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A 53-Base-Pair Inverted Repeat Negatively Regulates Expression of the Adjacent and Divergently Oriented Cytochrome $P450_{BM-1}$ Gene and Its Regulatory Gene, *bm1P1*, in *Bacillus megaterium*

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To study the role of the *cis*-acting element(s) in controlling the expression of the cytochrome $P450_{BM-1}$ gene and its upstream regulatory gene, *bm1P1*, in *Bacillus megaterium*, various deletion derivatives were constructed. A 53-bp inverted repeat located midway between the $P450_{BM-1}$ gene and *bm1P1* gene was found in vivo to negatively regulate the expression of both genes, the regulation of which may occur at the transcriptional level. The promoter of the $P450_{BM-1}$ gene was also identified and found to be similar to those recognized by the σ^A RNA polymerase of *Bacillus subtilis*. Possible mechanisms by which the 53-bp inverted repeat regulates the gene expression are discussed.

Bacillus megaterium ATCC 14581 can produce at least three distinct cytochrome P450s, i.e., $P450_{BM-1}$, $P450_{BM-2}$, and $P450_{BM-3}$, when grown in the presence of phenobarbital or other barbiturates (1, 12, 17). Genes coding for cytochrome $P450_{BM-1}$ (*CYP*106) and $P450_{BM-3}$ (*CYP*102) have been cloned (5, 23). Sequence analysis has shown that $P450_{BM-3}$ is the only prokaryotic P450 known to resemble the mammalian microsomal P450 monooxygenases (16). The three-dimensional structure of $P450_{BM-3}$ has been recently elucidated and has been proposed as the most suitable structure available to model mammalian cytochrome P450s (8, 14). Immediately upstream of the $P450_{BM-3}$ structural gene exists the *bm3R1* gene, which encodes a transcriptional repressor that negatively regulates the expression of the $P450_{BM-3}$ gene (18). $P450_{BM-1}$ shows a high degree of sequence similarity (63% identity, 76.5% similarity) to $P450_{meg}$, a steroid-15β-monooxygenase isolated from *Bacillus megaterium* ATCC 13368 (13). Upstream of and opposite in orientation to the $P450_{BM-1}$ gene exists the *bm1P1* gene, which encodes a putative positive regulator probably involved in barbiturate-mediated induction of $P450_{BM-1}$ expression (4). A 53-bp palindromic sequence comprising two 24-bp segments that form a perfect inverted repeat interrupted by a 5-bp segment (Fig. 1) is located midway between the divergently oriented $P450_{BM-1}$ and *bm1P1* genes. In vitro DNase I footprinting analysis has shown that the Bm3R1 protein, when used at micromolar concentrations, could weakly protect a region encompassing the 53-bp palindromic sequence (10). However, no functional assay for the role of the 53-bp inverted repeat in controlling the expression of the $P450_{BM-1}$ gene in vivo is available. In this study, we found that the 53-bp inverted repeat can act as a negative *cis*-acting element in controlling the expression of the divergently oriented $P450_{BM-1}$ and *bm1P1* genes. We also determined the transcription start site and identified the σ^A-like promoter of the $P450_{BM-1}$ gene.

The region spanning the translation start site of the $P450_{BM-1}$ gene and its upstream 135 bp was previously found to contain no promoter activity (3), a feature which we have subsequently confirmed (10a). Only when this region was extended 160 bp further upstream did a strong promoter activity appear. However, the putative $P450_{BM-1}$ promoter sequence was previously postulated solely on the basis of sequence analysis (5, 10). Since experimental evidence was needed to substantiate this assumption, we attempted to determine the precise transcription start site of the $P450_{BM-1}$ gene by primer extension analysis as previously described (18). RNA was prepared from log-phase *B. megaterium* cells harboring the plasmid pGS118 (see Fig. 3). A 21-mer oligonucleotide (5′-GTT ACTTTGTCGAATAACCTA-3′) complementary to a region extending from positions −108 to −88 (numbered relative to the translation start site of the $P450_{BM-1}$ gene) was used as the primer. Only one major extension product was detected (Fig. 2), indicating that the 5′ end of the mRNA for the $P450_{BM-1}$ gene is located at position −147 (Fig. 1). This transcription start site is at an appropriate distance from the putative promoter sequence postulated previously (5, 10), and the −35 and −10 regions of this promoter sequence are similar to those recognized by the σ^A RNA polymerase of *Bacillus subtilis* (11). The −10 region of this promoter and its downstream sequence overlap the 53-bp inverted repeat (Fig. 1).

