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Identification of a cytochrome P450 gene by reverse transcription–PCR using degenerate primers containing inosine

(C3H 10T½ cells/dimethylbenz[*a*]anthracene/benz[*a*]anthracene/aryl hydrocarbon hydroxylase)

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ABSTRACT **A cytochrome P450-like gene, tentatively named P450CMEF, was amplified by a mixed oligonucleotide-primed amplification of cDNA from C3H mouse embryo fibroblast cells, designated 10T½, that had been treated with 7,12-dimethylbenz[*a*]anthracene (DMBA) or benz[*a*]anthracene (BA). A set of inosine-containing degenerate primers that were targeted to two conserved regions of known cytochrome P450 cDNAs were used. One primer was coded for the well-described and conserved heme-binding region of P450 enzymes, and the second was designed based upon other considerations of homology among P450 molecules. One of the four PCR-amplified cDNA products hybridized to two major RNA bands, 4.2 and 5.3 kb, that were induced by DMBA or BA. The amino acid sequence of the fragment deduced from the base-sequence data indicate that the amplified cDNA has a 50–55% identity with the cytochrome P450 subfamily 1A. The induction of P450CMEF mRNA preceded the induction of aryl hydrocarbon hydroxylase activity after DMBA or BA treatment, suggesting that the product of P450CMEF is involved in the metabolism of these polycyclic aromatic hydrocarbons in 10T½ cells. From the partial sequence of the cDNA identified by this procedure, we propose that P450CMEF is a member of the P450 superfamily, possibly in a subfamily of family 1, that is induced in 10T½ cells by DMBA and BA. This method should be useful in identifying additional P450 genes and genes in other gene families.**

The cytochrome P450 enzymes form a superfamily of heme-containing proteins that are bound to the membranes of the endoplasmic reticulum and serve as the oxidation–reduction component of the mixed-function oxidase system (1, 2). *In vivo*, this system is involved in the oxidative metabolism of both endogenous compounds, such as steroids, and xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs; refs. 1 and 2). For PAHs, these enzymes catalyze a hydroxylation reaction; hence, they are also identified as aryl hydrocarbon hydroxylases (AHHs; ref. 2). More than 200 P450 genes have been described and classified into 36 families (3) according to their amino acid sequences. Within a single family, P450 proteins have >40% amino acid identity and, within a subfamily, usually >55% identity. The cysteine-containing region associated with the heme binding of a P450 protein is highly conserved among all eukaryotic species (1, 4–6). Families 1, 2, and 3 are involved in the metabolism of xenobiotics (6, 7). Two members have been identified in family 1, 1A1 and 1A2, that are involved in PAH metabolism (3, 7, 8).

The C3H mouse embryo fibroblast cell line, designated 10T½ (9), has been widely used to study PAH-induced neoplastic transformation (9–11, 14, 15). The induction of AHH in 10T½ cells is influenced by many factors. Several lines of evidence suggest that one or more P450 enzymes that, to our knowledge have not been previously described, rather than P4501A1 or P4501A2, are involved in PAH metabolism in these cells. (*i*) The profile of metabolites from 10T½ cells is different from that due to P4501A1/1A2 (16, 17). Also, the absence of an induced AHH activity in 10T½ cells by 7,12-dimethylbenz[*a*]anthracene (DMBA) and/or benz[*a*]anthracene (BA)—as measured by dealkylation of 7-ethyoxyresorufin, which is specific for several known P450 enzymes—suggests that 10T½ cells use a different gene (ref. 18 and R.L.W., unpublished data). (*ii*) 3-Methylcholanthrene, a PAH that can induce 1A P450 enzymes (19), is not able to induce AHH activity in 10T½ cells. Consistent with this observation, 3-methylcholanthrene is weakly cytotoxic in these cells; however, 10T½ cells are transformed by this PAH (refs. 10 and 20–23 and Wells *et al.*, unpublished data). (*iii*) When probes made from 1A1 and 1A2 genes were used to analyze the RNA from 10T½ cells, no expression of these genes was detected (ref. 24 and Z.S., unpublished data).

