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cDNA Cloning, Sequence Analysis, and Induction by Aryl Hydrocarbons of a Murine Cytochrome P450 Gene, *Cyp1b1*

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ABSTRACT

C3H mouse embryo fibroblast cells, designated 10T1/2, can be transformed by physical and chemical agents including polycyclic aromatic hydrocarbons. In a previous report (Shen *et al.*, Proc. Natl. Acad. Sci. USA 90, 11483-11487, 1993), we identified a cytochrome P450 gene induced by polycyclic aromatic hydrocarbons (PAHs) that is different from *1A1* or *1A2*, and which we tentatively named *P450CMEF*. Here, we report the entire cDNA sequence of *P450CMEF* (5,128 bp) and the amino acid sequence deduced from it (543 residues). A comparison of the latter sequence with known cytochrome P450s indicates that *P450CMEF* is in a new subfamily of family 1 of the P450 superfamily. Accordingly, the Committee on Standardized Cytochrome P450 Nomenclature designated the gene *Cyp1b1*. Exposure to various aryl hydrocarbons (2.5 hr) induced *Cyp1b1* mRNA in 10T1/2 cells to different degrees: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 7,12-dimethylbenz[*a*]anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, and β-naphthoflavone were strong inducers; α-naphthoflavone and 3-methylcholanthrene, were moderate inducers; and benzo[*e*]pyrene was a weak inducer.

INTRODUCTION

THE CYTOCHROME P450 ENZYMES are heme-thiolate proteins that reductively metabolize many endogenous as well as xenobiotic compounds. They are an important component of the mixed-function oxidase system. More than 200 *P450* genes have been documented (Nelson *et al.*, 1993), which have been grouped into 36 gene families according to their amino acid sequences and gene structure. All of the P450 proteins have a signature amino acid sequence, FXXGXXXCXG, in which cysteine is involved in heme binding (Nelson *et al.*, 1993). In most cases, P450s from the same family of genes have more than a 40% amino acid identity and those in the same subfamily more than 55% (Nelson *et al.*, 1993). The oxidases in the metabolism of aryl hydrocarbons are called aryl hydrocarbon hydroxylases (AHHs) because they catalyze hydroxylation reactions (Singer and Grunberger, 1983). Products of p450 genes in family 1—*i.e.*, *1A1* and *1A2*—are involved in polycyclic aromatic hydrocarbon (PAH) metabolism (Nebert and Gonzalez, 1987; Gonzalez, 1989; Gonzalez *et al.*, 1991; Nebert *et al.*, 1991) as, for example, in the case of 3-methylcholanthrene (3MC) (Gonzalez *et al.*, 1984; Kawajiri *et al.*, 1984; Kimura *et al.*, 1984).

The transformable cell line, C3H mouse embryo fibroblasts called 10T1/2, have been widely used in studying the induction of neoplastic transformation (focus formation) by physical and chemical agents (Reznikoff *et al.*, 1973; Han and Elkind, 1979; Nesnow *et al.*, 1981, 1989; Suzuki *et al.*, 1981; Landolph, 1985a,b; Wells *et al.*, 1992). Based on (i) the profiles of the metabolic products of 7,12-dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (BaP) in 10T1/2 cells (Pottenger *et al.*, 1991; Wells *et al.*, 1991); (ii) the lack of inducibility of AHH activity by 3MC in these cells (Gehly *et al.*, 1979; Ho *et al.*, 1983; Okey *et al.*, 1983); and (iii) the observation that probes from *Cyp1a1* and *Cyp1a2* did not yield detectable hybridizations with mRNA from 10T1/2 cells (Christou *et al.*, 1990; Shen, unpublished data)—it was concluded that a different *P450* gene was involved in PAH metabolism in these cells (Pottenger and Jefcoate, 1990; Shen *et al.*, 1993b).

The new *P450* gene to be described was tentatively named

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P450CMEF (for *P450* from C3H mouse embryo fibroblasts); the sequence of part of its cDNA was been reported (Shen *et al.*, 1993b). The latter cDNA fragment hybridized with a major ~5.3-kb and a minor ~4.2-kb transcript. Both transcripts were inducible by DMBA and benz[*a*]anthracene (BA); sequence comparisons suggested that the fragment came from a gene in family 1.

