Evidence against the Bm1P1 Protein as a Positive Transcription Factor for Barbiturate-mediated Induction of Cytochrome P450bm-1 in Bacillus megaterium*  

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Gwo-Chyuan Shaw‡, Chi-Chang Sung, Chang-Hsieh Liu, and Chia-Hung Lin  

From the Institute of Biochemistry, School of Life Science, National Yang-Ming University, Taipei 112, Taiwan, Republic of China  

The Bm1P1 protein was previously proposed to act as a positive transcription factor involved in barbiturate-mediated induction of cytochrome P450bm-1 in Bacillus megaterium. We now report that the bm1P1 gene encodes a protein of 217 amino acids, rather than the 98 amino acids as reported previously. In vitro gel shift assays indicate that the Bm1P1 protein did not interact with probes comprising the regulatory regions of the P450bm-1 gene. Moreover, disruption of the bm1P1 gene did not markedly affect barbiturate induction of P450bm-1 expression. A multicopy plasmid harboring only the P450bm-1 promoter region could increase expression of the chromosome-encoded P450bm-1. The level of expression is comparable with that shown by a multicopy plasmid harboring the P450bm-1 promoter region along with the bm1P1 gene. These results strongly suggest that the Bm1P1 protein is unlikely to act as a positive regulator for barbiturate induction of P450bm-1 expression. Finally, deletion of the Barbie box did not markedly diminish the effect of pentobarbital on expression of a reporter gene transcriptionally fused to the P450bm-1 promoter. This suggests that the Barbie box is unlikely to be a key element in barbiturate-mediated induction of P450bm-1.

At least three distinct cytochrome P450s, i.e. P450bm-1, P450bm-2, and P450bm-3, have been identified in Bacillus megaterium ATCC 14581 (1, 2). P450bm-1 (CYP106), whose function is still relatively unclear, is moderately induced by barbiturates (3). P450bm-2 (CYP102), a catalytically self-sufficient fatty acid monoxygenase, is strongly induced by barbiturates (4). The bm3R1 gene, located immediately upstream of the P450bm-3 structural gene, has been demonstrated to encode a transcriptional repressor that negatively regulates expression of the P450bm-3 gene (5). The in vitro inhibition by barbiturates on the interaction of Bm3R1 repressor with its operator near the P450bm-3 promoter has been correlated with their in vivo potency as inducers of P450bm-3 expression. This suggests that barbiturate inducers may function in vivo by releasing the binding of Bm3R1 repressor from its operator DNA (6).

Recently, Liang and Fulco (7) found that the bm3R1 repressor could also negatively regulate expression of P450bm-1 in B. megaterium. This repression was proposed to be mediated through the binding of Bm3R1 to some putative regulatory elements in the 5′-flanking region of P450bm-1 (7). However, gel mobility shift assays showed that barbiturates could not inhibit the binding of Bm3R1 to these regulatory elements (7), thus leaving open questions about how barbiturates can induce expression of P450bm-1. A Barbie box, located in the 5′-flanking region of the P450bm-1 gene, was also proposed by Liang et al. (8) to be a cis-acting element responsible for barbiturate-mediated induction of P450bm-1. In addition, the Bm1P1 protein, encoded by an open reading frame immediately upstream of the P450bm-1 gene and transcribed in the opposite direction, was proposed to function as a positive regulatory protein for barbiturate-mediated induction of P450bm-1. This was proposed to be a result of interference with the binding of Bm3R1 repressor to the Barbie box (9).

As an initial step toward understanding whether other adjacent genes are regulated by Bm1P1, we have cloned and sequenced the bm1P1 gene and its downstream DNA. We found some discrepancies between the sequence published by He et al. (9) and our sequencing data. In this report, we present the correct nucleotide sequence of the bm1P1 gene and provide evidence that the bm1P1 gene encodes a protein of 217 amino acids, rather than the 98 amino acids as reported by He et al. (9). Moreover, we provide evidence against Bm1P1 as a positive transcription factor involved in barbiturate-mediated induction of P450bm-1 and evidence against the Barbie box as a key element responsible for induction of P450bm-1 by barbiturates.