To determine whether another P450 is involved in the metabolism of DMBA or BA in 10T½ cells, an approach was used similar to the method of mixed oligonucleotide-primed amplification of cDNA (25, 26). We hypothesized that the product of an as yet unidentified PAH-inducible P450 gene should have a degree of homology with the products of known P450 genes. The P450 genes considered to be likely candidates were those in family 1 involved with PAH metabolism and those in families 2 and 3 whose products include enzymes involved in the metabolism of foreign chemicals. Degenerate PCR primers were designed to be targeted to relatively conserved regions of the proteins of these three families. Because of its ability to pair with any base, inosine was used to reduce the complexity of the mixture of primers (27, 28). Four bands were identified. The amino acid sequence that was deduced from the cDNA sequence of one of these bands was found to have a high degree of homology with the P450 proteins of subfamily 1A. In addition, using the cDNA from this band as a probe in Northern blot hybridization, two RNA bands were identified that were induced by DMBA or BA. Our results support the likelihood that, in 10T½ cells, DMBA or BA induces a P450 gene related to those in family 1.†

MATERIALS AND METHODS

Cell Culture and Cell Treatment. C3H mouse embryo fibroblast cells—designated 10T½, passages 9–15, and initially obtained from John Bertram (University of Hawaii)—were used in this study. Cells were cultured in Eagle's basal

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Abbreviations: BA, benz[*a*]anthracene; DMBA, 7,12-dimethylbenz[*a*]anthracene; AHH, aryl hydrocarbon hydroxylase; PAH, polycyclic aromatic hydrocarbon.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U02479).

minimum essential medium (BME) supplemented with 10% (vol/vol) fetal bovine serum (Intergen, Purchase, NY; BME-10) plus 0.02 M Hepes (Research Organics) and were incubated at 37°C in a humidified atmosphere of 2% CO_2/98% air. Other details of the culture and growth of the cells have been described (14).

Eighty thousand cells were plated into 100-mm plastic dishes (Corning) containing 15 ml of BME-10. After incubation for 3 days, 75 μl of DMBA or BA, dissolved in HPLC-grade acetone (Fisher Scientific), was added to the medium to 0.3 μM DMBA or 10 μM BA, concentrations in the range of those used for AHH induction and/or neoplastic transformation *in vitro* (14, 17, 24). Control cells were treated with an equal concentration of acetone alone, 0.5%, in 75 μl.

Measurement of AHH Activity. After rinsing with phosphate-buffered saline, cells were detached from dishes by scraping with a sterile rubber policeman, counted, suspended in 50 mM Tris·HCl (pH 7.5), and stored frozen at −100°C before the measurement of AHH activity. For the latter purpose, a rapid synchronous fluorescence method was used based upon measurements with cell lysates (17).

RNA Isolation. Isolation of total RNA from 1×10^7 cells was performed by the single-step method of Chomczynski and Sacchi (29). For mRNA isolation, 1×10^6 cells were treated with the Micro-FastTrack Kit (Invitrogen) by the manufacturer's protocol.

Mixed Oligonucleotide-Primed Amplification of cDNA. From 1×10^6 cells, mRNA was reverse-transcribed to cDNA using the cDNA cycle kit (Invitrogen), and then the cDNA was amplified with a set of inosine-containing degenerate primers (see *Results* for the sequences of the primers), which were targeted at two conserved regions of the products of P450 gene families 1, 2, and 3 (see below).