In this paper, we report the cDNA cloning, sequence analysis, and mRNA induction of *P450CMEF*. Based upon the amino acid sequence deduced from the foregoing, *P450CMEF* has been assigned to a new subfamily, *Cyp1b* (assignment by Committee on Standardized Cytochrome Nomenclature).

MATERIALS AND METHODS

Cell culture and chemical treatment

C3H mouse embryo fibroblasts, designated 10T1/2, were used. Details of their culture have been reported (Wells *et al.*, 1991, 1992; Shen *et al.*, 1993b). DMBA and BaP, were purchased from Aldrich Chemical Co. (Milwaukee, WI; α-naphthoflavone (αNF), βNF, and benzo[*e*]pyrene (BeP) from Sigma Chemical Co. (St. Louis, MO); 3MC from Eastman Kodak (Rochester, NY) and 2,3,7,8-tetrachlorodibenz-*p*-dioxin (TCDD) from Midwest Research Institute, NCI Chemical Carcinogen Repository (Kansas City, MO). All chemicals were dissolved in acetone, HPLC-grade (Fisher Scientific, Fair Lawn, NY), and stored at stock concentrations of 10 m*M*, or 100 μ*M* for TCDD only. For BaP, BeP, αNF, βNF, and 3MC, 15 μl of the stock solutions were added to 15 ml of Eagle's basal medium with 10% fetal calf serum (BME-10), which resulted in 10 μ*M* final concentrations. For DMBA and TCDD, the stock solutions were diluted with acetone before use to give final concentration of 0.3 μ*M* and 10 n*M*, respectively. Control cells were treated with equal volumes of acetone alone.

Library construction and screening

From subconfluent, exponentially growing cells that were treated with 0.3 μ*M* DMBA for 3 hr, mRNA was extracted with the FastTrack mRNA Isolation Kit (Invitrogen, San Diego, CA). About 5 μg of the mRNA was reverse-transcribed into cDNA using a combination of oligo(dT) and random primers in a first-strand cDNA synthesis. A cDNA library was constructed with the α-ZAP II cloning system (Stratagene, La Jolla, CA). A known fragment of *P450CMEF* (Shen *et al.*, 1993b) was labeled with [^{32}P]dCTP and used to screen the cDNA library by employing Duralon nylon membranes (Stratagene, La Jolla, CA) according to the manufacturer's manual.

DNA sequencing

A two-step cycle-sequencing protocol similar to that previously described (Shen *et al.*, 1993a) was used for sequencing. Specific sequencing primers, sense or antisense, were synthesized with an Applied Biosystems Synthesizer (Model 380B, Applied Biosystems, CA) according to the sequence of known regions of the cDNA. Primers were labeled with [^{35}S]dCTP and/or [^{35}S]dATP, along with unlabeled dTTP and/or dGTP, by omitting at least one of the above four dNTPs, but not both ^{35}S-labeled compounds, depending on the downstream flanking sequences of a particular sequencing primer. In this way, adequate labeling of at least 4 bases and an extension of about 10 bases were obtained before the sequencing primers were arrested. The labeled and extended primers were subsequently used in dideoxy-termination, linear polymerase chain reaction (PCR) cycle sequencing at an elevated annealing temperature. Other procedures were as already described (Shen *et al.*, 1993a,b) except that, in some instances, denaturing Long-Ranger Sequencing Gels (AT Biochem, Malven, PA) were run according to the suggestion of manufacturer. Single antisense strands were also sequenced to confirm the sequence of the coding region of the gene. GenBank's computer programs were used for sequence analysis.

Plasmid handling and other procedures

Positive phage plaques were purified and *in vivo* excision (EXASSIST/SOLR SYSTEM, Stratagene, La Jolla, CA) was used to make the pBluescript (SK$^-$) plasmid containing the cDNA inserts. Plasmid DNA was isolated from host strain XL-1 Blue for direct PCR cycle sequencing and restriction endonuclease digestion by using QIAGEN Plasmid Kits (QIAGEN, Chatsworth, CA). Antisense single-stranded plasmid DNA was also purified following the manufacturer's procedure for the pBluescript EXO/MUNG DNA sequencing system (Stratagene, La Jolla, CA). RNA isolation and Northern blot hybridization were done as previously described (Shen *et al.*, 1993b).