EXPERIMENTAL PROCEDURES

Materials—Various restriction enzymes, DNA-modifying enzymes, and Vent DNA polymerase used in polymerase chain reaction (PCR) were purchased from New England Biolabs. 32P-Labeled nucleotides and Sequenase version 2.0 DNA sequencing kit were from Amersham Pharmacia Biotech. Oligonucleotides used in PCR were ordered from Genemed Synthesis, Inc. BCA protein assay kit was from Pierce. Rabbit polyclonal antibodies against cytochrome P450bm were prepared as described previously (10). Goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase was obtained from Bio-Rad.

Growth of Bacteria—Luria-Bertani (LB) medium (15) was used for growing Escherichia coli and B. megaterium unless specified otherwise. Construction of a Genomic Library—Chromosomal DNA of B. megaterium ATCC 14581 was isolated according to the procedure described by Miura (11) and was partially digested with restriction enzyme Sau3AI to produce a majority of DNA fragments in sizes ranging from 3 to 6 kb. These fragments were ligated into the BamHI site of pBlueScript KS(+) (Stratagene). E. coli JM109 (12) was transformed with the ligated DNA to generate a genomic library.

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‡ To whom correspondence should be addressed. Tel.: 886-2-2826-7127; Fax: 886-2-2826-4843.

1 The abbreviations used are: PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; PB, phenobarbital; Bm1P1(His)6, six histidine residues added at the C terminus of Bm1P1; IPTG, isopropyl-β-D-thiogalactoside; bp, base pair; kb, kilobase; HTH, helix-turn-helix; PAGE, polyacrylamide gel electrophoresis.
Cloning of the bm1P1 Gene—A 395-bp DNA fragment containing the N-terminal sequence of the P450bm1 gene was amplified by PCR (13) using B. megaterium genomic DNA as a template and two oligonucleotides as primers. PCR was carried out as described previously (5). The upstream primer (5'-AACAGATGCTTATCCCTCGG-3') contains the sequence corresponding to nucleotide positions 4 to 23 of the P450bm1 coding region (14). The downstream primer (5'-TTTTTGATTGCTTC- TACAAGG-3') contains a sequence complementary to bases 378 to 398 of the P450bm1 coding region. This PCR-generated DNA fragment was gel purified, labeled by a random priming method (15) using Multiprime DNA labeling kit according to the instructions of the manufacturer (Amersham), and used as a probe to screen the genomic library according to the method described by Sambrook et al. (16).

DNA Sequencing and Sequence Analysis—DNA was sequenced by the dideoxy chain-termination method (16) using Sequenase version 2.0 (U.S. Biochemical) and a DNA sequencing kit and [α-35S]dATP. Alkaline-denatured double-stranded DNA (17) was used as the template. Synthesized oligonucleotides were used as specific primers to determine the sequences of bm1P1 and its downstream DNA. Various deletions were also made in the cloned DNA and its subcloned derivatives using a unidirectional nested deletion method with exonuclease III (18) to generate templates for sequencing reactions. DNA sequences were analyzed with the sequence analysis software package from Genetics Computer Group (Wisconsin).

Plasmid Constructions—For construction of various P450bm1 promoter-mutant chimeric fusions, PCR was used to generate variable-length segments of the P450bm1 regulatory region flanked by appropriate restriction sites. The upstream primers P85 (5'-GGCGGTCGGACCAAAGTTCTATGATCGATGCGGCGG-3'), P86 (5'-GGCGGTCGGACCCCTAAATATCTAGCTGTT-3'), and P90 (5'-GGCGGTCGGACCTGTTGCGGATGGCCCTAG-3') contain a SalI site (underlined) at their 5' ends, and the downstream primer P83 (5'-GGCGGACCTGATATAATGTCTCTTGCGA-3') contains a HindIII site (underlined) at its 5' end. After cleavage with HindIII and SalI, the PCR-generated DNA fragments were cloned between the SalI and HindIII sites of pUC18 (19) and transformed into E. coli. The transformants were selected for kanamycin resistance.

