Mutations That Increase the Activity of the Promoter of the Escherichia coli Melibiose Operon Improve the Binding of MelR, a Transcription Activator Triggered by Melibiose*

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MelR is an Escherichia coli transcription factor that activates expression of the melAB operon in response to the presence of melibiose in the environment. MelR stimulates transcription initiation at the melAB promoter by binding to four sites centered at positions −120.5, −100.5, −62.5, and −42.5 upstream of the transcript start point. In a previous study, we described a spontaneous mutant that exhibited increased melAB expression. Sequence analysis showed that this mutant carries five consecutive base changes at positions −49, −50, −51, −52, and −53 upstream of the melAB transcript start point. Here we show that these changes improve MelR binding to the target site centered at position −42.5 at the melAB promoter and that this improvement is responsible for increased promoter activity. Thus, the activity of the melAB promoter is fixed by the occupation by MelR of a DNA site that overlaps the −35 hexamer: MelR appears to be a typical class II-type transcription activator.

Melibiose metabolism by Escherichia coli is contingent on expression of the melA and melB genes, that are cotranscribed from a single promoter in response to the presence of melibiose in the growth medium: melA encodes an α-galactosidase, whereas melB encodes a melibiose transporter (1–5). An unusual feature of E. coli K12 strains is that they are able to metabolize melibiose at 30 °C, but they become progressively less able to metabolize melibiose at temperatures above 37 °C, due to thermal lability of the melAB melibiose transporter (6). In recent work (7), we have studied the W3133-2 mutant that is able to grow at higher temperatures with melibiose as the sole carbon source. Although we had expected that the melB gene in this mutant would be altered in some way, we found that the W3133-2 mutant carried five consecutive base changes in the melAB promoter. We showed that these changes led to an increase in expression of the melAB operon at all temperatures and that this increase, apparently, could compensate for the thermolability of the melB melibiose transporter (7). The principal objective of the work presented here has been to understand the effects of the base changes in the W3133-2 mutant.

Transcription initiation at the melAB promoter is totally dependent on MelR, a melibiose-triggered transcription activator (8). MelR is encoded by the melR gene, which is located immediately upstream of the melA gene, and transcribed from a promoter that is divergent from the melAB promoter (the transcription start points of the melAB and melR promoters are 237 bases apart; see Fig. 1) (9). MelR is a member of the AraC family of transcription factors and binds to 18-base pair target sequences (10). At the melAB promoter, MelR binds to four such target sequences: site 1, site 1, site 2, and site 2′ centered at positions −120.5, −100.5, −62.5, and −42.5, respectively, upstream of the melAB transcript start point (Fig. 1). Recent studies (11) have shown that the occupation of site 2′ by MelR is the most crucial for the activation of the melAB promoter, but that, of the four target sites, site 2′ binds MelR most weakly. We noted that the base changes in the melAB promoter in the W3133-2 mutant changed the DNA sequence of MelR binding site 2′ such that it corresponded better to the tighter binding site 1′, site 1, and site 2. This suggested that the increased melAB expression in the W3133-2 mutant could be due to better binding of MelR to site 2′. In the work reported here, we prove that this is indeed the case, by studying the binding of purified MelR and purified RNA polymerase holoenzyme (RNAP)1 to DNA fragments carrying the melAB promoter from both the wild type and W3133-2 mutant strain.

EXPERIMENTAL PROCEDURES

Subcloning of the mel Operon Regulatory Region—Plasmids pBM3133, carrying the entire wild type mel operon, and pBM3133-2, carrying the mel operon from the W3133-2 mutant, were described in our previous study (7). EcoRI-HindIII fragments, carrying the wild type or mutant mel operon regulatory region, were derived using polymerase chain reaction (PCR) using pBM3133 or pBM3133-2 as templates. The KK51 fragment, carrying an EcoRI site at position −312 with respect to the melAB transcript start, and a HindIII site at +36 was generated using the primers 5′-GGCTTTCATTGGGCTGAC-3′ and 5′-GGTGATGACGTCAATATAT-3′ (see Fig. 1). The shorter JK14 fragment, with an EcoRI site at position −94 with respect to the melAB transcript start, and a HindIII site at +36, was made with the primers 5′-GGATCCGGATCCGAC-3′ and 5′-GGATCCCTCGAG-3′ (see Fig. 1). PCR products carrying wild type or W3133-2 mutant sequences were restricted with EcoRI and HindIII and cloned either into plasmid vectors pSR (12) or pW50 (13).