RESULTS

cDNA cloning and sequencing

A λ-ZAP II cDNA library was constructed (Stratagene, La Jolla, CA) from the mRNA of DMBA-treated cells (see Materials and Methods). Using the fragment of cDNA that we had already sequenced as as probe, 75 putative *P450*-containing phage plaques were identified from among 4 × 10^5 pfu in the first round of screening. Ten of these selected at random were picked for a second round of screening. Three purified plaques were obtained, and these were cloned into the pBluescript (Sk$^-$) plasmid *via in vivo* excision of λ phage DNA (see Materials and Methods).

Phage DNA from these plaques was also PCR-amplified by using primers designed from the already known cDNA region of *P450CMEF* and the vector sequence to determine the length and orientation of the inserts *via* the protocol of Smith *et al.* (1990). Upon confirmation with restriction endonuclease digestions of the purified plasmids, it was determined that one of the three clones, #71, contained an insert of ~5.1 kb with its 5′ end linked to the T3 promoter and its 3′ end to the T7 promoter of the pBluescript (SK$^-$) vector.

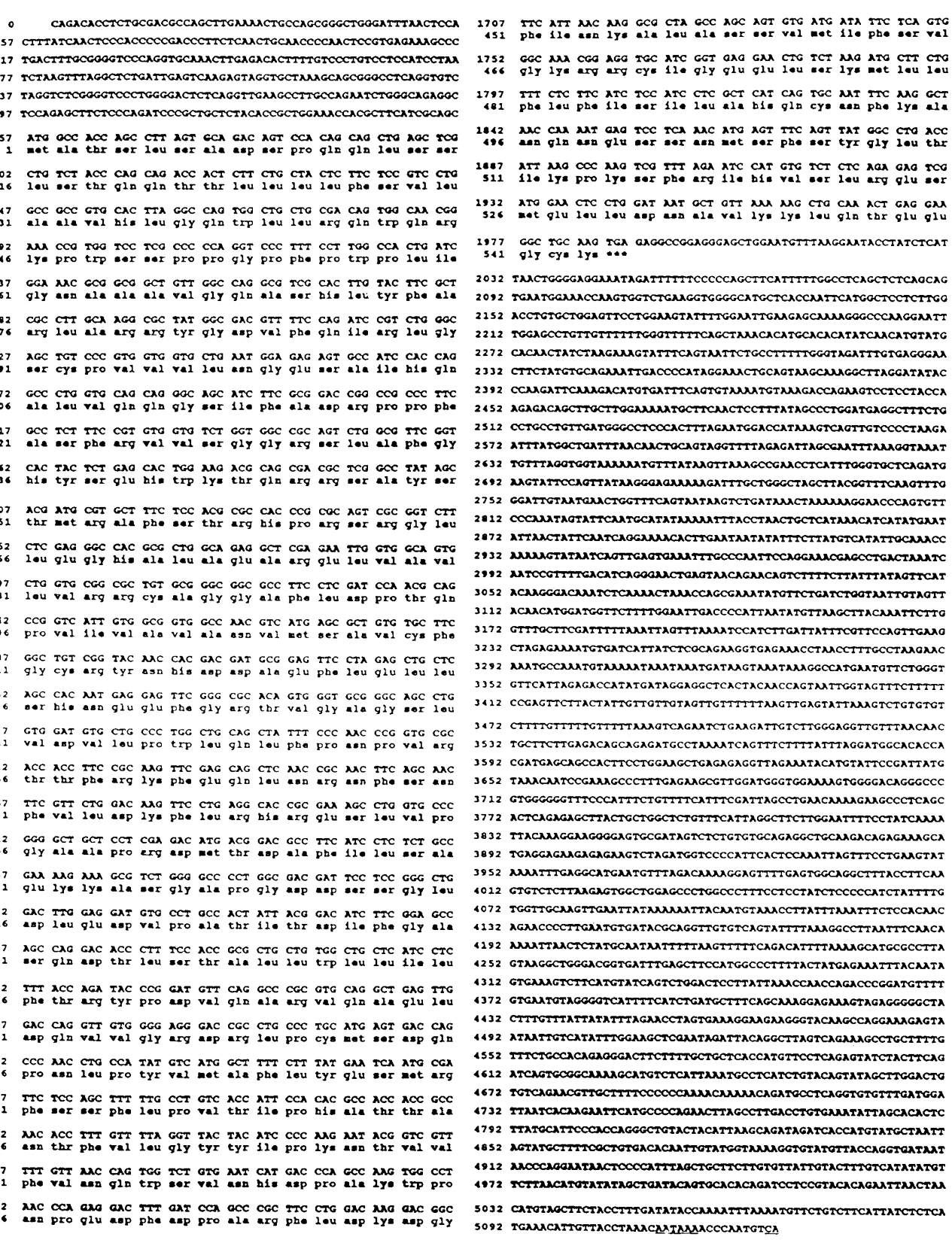

FIG. 1. The cDNA sequence and amino acid sequence of the open reading frame of the mouse *Cyp1b1* gene determined from a cDNA library made from actively growing C3H mouse 10T1/2 cells. The putative poly(A) addition signal (AATAAA), and the poly(A) addition site (CA) are identified (by underlining) according to the suggestions of Birnstiel *et al.* (1985).