A 900-bp EcoRI-HindIII fragment containing the bm1P1 promoter region plus the bm1P1 gene was amplified by PCR to construct plasmid pGS123 used for in vivo titration experiment. The upstream primer P75 (5'-GGCGGAAATCCGTAATGATCGATGCGGCGG-3') contains an EcoRI site (underlined) at its 5' end, and the downstream primer P71 (5'-GGCGGACCTGATATAATGTCTCTTGCGA-3') contains an HindIII site (underlined) at its 5' end. Following digestion with EcoRI and HindIII, this fragment was cloned into the corresponding sites of vector pUC18. Plasmid pGS119 was constructed to carry a sole copy of a 270-bp bm1P1 promoter region. This promoter was amplified using an upstream primer P75 and a downstream primer P88 (5'-GGCGGGAAT TCCCTTTAAATATCTAGTTT-3') that contains an EcoRI site (underlined) at its 5' end. Two primers flanking the bm1P1 gene were used in PCR for the construction of a 6×His-tagged Bm1P1-overproducing plasmid. The upstream primer P86, 5'-GGCGGAAATCCGTAATGATCGATGCGGCGG-3', contains an EcoRI site (underlined) at its 5' end, and the downstream primer, 5'-GGCGGAAATCCGTAATGATCGATGCGGCGG-3', contains a HindIII site (underlined) and sequence corresponding to 6×His (in lowercase). The PCR-amplified fragment was double digested with EcoRI and HindIII and cloned into vector pKK223-3 (20) to generate plasmid pGS104.

Overexpression of bm1P1 Protein Product in E. coli—The wild-type bm1P1 gene of B. megaterium was synthesized by PCR using two primers flanking the bm1P1 gene. Primers P66 and P71 were used as the upstream and downstream primers, respectively. The 690-bp EcoRI- HindIII digested PCR fragment was cloned into vector pKK223-3 (20) to generate plasmid pGS99. E. coli JM109 was transformed with pGS99. Induction of Bm1P1 production by addition of IPTG to E. coli cell cultures was performed as described previously (5). Bm1P1 overproduction was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) of whole cell extracts that were prepared as described previously (21).

Disruption of bm1P1—a 310-bp EcoRI-HindIII fragment carrying a partial N-terminal sequence of the bm1P1 gene was generated by PCR using two oligonucleotide primers. The upstream primer (5'-GGCGGAAATCCGTAATGATCGATGCGGCGG-3') contains an EcoRI site (underlined) at its 5' end, and the downstream primer (5'-GGCGGAAATCCGTAATGATCGATGCGGCGG-3') contains a HindIII site (underlined) at its 5' end. Following digestion with EcoRI and HindIII, this fragment was cloned into EcoRI- and HindIII-cut plasmid pH101 (23). The resulting plasmid pGS105 was transformed into E. coli JM109. Transformants were selected for chloramphenicol resistance. One clone was selected for Southern blot analysis (15) to confirm the disruption of the bm1P1 gene by using the 310-bp EcoRI-HindIII fragment as a probe.

Other Methods—Purification of Bm1P1(His)_6 protein by nickel ion-nitrilotriacetic acid affinity column chromatography was carried out as described previously (21). Gel mobility shift assays were performed according to the method of Fried and Crothers (24). Western blots were prepared as described by Townb et al. (25). Protein concentrations were determined by the BCA method according to the instructions of the manufacturer (Pierce) with bovine serum albumin as the standard. Transformation of B. megaterium was achieved by the protoplast method (27). CAT activity assays were performed by the colorimetric method of Shaw (26) with cell-free extracts of B. megaterium that were prepared by sonication as described previously (4).

RESULTS

Cloning of the bm1P1 Gene and Its Downstream DNA—Two oligonucleotides, corresponding to the published N-terminal nucleotide sequence of the P450bm1 gene (14) as described under “Experimental Procedures,” were used in PCR with B. megaterium chromosomal DNA as the template to synthesize the probe. B. megaterium chromosomal DNA was partially digested with Sau3AI, ligated into BamHI-cut vector pBlue-script KS1(+), and transformed into E. coli JM109. The transformants carrying recombinant plasmids were screened for inserts that hybridized to the probe. One positive clone, which showed strong hybridization to the probe, was identified. DNA sequencing was performed as described under “Experimental Procedures.” The sequencing strategy and restriction map are shown in Fig. 1. Nucleotide Sequence and Sequence Analysis—The nucleotide sequence of the bm1P1 gene and its flanking DNA are shown in Fig. 2. PCR with various pairs of synthetic oligonucleotides as primers and genomic DNA of B. megaterium as the template confirmed that the cloned bm1P1 gene and its downstream 5.3-kb DNA represent a continuous fragment of chromosomal DNA (data not shown). The nucleotide sequence that corresponds to the N-terminal 79 amino acids of the bm1P1 gene and its upstream region is consistent with that reported by He et al. (14). However, the nucleotide sequence that corresponds to the amino acid residues 218 to 310 of the bm1P1 gene and its downstream 380-bp region is different from that reported in another publication by He et al. (9) in many positions. The size of the Bm1P1 protein reported by He et al. (9) was 98 amino acids, whereas our sequencing data indicated that the bm1P1 gene encodes a putative protein of 217 amino acids. The reading frame of the bm1P1 gene reported by He et al. (9) seems to be incorrectly translated.
shifted after the 88th codon, resulting in premature termination of the open reading frame at the 99th codon.