In Vivo Expression Assays—The KK81 and JK14 EcoRI-HindIII fragments containing the wild type or mutant melAB promoters were cloned into pBluescript KS−(KpnI), a low copy number lac expression vector (13), to generate melAB::lac fusions. Recombinant plasmids were propagated in strain WAM131 (11), an mel− strain derived from GM-1 (14). β-Galactosidase levels in these cells were measured using the method of Miller (15): cells
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were grown in media either with or without meliobiose exactly as in our previous work (5).

Overexpression and Purification of His-tagged MelR—In the histidine-tagged variant used here (MelIR), the carboxyl terminus of the MelR is extended by 7 amino acid residues, including 6 tandem histidines; the full sequence of this extension is (in single-letter code) HHHHHH. To construct modified melR encoding the MelRH, we started with plasmid pBM3133 (7), in which the meliobiose operon was cloned into the EcoRI site of pBR322. To insert a unique NdeI site at the initiation codon site of the melR gene and to remove the termination codon of melR, we used PCR mutagenesis using the primer melRN-NdeI (5'-ACATATGAACTACGATCCTTAGTATG-3') and melRC (5'-GCCGCGGAAAACGTCTGGCGGCGG-3'). The amplified DNA fragment was cloned into the EcoRI site of plasmid pBR322. Site-directed mutagenesis was performed using the QuikChange Kit (Stratagene). The mutagenic primer was designed with the following sequence: 5'-CACGAACTACGATCCTTAGTATG-3'. The mutagenic primer was then used to transform E. coli strain DH10B, and the resulting plasmid was identified by restriction enzyme digestion.

**TABLE I**

| DNA fragment | β-Galactosidase activity |
|--------------|-------------------------|
|              | Meliobiose | + Meliobiose |
| KKS1 (wild type) | 3 | 44 |
| KKS1 (mutant) | 6 | 363 |
| JK14 (wild type) | 0 | 11 |
| JK14 (mutant) | 5 | 239 |

mm KCl, 6 mM guanidine-HCl, 10% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA, pH 8.3). After centrifugation at 22,500 × g for 20 min, the supernatant fraction was kept at −20 °C until use.

For protein purification, we used 0.5-ml bed-volume amounts of Ni2+-agarose (Qiagen). The resin equilibrated with the solubilization buffer was added to 9.5 ml of the solubilized protein and kept for 30 min with gentle shaking to allow for binding of MelIR. The mixture was then placed in a column, bound material was collected by gravity elution, and the column was washed with 10 ml of the solubilization buffer (pH 8.0). After nonspecifically bound material had been removed, MelIR was eluted with the solubilization buffer (pH 6.3). The eluted protein was dialyzed against a 50-fold volume of dialysis buffer (100 mM potassium phosphate, pH 7.4, 10% glycerol, 50 mM KCl, 10 mM 2-mercaptoethanol, 1 mM EDTA) four times. The dialyzed protein was concentrated by using Microcon 10 or Centricon 10 (Amicon). The purified protein was stored at −70 °C until use.

In **Vitrro** Transcription Assays—To measure in **vitrro** transcription initiation, we used the pSR plasmid vector (12), which carries the bacteriophage λ oop transcription terminator just downstream of the EcoRI and HindIII cloning sites. Transcription experiments were performed using the protocols described by Kolb et al. (12). The KKS1 or JK14 EcoRI-HindIII fragments containing the wild type or mutant melAB promoter sequences. The measured activities are indicative of the expression of melAB promoter:λ-ac fusions carried by the pRW50 derivatives. Cells were grown in minimal media with fructose as a carbon source either with or without meliobiose exactly as in our previous work (5). Data shown are the average values from three independent experiments.

**Fig. 1.** The *E. coli* mel operon regulatory region. A, a diagram of the intercistronic region between the divergently transcribed melA and melR genes; horizontal arrows indicate the corresponding transcription start sites. DNA sequences are numbered with respect to the melAB transcript start site +1. The melAB promoter −10 and −35 hexamers are indicated by open rectangles. The figure illustrates the KKS1 fragment bounded by an upstream EcoRI site at position −312 and a downstream HindIII site at position +36, and the JK14 fragment bounded by an upstream EcoRI site at position −94 and a downstream HindIII site at position +36. The locations of the DNA sites for MelR are indicated by triangles: each triangle indicates an 18-base pair sequence and its orientation, and the position of the center of each site is denoted. Filled triangles, sites 1 and 2, which have identical sequences; gray triangles, site 1′ and the related site 1″ that overlaps the melR promoter; open triangle, weak site 2′ that is discussed here. The locations of the −160 and −10 primers, which were used to generate ET1 probes for the gel retardation assays are shown. B, shows the DNA sequence around MelR binding sites (sites 1′, 1, 2, and 2′) in the wild type melAB promoter and in the W3133-2 mutant. The consecutive five-nucleotide mutation is shown with bold letters. The underlined sequences between sites 1′ and 2, and those between sites 1 and 2′, are identical. The location of the ET2 and ET3 probes used in the gel retardation assays are also indicated.