To further determine whether this promoter can drive the expression of the $P450_{BM-1}$ gene and whether the 53-bp inverted repeat plays a role in controlling the expression of the $P450_{BM-1}$ gene in vivo, a series of deletion derivatives were constructed. PCR-generated DNA fragments of various lengths located in the regulatory region of the $P450_{BM-1}$ gene were transcriptionally fused to a promoterless *cat* gene contained in multicopy plasmid pUB_{CAT} (Fig. 3) (18). PCR was carried out as described previously (18). The template used in the PCR was a 4.5-kb DNA fragment containing the N terminus of $P450_{BM-1}$ and its upstream sequence. The cloning and sequencing of this DNA fragment will be described elsewhere. The PCR-generated DNA fragments flanked by appropriate restriction sites (*Sal*I site on the upstream primer and *Hin*dIII site on the downstream primer) were cloned between *Sal*I and *Hin*dIII sites of pUB_{CAT} and transformed into *B. megaterium*. At least two independent clones from each construct were chosen for chloramphenicol acetyltransferase (CAT) activity assays. CAT activities were measured spectrophotometrically as described elsewhere (21). As shown in Fig. 3, deletion of half of the 53-bp inverted repeat resulted in a 2.5-fold increase

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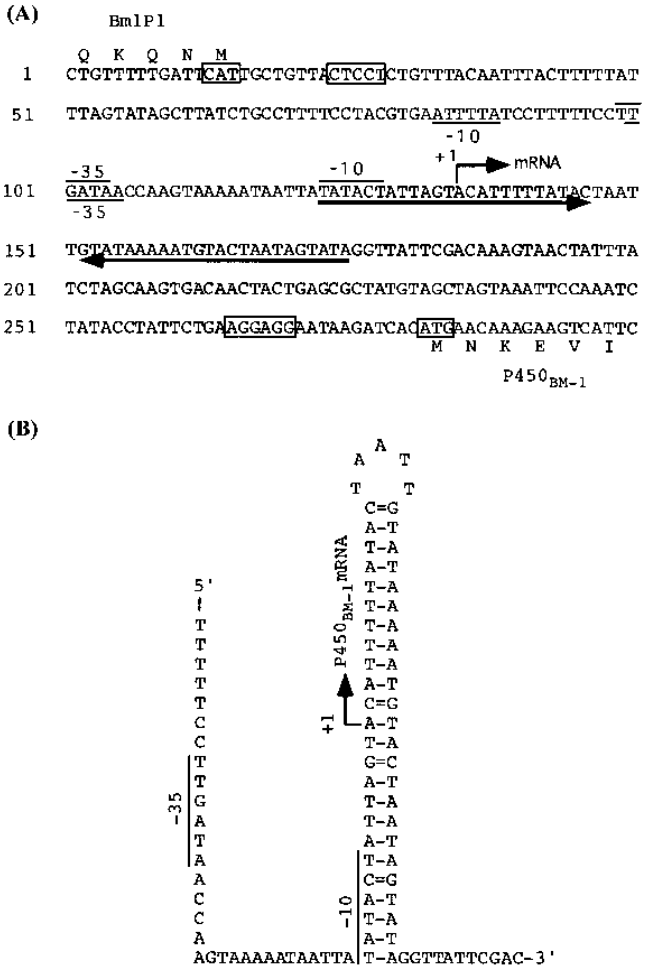

FIG. 1. (A) Regulatory region of the cytochrome $P450_{BM-1}$ gene. The 53-bp inverted repeat is indicated by a pair of inverted arrows. The transcription initiation site of the $P450_{BM-1}$ gene is marked +1. The σ^A-like sequence (including the −35 and −10 regions) is overlined for the $P450_{BM-1}$ promoter and underlined for the putative promoter of the *bm1P1* gene. The putative Shine-Dalgarno sequence and the translation start codon are boxed. (B) Potential secondary structure of the 53-bp inverted repeat.