The Swiss protein data base was examined for homologous sequences using the programs FASTA and BLAST. A group of proteins that showed significant similarity to the N-terminal region of the Bm1P1 protein was identified. These proteins are known or putative transcriptional regulators of a similar size to Bm1P1 and belong to the TetR/AcrR family of transcriptional regulators. Some of them, including TetC (197 amino acids) (28), AcrR (215 amino acids) (29), TcmR (226 amino acids) (30), BetI (195 amino acids) (31), MtrR (210 amino acids) (32), and Bm3R1 (192 amino acids) (5), are shown in Fig. 3. The N-terminal regions of these proteins were predicted to form a helix-turn-helix (HTH) DNA-binding motif (33). A segment of 20 amino acids near the N terminus of Bm1P1 (residues 26–45) was also strongly predicted to be an HTH DNA-binding motif according to the method of Dodd and Egan (34) (Fig. 3).

Sequence analysis of DNA within the 2.27-kb region downstream of bm1P1 revealed an open reading frame that can encode a putative protein of 539 amino acids (data not shown). This putative polypeptide showed distinct amino acid sequence similarity to a number of ATP-binding cassette transporters (35, 36) (data not shown). Its complete nucleotide sequence will be reported elsewhere.

Identification of the bm1P1 Gene Product—
To determine the size of the bm1P1 gene product, we constructed a Bm1P1-overproducing plasmid pGS99 by placing the bm1P1 gene under the control of the tac promoter as described under “Experimental Procedures.” Whole cell extracts prepared from E. coli transformants were analyzed by SDS-PAGE. A protein band with an apparent molecular mass of about 26 kDa was observed only in extract from E. coli carrying plasmid pGS99 grown in the presence of IPTG (Fig. 4, lane 5). The same band was not observed in non-IPTG induced cells carrying plasmid pGS99 and was not observed in cells carrying plasmid pKK223-3 as a control (Fig. 4, lanes 2–4). The estimated size of the overproduced Bm1P1 is in good agreement with that predicted from the nucleotide sequence. It should be noted that an IPTG-induced protein band with an apparent molecular mass of about 11 kDa as reported by He et al. (9) could not be
detected in this study.

DNA-binding Activity of Bm1P1—To determine whether Bm1P1 can bind to the 5′-flanking region, including the Barbie box, of the P450BM-1 gene, we used an E. coli strain bearing Bm1P1-overproducing plasmid pGS99 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. We also constructed a Bm1P1(His)6-overproducing plasmid for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Disruption of the bm1P1 Gene—To disrupt the bm1P1 gene in B. megaterium, we constructed an integrative plasmid pGS105 (Fig. 7) used as a probe in gel mobility shift assays. The results indicated that neither purified Bm1P1 nor cell-free extracts containing the overexpressed Bm1P1 protein could form a specific complex with DNA fragments carrying the 5′-flanking region of the P450BM-1 gene (data not shown).

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Evidence against Bm1P1 as a Regulator of the P450BM-1 Gene—To disrupt the bm1P1 gene in B. megaterium, we constructed an integrative plasmid pGS105 (Fig. 7) used as a probe in gel mobility shift assays. The results indicated that neither purified Bm1P1 nor cell-free extracts containing the overexpressed Bm1P1 protein could form a specific complex with DNA fragments carrying the 5′-flanking region of the P450BM-1 gene (data not shown).

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.