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E. Tamai, unpublished data.
The preincubations contained melibiose (10 mM) and different concentrations of purified MelR as indicated. The promoter sequences. The locations of the “constitutive” RNA I transcript and the transcript starting at the melAB by calibrated with Maxam-Gilbert sequence ladders.

Fig. 1. Before the PCR reaction, the primer (5′-TAACTTCATCATGGCAGGCGA-3′, 1, 2, and 2′) shown schematically in Fig. 2. The figure shows autoradiographs of gels on which RNA synthesized in vitro by E. coli RNAP was analyzed. The template is the pSR plasmid carrying the KK81 or JK14 inserts carrying the wild type or W3133-2 mutant melAB promoter sequences. The locations of the “constitutive” RNA I transcript and the transcript starting at the melAB promoter are indicated. The preincubations contained melibiose (10 mM) and different concentrations of purified MelR as indicated. The lower part shows quantification of the transcript starting at the melAB promoter in the different experiments relative to the RNA I transcript.

RESULTS

In Vivo Assays with the W3133-2 Mutant melAB Promoter—In our previous work, we described the W3133-2 mutant that results from five consecutive base changes in the melAB promoter (7). To study the effects of these changes in vitro, we subcloned EcoRI-HindIII DNA fragments carrying the wild type or W3133-2 mutant melAB promoter sequences (Fig. 1). First, using PCR, we amplified the KK81 fragment that carries the entire melAB promoter with all four MelR binding sites (sites 1, 1, 2, and 2′) that are known to be essential for optimal melAB promoter activity (10, 11). Second, we amplified the shorter JK14 fragment that carries the melAB transcript start site and only the MelR binding sites 2 and 2′. In preliminary experiments, we checked the activity of the melAB promoter carried by these fragments. To do this, the different EcoRI-HindIII fragments were cloned into the broad host range lac fusion vector, pRW50, and the resulting plasmids were transformed into WAM131, a Δlac mel+ strain derived from GM-1 (11, 14). Expression of the promoter melAB::lacZ fusions carried by the pRW50 recombinants was measured in transformants grown either with or without the inducer, melibiose. The results, listed in Table I, indicate that, with the longer KK81 fragment, expression from the melAB promoter is clearly induced by melibiose and that the W3133-2 mutant promoter is 8- to 9-fold more active than the wild type promoter. With the shorter JK14 fragment, the removal of MelR binding sites 1′ and 1 results in a 4-fold reduction in expression of the melAB promoter. However, in contrast, with the W3133-2 mutant, melAB promoter activity is only slightly affected by removal of MelR binding sites 1′ and 1. Thus, the base changes in the W3133-2 mutant result in a >20-fold increase in melAB promoter activity in the context of the shorter JK14 fragment. These results show that the effects of the base changes in the W3133-2 mutant are easily detected in short fragments and encouraged us to attempt in vivo studies.

Overexpression and Purification of His-tagged MelR—To perform in vitro study, we needed to purify MelR. However, the published method for purification of MelR required a large
amount of medium and a complicated column works (20). Therefore, we employed Ni\textsuperscript{2+} affinity chromatography for the purification and T7 expression system for overexpression of MelR. First we constructed a plasmid that carries a derivative melR encoding MelR with the carboxyl-terminal hexahistidine tag (MelRH). The derivative melR gene is located downstream from the T7 promoter. To confirm that the MelRH is functional in vivo, a XbaI-EcoRI fragment, which carries a Shine-Dalgarno sequence was subcloned from pETRH or pETR into pMW119, in which expression of melRH or melR is controlled by the lac promoter. A MelR-defective mutant, Y1089 (DmelR) (19), harboring either one of the constructed plasmids, formed similar red colonies on a MacConkey-melibiose (5 mM) plate (data not shown). This result indicates that the MelRH worked as a transcription activator for the melibiose operon in vivo.

Other results from gel retardation assay and in vitro transcription assay done with MelR or MelRH indicated that MelRH had almost the same DNA binding affinity and transcription activation activity as wild type MelR (data not shown). For overexpression of MelRH, we used the T7 system. We tested several media and found that M9 medium increased the expression level of our target protein and reduced background proteins. The MelRH protein produced in BL21(DE3)/pETRH after addition of isopropyl \(\beta\)-D-thiogalactopyranoside reached about 10–20% of total cell protein. However, most of the overproduced MelRH protein formed inclusion body. The advantage of this preparation was that the MelRH in the inclusion body was more than 80% pure without any column works. The inclusion body was solubilized with 6 M guanidine buffer and purified using Ni\textsuperscript{2+}-agarose. The purified protein was renatured by dialysis and then concentrated by ultrafiltration. When concentration of the purified MelRH was high, precipitation occurred during dialysis and most protein bound to filter during ultrafiltration. Our protocol yielded 500 \(\mu\)g of >95% pure His-tagged MelR from 200 ml of culture. This amount is enough for in vitro analysis. The renatured MelRH showed almost identical transcription activation activity as that ob-