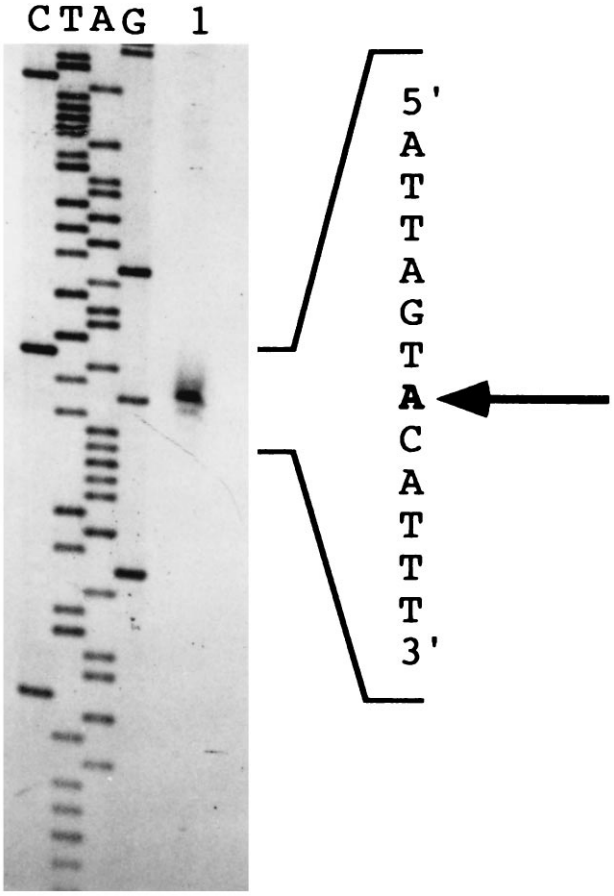

FIG. 2. Primer extension analysis of $P450_{BM-1}$ transcription. A dideoxy sequencing ladder obtained with the same primer used for primer extension analysis was resolved on a 6% polyacrylamide sequencing gel in parallel with the primer extension product by using RNA from *B. megaterium* cells harboring plasmid pGS118 (lane 1). A sequence complementary to that read from the ladder is shown at the right with the transcription start site indicated by an arrow.

in CAT activity. Further deletion of the −10 region of the putative promoter of the $P450_{BM-1}$ gene yielded a dramatic decrease in CAT activity. This result indicates that the predicted promoter is indeed the major $P450_{BM-1}$ promoter. It also appears that the 53-bp inverted repeat is important for negative regulation of the $P450_{BM-1}$ gene and the regulation may occur at the transcriptional level.

To determine whether the 53-bp inverted repeat is also involved in the regulation of the divergently oriented *bm1P1* gene in vivo, we constructed a series of deletion derivatives (Fig. 4) by PCR and cloned these DNA fragments between *Eco*RI and *Hin*dIII sites of shuttle vector pHY300PLK (Takara Shuzo Co. Ltd., Kyoto, Japan). Whole-cell extracts (20) of *B. megaterium* cells harboring these plasmids were subjected to sodium dodecyl sulfate (SDS)–13% polyacrylamide gel electrophoresis (PAGE) (7) and Western blot analysis (22). To prepare the anti-Bm1P1 antibody, histidine-tagged Bm1P1 was overexpressed and purified in a manner similar to that previously described (4) and used to immunize rabbits by the method of Harlow and Lane (2). As shown in Fig. 4, expression of the *bm1P1* gene in *B. megaterium* cells harboring plasmid pGS112 or pGS141 was barely detectable. Partial or complete deletion of the 53-bp inverted repeat caused a dramatic increase in the level of expression of *bm1P1*. This result is consistent with the elimination of a negative regulatory site resulting in complete derepression of expression. Further deletion of the region between the 53-bp inverted repeat and the translation start site of *bm1P1* completely eliminated the expression, suggesting that the *bm1P1* promoter is located within this 81-bp segment. The transcription start site of the *bm1P1* gene was previously identified by Fulco and coworkers (4). The location of the putative promoter within the 81-bp region as shown in Fig. 1 is in agreement with our result.