Effect of bm1P1 Disruption on Induction of P450BM-1, by Pentobarbital—To examine the role of bm1P1 in barbiturate-mediated induction of P450BM-1, we used an E. coli strain bearing Bm1P1(His)6-overproducing plasmid pGS105 for the preparation of cell-free extracts containing the overexpressed Bm1P1 protein. The other lanes contain whole cell extracts from E. coli carrying the control vector pKK223-3 grown in the absence (lane 2) or presence (lane 3) of IPTG and extracts from E. coli carrying the plasmid pGS99 grown in the absence (lane 4) or presence (lane 5) of IPTG. The position of the overproduced Bm1P1 is indicated by an arrow at the right.
Evidence against Bm1P1 as a Regulator of the P450BM-1 Gene

DISCUSSION

In the present study, we have determined the nucleotide sequence of the bm1P1 gene and its flanking region. The nucleotide sequence presented in this paper is different from that published by He et al. (9) in many positions. Our sequence analysis indicated that bm1P1 encodes a putative protein of 217 amino acids with a calculated molecular weight of 25,405, rather than the 98 amino acids as reported by He et al. (9). As stated previously, sequencing errors may have resulted in a reading frameshift of bm1P1 after the 88th codon, resulting in premature termination of the open reading frame at the 99th codon. To examine the size of Bm1P1 protein, He et al. (9) constructed a Bm1P1-overproducing plasmid by using two PCR primers flanking the bm1P1 coding region to synthesize the bm1P1 gene. Although the estimated size of the overexpressed Bm1P1 (about 11 kDa) was in agreement with what they predicted, the overexpressed Bm1P1 was actually a truncated form of the full-length 217-amino acid Bm1P1. If He et al. had used a PCR primer complementary to a sequence further downstream of the truncated Bm1P1 to construct a Bm1P1-overproducing plasmid, they probably would have identified Bm1P1 as a protein with higher molecular weight. In fact, one of the PCR primers we used for construction of a Bm1P1-overproducing plasmid was complementary to the C terminus of the full-length Bm1P1. So, the IPTG-induced protein band was detected at a position corresponding to a protein with an apparent molecular mass of about 26 kDa rather than 11 kDa. It is reasonable to question whether the coding region of bm1P1 we identified may still extend further into its downstream region. To resolve this question, we used PCR primers complementary to sequences about 150 or 500 bp further downstream of bm1P1 to construct Bm1P1-overproducing plasmids. The results indicated that the apparent molecular weight of the resulting Bm1P1 was identical to that of the 217-amino acid Bm1P1 (data not shown), suggesting that the size of Bm1P1 we determined is its full size.

By using either purified 6×His-tagged Bm1P1 or cell-free extract containing overexpressed wild-type Bm1P1 in gel mobility shift assays, we did not detect any specific protein binding to the 5'-flanking region, including the Barbie box, of the P450BM-1 gene. Moreover, disruption of the bm1P1 gene did not markedly affect barbiturate induction of P450BM-1 expression. This further suggests that Bm1P1 is not required for barbiturate-mediated induction of P450BM-1. The previous proposition by He et al. (9) that Bm1P1 was a putative positive regulator of P450BM-1 gene expression was simply based on the following observations: (i) Bm1P1 was shown to interfere with the binding of Bm3R1 repressor to the 5'-flanking region of the P450BM-1 gene in gel mobility shift assays; and (ii) B. megaterium cells transformed by a multicopy plasmid containing the bm1P1 gene and its promoter region showed a marked increase in chromosomal P450BM-1 level when compared with B. megaterium transformed by the vector alone. However, our data indicated that B. megaterium transformed by a multicopy plasmid containing the bm1P1 promoter region alone could still exhibit a comparable increase in chromosomal P450BM-1 level. Alternative to the previously proposed positive regulatory role of the Bm1P1 protein, a mechanism involving competitive binding between plasmid- and chromosome-encoded promoters for a putative negative regulatory protein is suggested. This mechanism may lead to the observed derepression of the chromo-

Fig. 6. In vivo titration effect on expression of the chromosomal P450BM-1 gene. B. megaterium cells were transformed by plasmids pLC4 (vector control), pGS148 (pLC4 carrying the bm1P1 promoter region alone), and pGS132 (pLC4 carrying the bm1P1 promoter region plus the bm1P1 gene), respectively, and grown to stationary phase. Equal numbers of cells were harvested, and whole cell extracts were subjected to SDS-PAGE and Western blot analysis with anti-P450BM-1 antibodies. Lane 1, vector control; lane 2, pGS148; lane 3, pGS132.