**FIG. 3.** Gel retardation studies. Autoradiographs of gel retardation experiments to measure the binding of purified MelR to different DNA fragments. A, binding to ET1 probes carrying MelR binding sites 1', 1, 2, and 2' from the wild type or W3133-2 mutant melAB promoter. B and C, binding to the ET2 or ET3 probes from the wild type or W3133-2 mutant melAB promoter. The quantities of MelR used in each incubation are indicated.

**FIG. 4.** DNase I footprint analysis of MelR binding. An autoradiograph of a sequencing gel run to analyze products after DNase I cleavage of complexes formed by MelR at the JK14 fragment from the wild type or W3133-2 mutant melAB promoter. The quantities of MelR used in each incubation are indicated. Maxam-Gilbert sequencing reactions were used to calibrate the gel. The calibration that is shown takes the melAB transcript start point as +1, and the vertical bars indicate the locations of MelR binding sites 2 and 2'.
served with MelR purified by the method reported previously (19) without denaturation (data not shown).

**Activation of the melAB Promoter in Vitro using Purified MelR**—It is important to test whether the results obtained in the *in vivo* system (Table I and Ref. 7) are reproducible in the *in vitro* system. To do this, we cloned EcoRI-HindIII DNA fragments carrying the wild type or W3133-2 mutant melAB promoter sequences into the pSR vector plasmid, which carries the bacteriophage *λ* oop transcription terminator just downstream of the HindIII site (12). The resulting recombinant plasmids were used to monitor MelR-dependent activation at the melAB promoter; i.e. transcripts initiating at the melAB promoter run to the *λ* oop terminator and give discrete transcripts that are easy to detect by gel electrophoresis. In addition, because pSR contains a ColE1 replication origin, the RNA I transcript can be used as an internal control to aid quantification of MelR-dependent transcripts. Fig. 2 shows the results from a typical experiment, where purified RNAP, together with purified MelR and melibiose, was preincubated with purified circular pSR plasmid carrying the JK14 or KK81 insert, prior to the addition of labeled nucleoside triphosphates. Each incubation produces the control RNA I transcript, prior to the addition of labeled nucleoside triphosphates. The results (Fig. 2) show that, with the wild type melAB promoter sequence, MelR affords clear protection of the segment of DNA corresponding to MelR binding site 2. However, even in the presence of melibiose and higher concentrations of MelR, very poor protection of the segment of DNA corresponding to MelR binding site 2’ is observed. However, with the JK14 fragment derived from the W3133-2 mutant, clear protection of both site 2 and site 2’ is seen, even in the absence of melibiose.

**DisCUSSION**

From our results, we conclude that, as predicted, the five base changes in the melAB promoter of the W3133-2 mutant improve the binding of MelR to site 2’. We suppose that this improvement in MelR binding is responsible for the increased melAB promoter activity in the W3133-2 mutant, although we cannot prove that it is solely responsible. In our previous paper, we noted that the five base changes in the W3133-2 mutant create an extended 28-nucleotide inverted repeat at the melAB promoter (7). We suggested that this inverted repeat might facilitate the formation of a stem-loop structure and that this may be responsible for the increased melAB promoter activity in the W3133-2 mutant. However, our observation that the increased melAB promoter activity is observed even in the absence of MelR binding sites 1’ and 1 (i.e. with the JK14 fragment, which lacks the extended inverted repeat) proves that this cannot be the case. Indeed, the effects of the five base changes are greater with the JK14 fragment: this appears to be because MelR binding to site 2’ is aided by MelR binding to sites 1’ and 1, and thus, in the absence of sites 1 and 1’, improvement of site 2’ has a proportionately greater effect.

Taken together, our results show that the activity of the melAB promoter is limited by the binding of MelR to site 2’. Because this site overlaps the −35 hexamer of the melAB promoter, it appears that MelR, like AraC and many other members of the AraC family, may be a typical “Class II” transcription activator (17). Our results suggest that the principal function of the upstream sites 1’ and 1 is to help the occupation of site 2’. Thus, the requirement of sites 1’ and 1 for melAB promoter activity is greatly reduced when MelR binding site 2’ is improved (Table I). It is known that Class II transcription activators function by making a direct contact with Region 4 of the RNAP σ subunit (18). Thus, the challenge now is to understand the molecular details of the MelR-σ subunit interactions.

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