To examine whether the effect of the 53-bp inverted repeat on the expression of the *bm1P1* gene was also exerted at the transcriptional level, two new plasmids were constructed. The inserts removed from plasmids pGS141 and pGS142 (Fig. 4) by restriction digestion with *Eco*RI and *Hin*dIII were recloned between *Eco*RI and *Hin*dIII sites of promoter-probing vector pLC4, which contains a promoterless *xylE* reporter gene (15), resulting in plasmids pGS163 and pGS165, respectively (Fig. 5). Expression of the *bm1P1-xylE* transcriptional fusions in *Escherichia coli* JM109 (24) was determined spectrophotometrically by measurement of catechol dioxygenase (XylE)

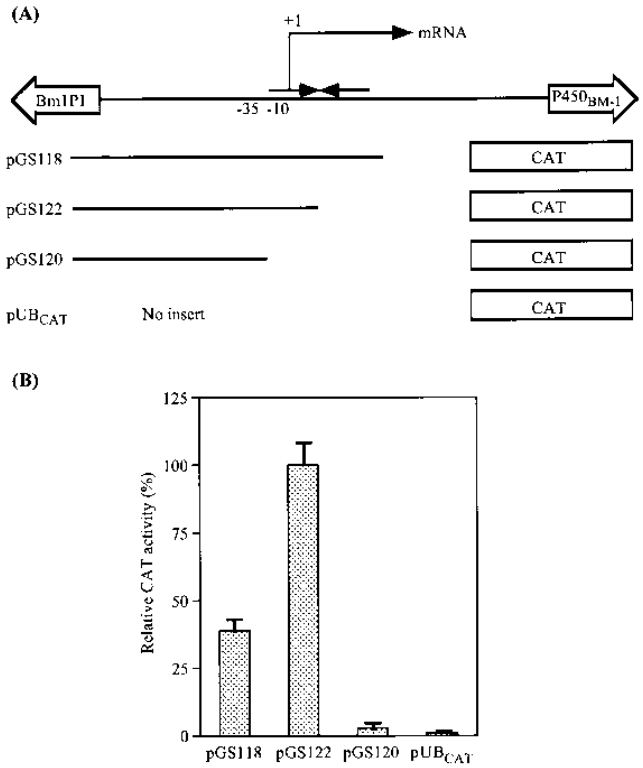

FIG. 3. CAT activities directed by various deletion derivatives of the $P450_{BM-1}$ promoter region. (A) The pUB_{CAT}-based vectors used, pGS118, pGS122, and pGS120, contain a *cat* reporter gene preceded by 262, 206, and 174 bp, respectively, of the DNA sequence downstream from position −341, which is numbered relative to the translation start site of the $P450_{BM-1}$ gene. (B) Relative CAT activities of *B. megaterium* cells harboring the above plasmids. *B. megaterium* cells were grown at 37°C to an optical density at 600 nm of 1.0. The specific activity of CAT obtained from pGS122 was assigned a relative value of 100%. Each value is the mean of at least four trials in separate experiments. Each error bar indicates the standard error of the mean.