Preparation of Labeled Probes for Northern Blot Hybridization. β-Actin, which is not inducible by PAHs (ref. 24 and Z.S., unpublished data), was used as an internal control and to normalize the mRNA results. To obtain a β-actin probe, 5×10^5 cells were lysed (30 min, 37°C) with 5 μl of 10% (vol/vol) Nonidet P-40 and 5 μl of proteinase K (2 mg/ml) in a total volume of 100 μl. The lysate was heated for 5 min at 95°C to denature the proteinase K and then centrifuged at $4000 \times g$ for 5 min. Supernatant (10 μl) was used to amplify part of the β-actin gene in 50 μl with the following primers: (*i*) upstream sense primer, 5′-GAGACCTTCAACACCCCA-3′; (*ii*) downstream antisense primer, 5′-ATCTCCTTCTG-CATCCTGTC-3′. The amplified band for β-actin, as well as other PCR bands amplified separately for other purposes, were cut out of the gel and purified with a gel purification kit (Qiagen, Chatsworth, CA) by the manufacturer's instructions except that the elution was done with 0.1 × TE (1 mM Tris·HCl, pH 8.0/0.1 mM EDTA). Purified fragments were labeled with ^{32}P as noted below.

All of the probes used in Northern blot hybridizations were labeled with [α-^{32}P]dCTP (specific activity, ≈3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) by linear PCR amplification. Appropriate amounts of antisense primers and templates were added to 5.0 μl of [α-^{32}P]dCTP, 1.0 μl of 1.0 mM dTTP/1.0 mM dATP/1.0 mM dGTP, 0.2 μl of *Taq* enzyme (5 units/μl; Perkin–Elmer/Cetus or Boehringer Mannheim), an appropriate amount of PCR buffer (provided with the *Taq* enzymes), and sterile distilled H_2O to make 20 μl. Ten cycles of PCR amplification used the same sequence of steps and temperatures as in exponential PCR amplification, depending on the probes and primers involved. The labeled single-stranded probes that resulted were purified with Sephadex G-50 Quick Spin columns (Boehringer Mannheim), denatured, and used for Northern blot analysis for hybridization procedures (see Fig. 5).

Direct-Cycle DNA Sequencing. To sequence the amplified cDNA, a cycle-sequencing method was used that had been found to be suitable for inosine-containing highly degenerate primers (30). Based upon the tentative sequence obtained with these degenerate primers, internal primers were synthesized and used to confirm the sequence by sequencing both strands. Parallel reactions with the substitution of dGTP and dideoxyguanosine 5′-triphosphate (ddGTP) with 7-deaza-dGTP and 7-deaza-ddGTP, respectively, were also done to minimize the likelihood of artifacts due to gel compression. Reagents called for in the TAQuence Version 2.0 kit (United States Biochemical) were used.

Other Molecular Procedures. A plasmid containing the P4501A2 gene, which was used in optimizing the conditions for PCR amplification and DNA sequencing, was kindly provided by D. W. Nebert and T. Petersen (University of Cincinnati Medical Center). The plasmid was grown in the bacterium HB101 and then isolated (31). The plasmid was cut with *Xho* I to release a linear partial fragment of the P4501A2 cDNA for use in the optimization of the PCR steps. The linearized plasmid was also amplified with the same set of degenerate primers referred to above. The resultant product was purified and used to optimize the sequencing procedure.

All primers were synthesized in an Applied Biosystems synthesizer, model 380B (Applied Biosystems). To search for homologies between the DNA fragment of the cDNA that was induced in 10T½ cells by DMBA or BA and known sequences, the EUGENE program at GenBank was used.

RESULTS

Design of the Degenerate Primers. For the PCR amplification of the presumably unknown P450 gene inducible by DMBA or BA, degenerate primers were designed based upon known sequences of P450 cDNAs—in families 1, 2, and 3 from human, rat, and mouse cDNAs—that were retrievable from GenBank (Table 1). From the cDNA sequence of the well-described cysteine-containing heme-binding region (1, 4–6), the following downstream antisense degenerate primers were designed: 5′-TCICCIAWRCAIHDICKYTTICC-3′, where W is A or T; R is A or G; H is C, A, or T; D is G, T,