grown in the absence of pentobarbital, showed a dramatic increase in expression of the chromosome-encoded P450BM-1. The expression could be further increased when grown in the presence of pentobarbital (see Fig. 2 in Ref. 9). These observations led them to the proposition that Bm1P1 might function as a positive transcription factor to activate P450BM-1 expression. As our data from bm1P1 disruption indicated that Bm1P1 is unlikely to act as a positive regulator for P450BM-1 expression, those results obtained by He et al. may be open to other interpretations. We reexamined those results by construction of a multicopy plasmid containing the bm1P1 promoter alone (i.e. without the bm1P1 gene) in addition to a multicopy plasmid containing the bm1P1 promoter region plus the bm1P1 gene. As shown in Fig. 6, B. megaterium cells transformed by the multicopy plasmid pGS132 containing the bm1P1 promoter region plus the bm1P1 gene indeed exhibited a marked increase in P450BM-1 level as compared with cells transformed by the control vector alone. However, when B. megaterium cells were transformed by pGS148 containing the bm1P1 promoter alone, a comparable increase in P450BM-1 level was also observed. These results suggest that the previously observed increase in P450BM-1 level may be due to derepression rather than activation by the Bm1P1 protein per se. This derepression is presumably caused by competitive binding between the multicopy plasmid-encoded P450BM-1 promoter and the chromosome-encoded P450BM-1 promoter for a putative negative regulatory protein. Our data further support that Bm1P1 is not a positive transcription factor for P450BM-1 gene expression.

Effect of Barbie Box Deletion on Induction of P450BM-1 by Pentobarbital—To further test whether the Barbie box located in the coding region of bm1P1 was important for barbiturate-mediated induction of P450BM-1, as reported by Liang et al. (8), we synthesized a series of deletion derivatives of the P450BM-1 regulatory region by PCR and ligated these fragments into a promoter probe vector pUBCAT to generate various P450BM-1 promoter-cat transcriptional fusions. B. megaterium cells transformed with these constructs were selected for kanamycin resistance. At least two independent clones for each construct were chosen for CAT activity assays to obtain consistent results. As shown in Fig. 7, deletion of part of the Barbie box did not result in a marked decrease in the observed induction of P450BM-1 by pentobarbital. Similar results were obtained when the complete Barbie box was deleted. These data suggest that the Barbie box is unlikely to be the key element responsible for barbiturate-mediated induction of P450BM-1. However, our results did confirm the previous observation (8) that the sequence within the Barbie box was important for negative regulation of P450BM-1 gene expression in the absence of pentobarbital.
Liang and Fulco (7) previously demonstrated that the Bm3R1 repressor could negatively regulate expressions of both P450BM-1 and P450BM-3 genes in B. megaterium coordinately. It should be noted that the underlying molecular mechanisms for negative regulation of P450BM-1 and P450BM-3 expressions may be somewhat different. It has been previously demonstrated that Bm3R1 repressor exerts repression on P450BM-3 gene expression by direct binding to a 20-bp palindromic operator near the transcription start site of the P450BM-3 gene (5, 6). However, Bm3R1 may regulate P450BM-1 gene expression in an indirect manner rather than by direct binding to the 5'-flanking region of the P450BM-1 gene. This hypothesis is supported by the following observations. (i) When using purified Bm3R1 protein in gel mobility shift assays, Liang and Fulco (7) could not detect formation of the specific complex with the 5'-flanking sequence, including the Barbie box, of the P450BM-1 gene. When the E. coli cell-free extract containing the overexpressed Bm3R1 was used, they could detect formation of protein-DNA complexes. However, the footprints protected by Bm3R1 were barely visible (7). Gel mobility shift assays further showed that pentobarbital could not disrupt these protein-DNA complexes in vitro (7), thus casting some doubt on the identity of the protein component(s) in these complexes. (ii) No significant consensus sequence was identified among these putative Bm3R1 binding sites, especially when compared with the well-established Bm3R1 operator sequence near the P450BM-3 promoter. (iii) An in vivo titration effect is exerted by the multicopy plasmid-encoded P450BM-1 promoter region on expression of the chromosome-encoded P450BM-1 but not on expression of the chromosome-encoded P450BM-1 (see Fig. 2 in Ref. 9). This suggests that a putative negative regulator was titrated out by complexing to the multicopy plasmid-encoded P450BM-1 promoter region and is unlikely to be the Bm3R1 repressor. Otherwise, the plasmid-borne P450BM-1 promoter region would have increased the chromosomal P450BM-3 level by titrating out Bm3R1 bound to the operator site near the P450BM-3 promoter. These observations imply that Bm3R1 probably regulates P450BM-1 gene expression not by direct binding to the P450BM-1 regulatory region but in an indirect manner.