About 0.1-0.2 μg of double-stranded plasmid DNA derived from clone #71 was used for direct DNA sequencing to obtain the whole cDNA sequence (see Materials and Methods). Parts of the inserts in clones #7 and #41 were also sequenced, but only revealed sequences identical to those in the corresponding regions of clone #71.

Sequence analysis

The cDNA sequence of clone #71 is shown in Fig. 1. It is 5,128 bases in length ending with CA. From 1,437-1,753 bp is the fragment that was previously reported (Shen *et al.*, 1993b). Ten base pairs upstream from the 3′ *CA* is the location of a putative poly(A) addition signal sequence, *AATAAA*. Birnstiel *et al.* (1985) reported that most poly(A) signals are added to a CA located 9-30 bp downstream of the poly(A) addition signal. Accordingly, it is likely that the cDNA sequence in Fig. 1 contains the 3′ end of *P450CMEF* cDNA except for the poly(A) itself. Further, if we assume that the average size of a poly(A) site is 260-300 bp (Birnstiel *et al.*, 1985), we would estimate that the entire length of the mRNA corresponding to the cDNA in Fig. 1 is ~5.4 kb. The latter length agrees with what we have observed with Northern blot hybridization, ~5.3 kb (Shen *et al.*, 1993b). Hence, the sequence in Fig. 1 probably is the entire cDNA sequence of *P450CMEF*.

The regions corresponding to the degenerate primers that were used in the original identification of this gene (Shen *et al.*, 1993b) are upstream, 1,419-1,438 bp, and downstream, 1,753-1,774. Accounting for the degeneracies that were designed into the primers and the use of inosine, which is nonspecific in its base-pairing, the downstream primer has turned out to completely match the sequence. However, the upstream degenerate primer contained five mismatches (25%).