Table 1. P450 genes used in designing degenerate primers

Gene symbol (trivial name)	GenBank locus no.	Primer regions, bp		Expected size, bp
		Upstream	Downstream	
CYP1A1 (P-1)	HUMCYP145	1110–1129	1443–1465	356
CYP1a-1 (P1)	MUSCYP145X	1137–1156	1470–1492	356
CYP1A2 (P3)	HUMVYP345	1032–1051	1368–1390	359
CYP1a-2 (P3)	MUSCYP34A	1078–1097	1414–1436	359
CYP2A1 (P-450a)	RATCYP45F	989–1008	1316–1338	350
CYP2A3 (IIA3)	J02852	955–974	1282–1304	350
CYP2A6 (IIA3)	HUMCPIIA3	985–1004	1312–1334	350
CYP2A7 (IIA4)	HUMCYIIA4A	985–1004	1315–1337	353
CYP2B6 (IIB)	HUMCYP2BA	973–992	1300–1322	350
CYP2C6 (PB1)	RATCYPPB	949–968	1276–1298	350
CYP2C7 (PB1)	RATCYPPBA	949–968	1276–1298	350
CYP2D1 (IID1)	RATCY2DA	1048–1067	1375–1397	350
CYP2D3 (IID3)	RATCY2DB	1052–1071	1379–1401	350
CYP2D5 (IID5)	RATCY2DC	1013–1032	1340–1362	350
CYP2d-9 (16-α)	MUSTHAA	1068–1087	1395–1417	350
CYP2E1 (j)	RATCYPJ	982–1001	1309–1331	350
CYP2E1 (j)	HUMCYPJ	982–1001	1309–1331	350
CYP2F1 (2F)	HUMCYPIIF	1022–1041	1349–1371	350
CYP3A1 (PCN)	RATCYP45P	1072–1091	1396–1418	347
CYP3A2 (PCN2)	RATCYPCN2	1057–1076	1381–1403	347
CYP3A4 (PCN1)	HUMCYPNOA	1078–1097	1402–1424	347
CYP3A5 (PCN3)	HUMCYPCN	1075–1094	1396–1418	344

Gene symbol nomenclature recommended by Nebert *et al.* (8) was used.

```
      5'AAAITCCAIGAGGAGCTIGA3'
         GG  G   A A  AA
                 C
m1a1: AAGATCCAGGAGGAACTAGA
h1a1: --------A-----G-----
h1a2: ---------A----G--G--
h2a6: ---G----T-----GA-T--
h2b6: -GAG--T-CA----GA-T--
h2e1: ---C----T--A---A-T--
h2f1: CGCG-G--------GA-C--
h3a4: --AC-G---------A-T--
h3a5: --AC-G--AA----GA-T--
m1a2: --------T-----G--G--
m2d9: -GAG----AC-A---A-C--
r2a1: ---G----T------A-T--
r2a3: ---G----C-----GA-T--
r2c6: --AG-------A--GA-T--
r2c7: --AG-------A--GA-T--
r2d1: -GAG----AC-A--GA-T--
r2d3: CGAG-A--AC-----A-C--
r2d5: -GAG----AC-A--GA-T--
r2e1: --AC-T--T--A---A-T--
r3a1: --AC-G--------GA-C--
r3a2: --AC-G--------GA-C--
      |||  |  gene
      ||   |  subfamily
      |    |  family
      |    |  species
```

FIG. 1. Conserved regions of P450 genes from which the upstream primers were designed. This region is 321–336 bp upstream from the conserved heme-binding region. The regions are aligned relative to mouse *CYP1a1* gene, indicated as "m1a1" in the figure. Dash, base identical to m1a1. Degenerate primer sequences designed are also shown at the top. When all four bases at one position were possible, inosine (I) was used to reduce the number of primers required in the mixture. h, Human; r, rat; m, mouse.

or A; K is T or G; Y is T or C; the underlined triplet codes for cysteine; and inosine is I. The cDNA region in question is in exon 7 of the mouse *CYP1a1* gene and exon 9 of some of the members of family 2. When the P450 cDNA sequences in Table 1 were compared, a relatively conserved region, 321–336 bp upstream from the heme-binding regions, was identified as in Fig. 1. This region was used to design the following upstream primers: 5'-ARRITSCAIVARGA-RMTIGA-3', where S is G or C, V is C, A, or G, and M is A or C. The preceding sequence, which overlaps with the end of exon 4 and the beginning of exon 5 in the mouse *CYP1a-1* gene, is located in exon 7 of some members of family 2. With this set of upstream primers, we anticipated that PCR products differing in size and, therefore, readily distinguishable would be obtained from cDNA vs. genomic DNA.