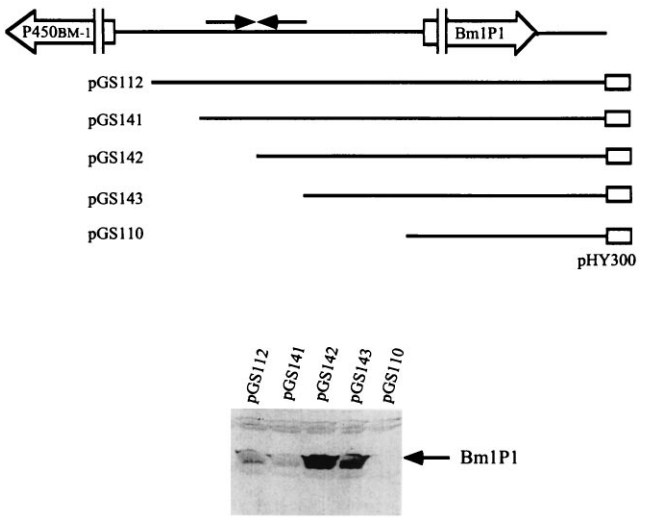

FIG. 4. Western blot analysis of the effect of the 53-bp inverted repeat on expression of the *bm1P1* gene. The pHY300PLK-based vectors used, pGS112, pGS141, pGS142, pGS143, and pGS110, contain the *bm1P1* gene followed by 364 bp of its 3′-flanking sequence, as well as preceded by 229, 187, 131, 99, and 18 bp of its 5′-flanking sequence, respectively. *B. megaterium* cells harboring the above plasmids were grown at 37°C to stationary phase. Equal numbers of cells were harvested, and whole-cell extracts were subjected to SDS-PAGE and Western blot analysis with an anti-Bm1P1 antibody as the probe.

activity as described previously (15). As shown in Fig. 5, deletion of half of the 53-bp inverted repeat resulted in a marked increase in the level of XylE activity, indicating that the negative regulation of *bm1P1* expression by the inverted repeat may occur at the transcriptional level.

How the 53-bp inverted repeat can regulate the gene expression is an interesting question. The location of the transcription start site of the $P450_{BM-1}$ gene within the inverted repeat (Fig. 1) makes the inverted repeat unsuitable as a transcriptional terminator since the 5′ end of the $P450_{BM-1}$ mRNA could not form a stable stem-loop conformation. The fact that the 53-bp inverted repeat not only overlaps the promoter sequence of the $P450_{BM-1}$ gene but also lies adjacent to the promoter of the *bm1P1* gene makes the inverted repeat suitable as an operator for both genes. A *trans*-acting negative regulator may bind to this operator to exclude RNA polymerase from binding to either promoter. If this were the case, it would be very unusual, since most of the operators identified in prokaryotes are less than 40 bp long. A 20-bp perfect palindromic sequence between the $P450_{BM-3}$ gene and its promoter has been clearly demonstrated to be the binding site for the purified Bm3R1 repressor by in vitro DNase I footprinting experiments (19). However, the Bm3R1 protein purified in the same way could not form a stable complex in vitro with a DNA fragment containing the 53-bp inverted repeat (9). Only when using crude extracts of *E. coli* containing the overexpressed Bm3R1 protein at micromolar concentrations could a region encompassing the 53-bp inverted repeat be weakly protected from DNase I digestion (9). This result led to the tentative assumption that the 53-bp inverted repeat might be another DNA-binding site for the Bm3R1 repressor, although no significant consensus sequences exist between these two inverted repeats. In fact, the results shown in Fig. 5 could be used to elucidate whether the level of XylE activity was influenced by the presence or absence of the 53-bp inverted repeat in a genetic background without *bm3R1*. As shown in Fig. 5, deletion of half of the 53-bp inverted repeat still elicited a dramatic increase in the level of XylE activity, even in a genetic background without *bm3R1*, suggesting that either the Bm3R1 protein is not the *trans*-acting factor that can interact with the 53-bp inverted repeat or some other DNA-binding protein(s) produced in *E. coli* recognizes the 53-bp inverted repeat and functions like the Bm3R1 protein. It has been previously shown that a 50-bp inverted repeat located in a plasmid and composed largely of A-T base pairs, spanning the region from −23 to +27 with respect to the transcription start site, could be found in the form of a cruciform DNA structure within *E. coli* cells (6). Transcription from this promoter by RNA polymerase in vitro was repressed as the cruciform structure was extruded by increasing negative DNA supercoiling. Transcription in vivo was induced as supercoiling was relaxed by growth under conditions that inhibit DNA gyrase (6). However, we could not detect induction of $P450_{BM-1}$ expression by the topoisomerase inhibitor novobiocin.