Analysis of the sequence in Fig. 1 revealed an open read-

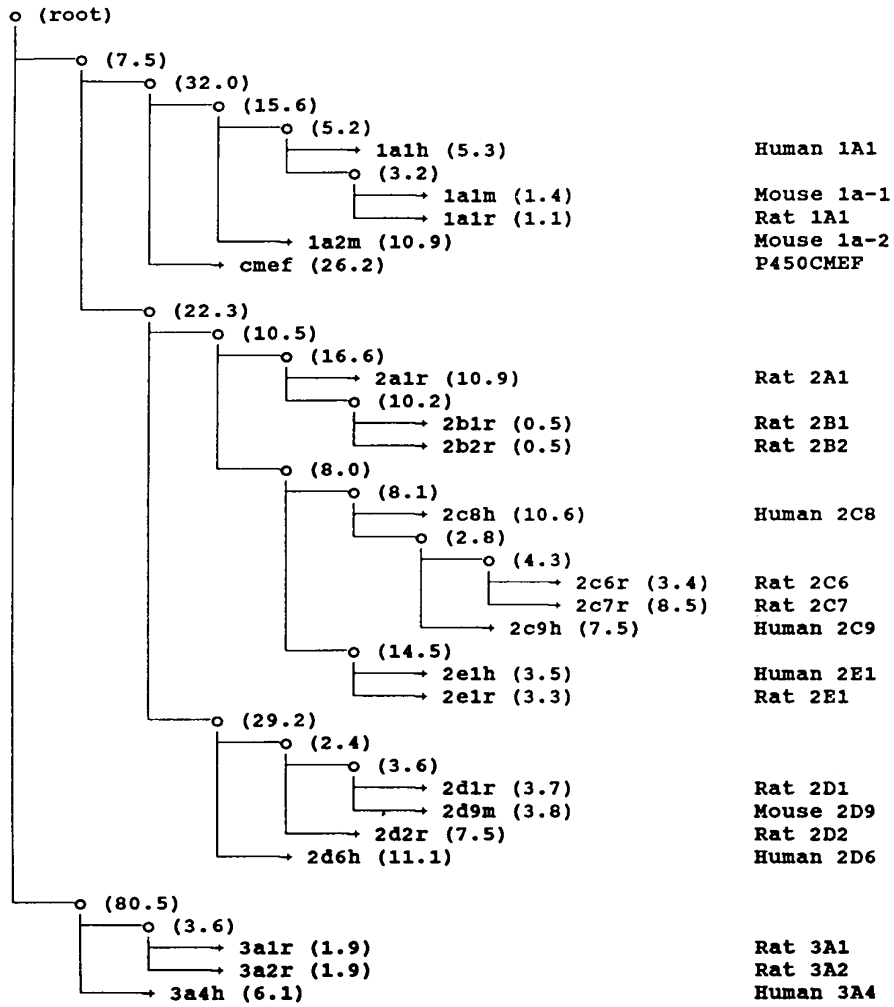

FIG. 2. Phylogenetic tree generated by progressive alignment according to Feng and Doolittle (1987), which shows the relationship of P450CMEF with other P450 proteins. (○) Node or branching point; (→) a terminal sequence; and (number) distance to previous node in arbitrary units. The names on the right indicate the species and specific P450 sequences that were compared.

ing frame starting at position 375, which would code for a putative sequence of 543 amino acids having a molecular weight of ~60 kD. Accordingly, we propose that 357–359 bp (Met^1) should be the translation start codon because: (i) in the same reading frame, there is a stop codon at 237–239 bp (*i.e.*, 120 bp upstream of this assigned start codon); (ii) the next possible start codon would be 810–812 bp which would code for Met^{152}; and (iii) the proposed sequence of amino acids between Met^1 and Met^{152} is a region that is highly homologous to Cyp1a suggesting that it is unlikely that 810–812 bp is the start codon.

The sequence of the open reading frame has a larger degree of homology with P450s in subfamily 1A than with any other. By using the GenBank alignment program (Altschul and Erickson, 1986), with a Dayhoff cost matrix and a penalty factor of 2.5 for opening a gap and an incremental penalty factor of 0.5, the sequence was found to have a 38–39% amino acid identity with subfamily 1A P450s. However, when the FASTA program in GenBank was used (Pearson and Lipman, 1988), which only aligns certain primary regions, a homology of 40–41% with 1A P450s was found. The preceding comparisons suggest at least that *P450CMEF* should be in a different subfamily from that of 1A.

In view of the preceding uncertainties, the classification of *P450CMEF* to a specific *P450* family was guided by the phylogenetic tree-building program in GenBank of Feng and Doolittle (1987). By comparing the homologies of some 20 amino acid sequences in families 1, 2, and 3 with the putative sequence of P450CMEF, a phylogenetic tree was constructed (Fig. 2). It is evident from Fig. 2 that, although *P450CMEF* should be in a new subfamily, it still belongs in family 1. Based on the open reading frame discussed above, the Committee on Standardized Cytochrome P450 Nomenclature has assigned this gene to a new subfamily called *Cyp1b1* (D. Nelson, D. Nebert, personal communications, 1993), a designation that we will also use in the remainder of this report.

Induction of Cyp1b1 *by PAHs in 10T1/2 cells*

The ability of BA and DMBA to induce *Cyp1b1* mRNA has been previously described (Shen *et al.*, 1993b). Other reports, as well as our own results (unpublished data), have shown that in 10T1/2 cells, 3MC and BeP do not induce AHH activity (Gehly *et al.*, 1979; Ho *et al.*, 1983). TCDD (Ho *et al.*, 1983), BaP (Gehly *et al.*, 1979), and βNF do induce AHH activity, and αNF frequently is an inhibitor.

With regard to the *Cyp1b1* mRNA, the action of a number of aryl hydrocarbons has been examined. Subconfluent 10T1/2 cells were exposed for 2.5 hr to 10 μ*M* of BaP, BeP, αNF, βNF, or 3MC, or to 10 n*M* of TCDD. Total RNA was used for Northern blot hybridization. The results from a single experiment are shown in Fig. 3a. Compared to acetone-treated controls, the averages (and standard deviations) of the relative mRNA levels induced by these treatments in several replicate experiments are shown in Fig. 3b (see legend for the details). It is evident that BA, BaP, βNF, and TCDD are strong inducers; αNF and 3MC are moderate inducers; and BeP induces to only a minor degree.