The upstream primer mixture had 192 20-mers containing three inosines. Because inosine can bind to any base, theoretically these primers could anneal to some 12,000 sequences. The downstream primer mixture had 144 23-mers containing five inosines. Theoretically, these could have annealed to some 147,000 sequences. As demonstrated (25), up to a 20% primer mismatch might not affect the amplification of an unknown gene when annealing conditions of only moderate stringency are used. For a few of the members of family 3 (Table 1), the preceding primer sets may have contained up to six mismatches.

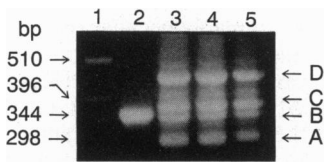

FIG. 2. Electrophoretic bands (ethidium bromide fluorescence) produced by the method of mixed oligonucleotide-primed amplification of cDNA. cDNA from 1×10^6 10T½ cells was amplified. PCR amplification conditions: denaturation at 95°C, 1 min; annealing at 50°C, 1.5 min; strand extension at 72°C, 2 min plus a 4-s extension per cycle. A total of 30 cycles was used. PCR products were loaded into a 1.5% agarose gel in TBE buffer containing ethidium bromide (0.5 μg/ml) and then electrophoresed. Lanes: 1, DNA ladder; 2, amplified *CYP1a2* gene cDNA; 3, amplified from cDNA from 10T½ cells after a 4-h treatment with 10 μM BA; 4, amplified cDNA from 10T½ cells after a 4-h treatment with 0.3 μM DMBA; 5, amplified cDNA from 0.5% acetone-treated cells. Estimated sizes of the four major bands: A, ≈305 bp; B, ≈357 bp; C, ≈374 bp; D, ≈457 bp. The DNA from these bands was purified and used as probes in Northern blot hybridization and for direct DNA sequencing (see Fig. 5).

```
GAC CAG GTT GTG GGG AGG GAC CGC CTG CCC TGC ATG AGT GAC CAG
    gln val val gly arg asp arg leu pro cys met ser asp gln

CCC AAC CTG CCA TAT GTC ATG GCT TTT CTT TAT GAA TCA ATG CGA
pro asn leu pro tyr val met ala phe leu tyr glu ser met arg

TTC TCC AGC TTT TTG CCT GTC ACC ATT CCA CAC GCC ACC ACC GCC
phe ser ser phe leu pro val thr ile pro his ala thr thr ala

AAC ACC TTT GTT TTA GGT TAC TAC ATC CCC AAG AAT ACG GTC GTT
asn thr phe val leu gly tyr tyr ile pro lys asn thr val val

TTT GTT AAC CAG TGG TCT GTG AAT CAT GAC CCA GCC AAG TGG CCT
phe val asn gln trp ser val asn his asp pro ala lys trp pro

AAC CCA GAG GAC TTT GAT CCA GCC CGC TTC CTG GAC AAG GAC GGC
asn pro glu asp phe asp pro ala arg phe leu asp lys asp gly

TTC ATT AAC AAG GCG CTA GCC AGC AGT GTG ATG ATA TTC TCA GTG GG
phe ile asn lys ala leu ala ser ser val met ile phe ser val
```

FIG. 3. Base-pair sequence and the amino acid sequence derived from the open reading frame of the fragment of P450CMEF cDNA that was sequenced. The underlined bases GA come from the 3' end of the upstream primer, and the underlined bases GG complement the 3' end of the downstream primer. If the two primers were added (43 bp), the total length would be 356 bp.