The molecular mechanism(s) for induction of various mammalian cytochrome P450s by phenobarbital (PB) have been the subject of extensive studies. Padmanaban and co-worker (37) first identified by DNase I footprinting analysis a 32-bp region (−288 to −256) upstream of the CYP2B1/CYP2B2 genes critical for mediating PB transcriptional activation. Based on sequence comparisons and in vitro protein binding assays, Fulco and colleagues (8, 38) then identified a sequence (so-called Barbie box) conserved among the B. megaterium P450 genes and the rat CYP2B1/CYP2B2 genes. However, it should be noted that
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PB treatment reduced binding of the bacterial protein(s) to the Barbie box but increased binding of the mammalian proteins to the same element. This suggests that barbiturate-mediated induction of B. megaterium P450BM1 may be mechanistically different from induction of mammalian P450s. Although some reports support the role of the Barbie box in PB responsiveness (39, 40), more data argued against a PB-inducible role for the Barbie box (41–45). These discrepancies suggest that the role of the Barbie box in PB-mediated regulation of mammalian P450s and other genes remains questionable.

On the other hand, the main mechanism responsible for barbiturate-mediated induction of P450BM3 in B. megaterium has been previously elucidated (6). Barbiturates can function as inducers to prevent binding of Bm3R1 repressor to its operator site, a 20-bp inverted repeat near the transcription start site of the P450BM3 gene. The in vivo potency of barbiturates as inducers of P450BM3 strongly correlates with their ability to inhibit interaction of Bm3R1 with its operator DNA in vitro. However, the mechanism by which barbiturates can moderately induce P450BM1 remains unresolved.

Liang et al. (8) proposed a role of the Barbie box in the induction of P450BM1 by pentobarbital. In contrast, our data indicated that deletion of the Barbie box did not markedly affect pentobarbital-mediated induction of P450BM1. In fact, there are conflicting data presented in previous publications by Liang et al. (7, 8). They reported that mutation of the Barbie box upstream of the P450BM1 promoter abolished pentobarbital-mediated induction of CAT activity in B. megaterium bearing a P450BM1-cat transcription fusion vector (see Fig. 3 in Ref. 8). However, gel mobility shift assays showed that both wild-type and mutant Barbie boxes exhibited similar binding activity toward E. coli cell-free extract containing overexpressed Bm3R1 (see Fig. 4 in Ref. 8 and Fig. 2 in Ref. 7). Furthermore, as shown in Fig. 9 of Ref. 8, deletion of a sequence further upstream of the Barbie box resulted in a lower level of induced CAT activity by pentobarbital compared with that after deletion of the Barbie box. These results suggest that the Barbie box located in the 5′-flanking region of the P450BM1 gene may not be crucial for pentobarbital-mediated induction of P450BM1. On the other hand, our data have confirmed that the sequence within the Barbie box is indeed important for negative regulation of P450BM1 gene expression in the absence of pentobarbital.

At least two cis-acting elements important for negative regulation of P450BM1 gene expression have been identified. One is the sequence within the Barbie box upstream of the P450BM1 gene as mentioned above. The other is the 53-bp inverted repeat located midway between the P450BM1 gene and the bm1P1 gene (46). Our preliminary data indicated that deletion of the 53-bp inverted repeat had no marked effect on induction of P450BM1 by pentobarbital (data not shown).

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Evidence against the Bm1P1 Protein as a Positive Transcription Factor for Barbiturate-mediated Induction of Cytochrome P450 BM-1 in Bacillus megaterium

Gwo-Chyuan Shaw, Chi-Chang Sung, Chang-Hsiesh Liu and Chia-Hung Lin

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