The Bm1P1 protein, which contains a typical helix-turn-helix DNA-binding motif at its N terminus, has been proposed to be a putative positive regulator of $P450_{BM-1}$ expression (4). Based on the data presented in this report, it is relevant to ask if the presence of Bm1P1 is necessary to see the relative differences in reporter enzyme activity between the constructs with either all or half of the 53-bp inverted repeat. Two lines of evidence suggest that Bm1P1 is not involved in the observed induction of $P450_{BM-1}$ and *bm1P1* expression by deletion of half of the

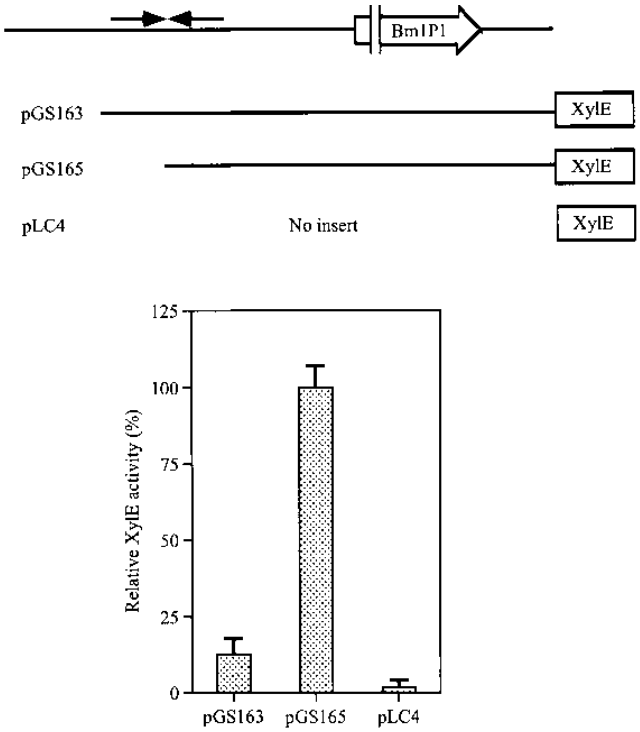

FIG. 5. Effect of the 53-bp inverted repeat on the expression of *bm1P1-xylE* transcriptional fusions. The pLC4-based vectors used, pGS163 and pGS165, contain a *xylE* reporter gene preceded by the *bm1P1* gene and its downstream 364-bp and upstream 187- and 131-bp DNA sequences, respectively. *E. coli* cells harboring the above plasmids were grown at 37°C to an optical density at 600 nm of 1.0. The specific activity of XylE obtained from pGS165 was assigned a relative value of 100%. Each value is the mean of at least four trials in separate experiments. Each error bar indicates the standard error of the mean.

53-bp inverted repeat. First, we have constructed a *B. megaterium* mutant in which the *bm1P1* gene has been disrupted. The details for the construction of this mutant and confirmation by Southern blot hybridization will be described elsewhere. Crude extracts from this *B. megaterium* mutant transformed with plasmids pGS118 and pGS122 (shown in Fig. 3), respectively, were assayed for reporter CAT activity. The results show that in the *bm1P1* mutant background, deletion of half of the 53-bp inverted repeat still resulted in a remarkable increase in expression from the $P450_{BM-1}$ promoter (data not shown). Second, to examine the relative differences in reporter XylE activity between plasmids pGS163 and pGS165 (shown in Fig. 5) in the absence of Bm1P1, we changed the translation start codon of the *bm1P1* gene in plasmids pGS163 and pGS165 from ATG to ACG by PCR. The desired mutation was confirmed by DNA sequencing, and the loss of Bm1P1 production was confirmed by SDS-PAGE. *E. coli* cells harboring the mutated plasmid with half of the inverted repeat still exhibited remarkably higher reporter XylE activity than those with the intact inverted repeat (data not shown), suggesting that Bm1P1 is not necessary for the negative regulatory effect of the 53-bp inverted repeat, even in *E. coli*. The mechanism responsible for negative regulation by the 53-bp inverted repeat remains to be characterized.

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