Amplification of a cDNA Fragment with Mixed Primers. After the mRNA from cells was reverse-transcribed into cDNA, the latter was amplified with the set of degenerate primers described above. PCR amplification resulted in four bands in the electrophoresis gel (Fig. 2). To screen for a possible P450 cDNA fragment, each band in Fig. 2 was cut out, purified, labeled with [^{32}P]dCTP, and hybridized with RNA extracted from DMBA- or BA-treated cells. Band B hybridized to two DMBA- or BA-inducible RNA bands, ≈4.2 and ≈5.3 kb (as illustrated in Fig. 5). Also, the size of band B in Fig. 2 (350–360 bp) is close to what was to be expected from the sequences of the known P450 genes (Table 1). Only band B is discussed further.

DNA Sequences. Fig. 3 shows the 313-bp sequence of DNA between the degenerate primers. To account for the lengths of the primers (23 + 20 = 43 bp), the length of band B in Fig. 2 is the same as that expected from the known 1A1 genes in Table 1. Thus, as in Fig. 3, a peptide of 104 amino acids was deduced and used in comparisons with other sequences. After the cDNA fragment was sequenced, the sequences of the primers in the degenerate mixture that were specific and had been incorporated into the amplified product were obtained. But, as noted, mismatches are possible when degen-

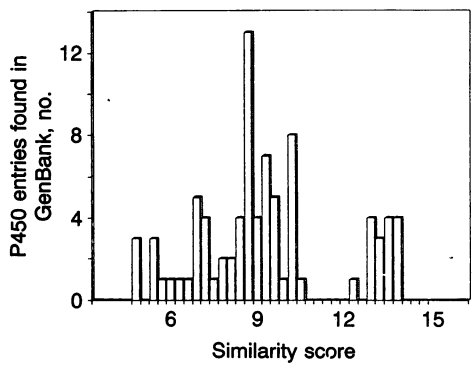

FIG. 4. Distribution of similarity scores of the P450CMEF fragment with known P450 amino acid sequences in GenBank. The higher the similarity score, the greater the homology.