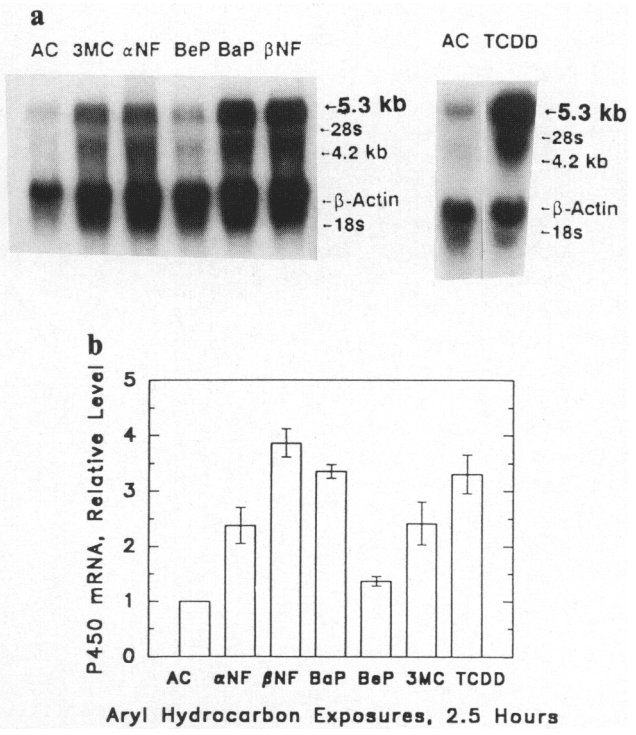

FIG. 3. a. Northern blot hybridization of *P450CMEF* (*Cyp1b1*) with total RNA from TCDD-treated C3H-10T1/2 cells and cells treated with different PAHs. Subconfluent cells were incubated with 10 μ*M* of αNF, βNF, BaP, BeP, 3MC, or 10 n*M* of TCDD for 2.5 hr. Acetone-treated cells (AC) were used as the controls. The label at the top of the figure indicates the treatment. Bases 979–1,404 (Fig. 1) was amplified from a cDNA plasmid, purified with the QIAGEN gel purification kit (QIAGEN, Chatsworth, CA); the antisense strand was labeled with [^{32}P]dCTP by linear PCR and used for Northern blot hybridization of *P450CMEF* (*Cyp1b1*) mRNA. After the radioactivity of the membrane had been scanned with an Ambis Radioanalytical Image System (San Diego, CA) and an X-ray film had been exposed, the membrane was stripped and rehybridized with a β-actin probe (Shen *et al.*, 1993b) for the normalization of RNA loading, and a second X-ray film was exposed. b. Induction of *P450CMEF* (*Cyp1b1*) mRNA in C3H mouse 10T1/2 cells. Quantitation was obtained from the relative counting rates of ^{32}P-labeled probes retained on the membranes. Averages of three to five hybridization experiments, including the results in Fig. 3a, and their standard deviation are shown.

DISCUSSION

The 5,128-bp sequence that we have described for *P450CMEF* has an open reading frame on the basis of which this gene has been assigned to a new rodent subfamily with the designation *Cyp1b1*. This P450 gene was first detected by the application of highly degenerate primers and PCR amplification (Shen *et al.*, 1993b). This method proved to be successful even though the upstream degenerate,

inosine-containing primer that was used proved to contain five mismatches (25%), illustrating the versatility of the method.

A 55-kD P450 protein, called P450-EF had been identified from BA-treated 10T1/2 cells (Pottenger *et al.*, 1991). Western blots showed that P450-EF has no cross-reactivity with P450 proteins in subfamily 1A. Although P450-EF might belong in a new family as suggested by Pottenger *et al.* (1991), because sequence information is not available, the relationship between the genes *P450-EF* and *Cyp1b1* is not known.

In our previous report, two mRNA bands, ~5.3 kb and ~4.2 kb, were observed when a probe from bases 1,419–1,774 in Fig. 1 was used. The same two bands were observed (Fig. 3a) when a probe from bases 979–1,404 was used. In addition to the possible reasons for two mRNAs that have already been discussed (Shen *et al.*, 1993b), because 10T1/2 cells are immortal we note that a transcript from a mutated copy of the gene also is a possibility.

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Sequence reported in this paper has been deposited in GenBank under accession number U03283.

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