 μ M melibiose, the melibiose requirement for five of the six mutants (GS119, KE123, QR128, ST167 and KR182) was lowered by 2–10-fold. In the sixth case, KN218, the melibiose requirement was increased to 60 nM. This result suggests that five out of six of the MelR substitutions result in a protein that has a higher affinity for melibiose, presumably because the conformation is altered in some subtle way.

In previous work we had noted that MelR-dependent induction of *pmelAB* *in vivo* is dependent on melibiose, but that weak

Table 4 Melibiose-dependence of activation by wild-type and mutant MelR

The Table shows data obtained with M182 cells carrying *pmelAB* on the KK43 fragment cloned in pRW50 and pJW15 carrying wild-type *melR* or mutant derivatives as listed. Cells were grown in minimal media with fructose as the carbon source (Caswell et al., 1992b) plus different concentrations of melibiose. β -Galactosidase activities were measured and the concentration of melibiose required for half-maximal induction of *pmelAB* was deduced empirically.

MelR derivative	Melibiose concn. required for 50% induction of <i>pmelAB</i> (μ M)
Wild-type (in pJW15)	30
GS119	8
KE123	12
QR128	3
ST167	6
KR182	6
KN218	60

Figure 6 Activation of MelR by arabinose

The figure illustrates the activation of *pmelAB* as a function of arabinose (●, ▲, ■) or melibiose (○) concentration. Activation was judged by β -galactosidase expression in M182 cells carrying pRW50 with the KK43 *EcoRI*–*HindIII* fragment. As a source of *melR* the cells also contained pJW15 encoding wild-type *melR* (●, ○), the KR182 substitution (■) or the KN218 substitution (▲). Activities were normalized, with 100 % taken as the normal activity with wild-type MelR plus melibiose.

induction is possible with high concentrations of some other sugars (C. Webster and J. Williams, unpublished work). For example, the inclusion of 25 mM arabinose resulted in 10 % induction of *pmelAB* in the presence of wild-type *melR*. Similar assays showed that the GS119, KE123, QR128, ST167 and KR182 substitutions resulted in an increase in this arabinose-dependent activation. An example of the data is illustrated in Figure 6, which shows a plot of *pmelAB* expression as a function of increasing arabinose concentration. Arabinose-dependent induction with wild-type MelR was weak but was improved several-fold with the KR182 substitution. Interestingly, the KN218 substitution in helix-turn-helix 1 had little effect on arabinose-

dependent induction. On the basis of these results, we suggest that the GS119, KE123, QR128, ST167 and KR182 substitutions cause a change in MelR that alters its inducibility with different sugars, whereas the KN218 change is likely to alter DNA-binding preferences.

Conclusions

MelR is a member of the AraC/XylS family of transcription regulators, a group of at least 27 proteins that, with two exceptions, are all transcription activators. Most members of this family appear to consist of two domains, with the C-terminal segment involved in DNA binding and the N-terminal segment responsible for signal-induced triggering (Gallegos et al., 1993). In most cases these proteins bind to tandem copies of 16–18 bp operators and activity is triggered by small ligands. Since there is no structural information on any family member, our understanding of how the operator sequences are recognized and how activity is triggered is sketchy. In the case of *pmelAB*, transcription is dependent on MelR, which binds at two identical 18 bp sequences, organized as an inverted repeat separated by 20 bp (Figure 1).

The first aim of the present work was to investigate the sequence requirements for MelR binding by finding mutations that suppressed MelR binding. This has proved difficult and, to date, we have failed to find simple single mutations that suppress binding; a possible reason for this could be the extended nature of MelR-binding sites. Our results (Table 2) show that most changes have a larger effect in the context of the downstream Site 2 than in the context of Site 1. The likely explanation for this is that MelR binding to Site 1 is tighter than to Site 2, and substantial residual MelR binding is possible to mutated versions of Site 1. At first view this is surprising, since both Sites 1 and 2 have identical sequences. However, it is likely that flanking sequences contribute to binding stability, and Caswell et al. (1992b) argued that MelR binding to Site 1 but not Site 2 can be stabilized by wrapping of bendable upstream sequences. A further complication in the analysis of binding site mutations comes from the observation (Table 2) that the effects of mutations are dependent on the *melR* copy number. This is probably because, in some cases, the product of a single copy of *melR* is insufficient to saturate the two operators, presumably because expression of *melR* is not autoregulated (Webster et al., 1988). From the data in Tables 2 and 3 we can estimate that the MelR concentration *in vivo* must vary in the range 10–100 nM.

A second major aim of this work was to exploit mutations that suppressed MelR binding, to provide a tool for selecting substitutions in MelR that gave altered DNA binding preferences. Because simple mutations in the MelR operators gave only small effects, we were obliged to use symmetrically multiply mutated derivatives of *pmelAB* to select for suppressors in MelR. Previous

attempts with this type of approach have resulted in DNA-binding proteins with relaxed, rather than new, sequence specificity (Ebright, 1991). However, of the six mutations that we characterized, only one, KN218 in helix-turn-helix 1, appears to directly affect operator binding. The simplest explanation for this is that helix-turn-helix 1 is responsible for interactions with the –59/–104 parts of the MelR operator, and that the KN218 substitution partially relieves specificity for the wild-type sequence in this region. However, it is important to realise that, to date, we have no direct evidence for this; other explanations are possible, and definitive proof will require *in vitro* studies with purified MelR carrying the KN218 substitution.

The most surprising result from this work was the identification of a set of substitutions at disparate positions in the central portion of MelR that increase the sensitivity of MelR to activation by melibiose and arabinose. The simplest explanation for the effects of these changes is that they alter the conformation of MelR to bind more tightly to melibiose and to relax the specificity of inducer binding. To explain the variety of locations of these substitutions, we suppose that some kind of conformational restraint is being removed from MelR. Interestingly, Ramos et al. (1990) found that diverse substitutions in the central zone of XylS altered the range of benzoate derivatives that could trigger XylS-dependent transcription activation. Surprisingly, none of our mutant MelR derivatives had lost the requirement for melibiose to trigger induction. Triggering of MelR activity must therefore be a complex multi-step process. Further progress will require more sophisticated genetic analysis, the development of an *in vitro* system to study activation and, ultimately, structural data with information on the cofactor binding site.

This work was funded by the U.K. Science and Engineering Research Council with Project Grant GRF 75322. C.M. was supported by the Spanish Ministry of Education, and S.B. is a Royal Society EPA Fund Research Fellow.

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