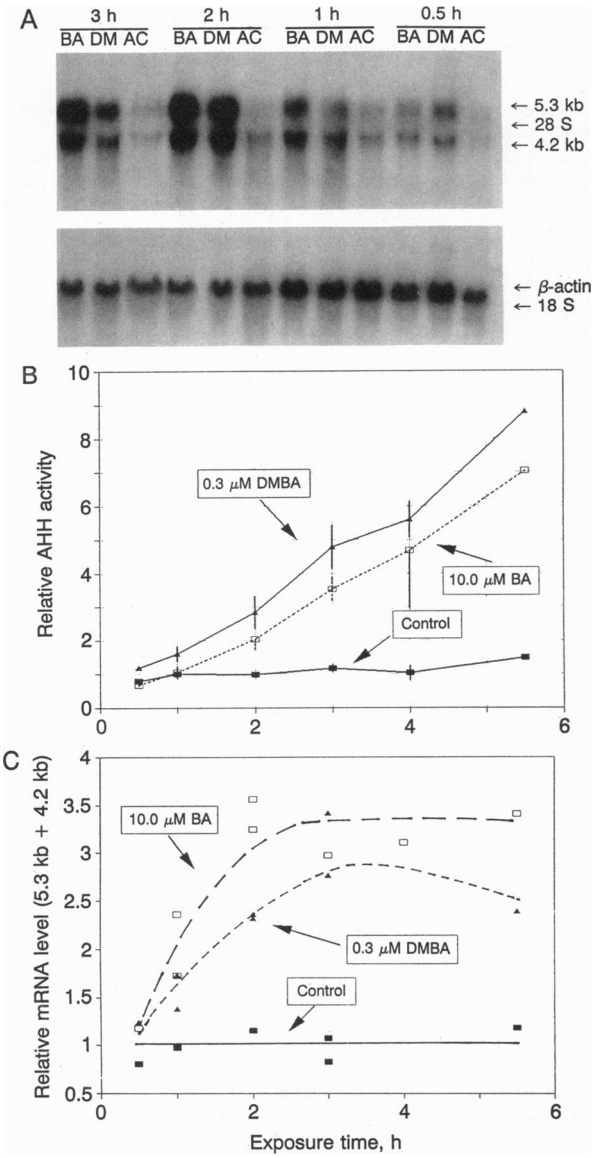

FIG. 5. Time course of the induction of AHH activity and P450CMEF mRNA in 10T½ cells by DMBA (DM) or BA. (*A*) Northern blot hybridization with P450CMEF and β-actin cDNA probes. An unidentified amount of mRNA, isolated from 1.0×10^6 cells after various periods of treatment, was loaded into a 1.0% formaldehyde denaturing gel, electrophoresed, transferred to a Zeta-Probe GT nylon membrane (Bio-Rad) using a BIOS blotting unit (Bios, New Haven, CT), and UV-cross-linked with an exposure of 160 mJ/cm² using a Stratalinker, model 2400 (Stratagene). The membranes were treated with [α-^{32}P]dCTP-labeled single-strand probes at high stringency [50% (vol/vol) formamide, 43°C] by incubating them overnight in a hybridization incubator (Robbins Scientific, Mountainview, CA) and then washing as described for Zeta-Probe membranes. Membranes were exposed to Kodak XAR-5 film and developed. After blotting, the membrane was striped and rehybridized with a β-actin probe, as an internal control for the amount of RNA analyzed. The sizes of the two bands probed with the DNA from band B (Fig. 2) were estimated from the sizes and positions of 18S and 28S rRNA. By using an RNA ladder (Bethesda Research Laboratories Life Technologies), the sizes of 18S and 28S rRNAs were estimated to be 1.8 and 4.7 kb, respectively (data not shown). AC, acetone treatment only. (*B*) AHH activity as function of time after cell treatment with DMBA or BA. Cells were treated with acetone only control (■), 0.3 μM DMBA (▲), or 10 μM BA (□). Data are from the two experiments shown in *C* plus three other experiments. Uncertainties are SEMs. (*C*) Relative induction of 4.2- and 5.3-kb mRNAs (intensities combined). Symbols are as for *b*. ^{32}P in the membranes was measured with an Ambis radioanalytic imaging system (AMBIS Systems, San Diego) to locate the hybridized bands and to quantitate their radioactivities. The counting rates were normalized to those of the β-actin bands. Data shown are the combined results of the bands in *A* and another experiment (autoradiographs not shown). Two points plotted at the same time were from different experiments.

erate primers are used (25). For that reason, only the sequence between the primers is shown in Fig. 3.

By using the FASTA program in GenBank for evaluating similarities in DNA sequences (32–35), the sequences in Fig. 3 were compared with DNA and peptide sequences in the data base. The following information was obtained. (*i*) Relative to the sequence shown in Fig. 3, all of the DNA entries in GenBank, which have similarity scores >6.0 and homologous regions >100 bp, were P450 sequences. (Homology increases with increasing similarity score.) (*ii*) All of the peptides with similarity scores >4.5 were P450 peptides. (*iii*) None of the known P450 genes in GenBank was identical with the sequence in Fig. 3. The highest nucleic acid similarities were with DNA sequences that are 55–60% homologous and are in subfamily 1A. The highest amino acid similarities, 50–55%, were with proteins also in subfamily 1A.

Fig. 4 shows the similarity scores between the amino acid sequence in Fig. 3 and the P450 amino acid sequences that can be found in GenBank. It is evident that one group of entries, 16 entries in all, have noticeably higher similarity scores of 12 or more and is well separated from the scores of the remainder. All of the genes within the group of higher scores are members of subfamily 1A. Tentatively, we have named the gene, of which the sequence in Fig. 3 is a fragment, P450CMEF for the P450 from C3H mouse embryo fibroblasts.

When the amino acid sequence of P450CMEF was aligned with some other P450 amino acid sequences, we found mouse 1a1 and human 1A1 have 55 identical amino acids with the fragment of P450CMEF; mouse 1a2 has 53; human 1A2 has 57; and rat 2A1 has 39. Mouse 1a2 and human 1A2 have one more amino acid than the peptide in Fig. 3. The preceding data support the likelihood that band B in Fig. 2 was amplified from the cDNA of another P450 gene that was induced in 10T½ cells. Our data suggest that P450CMEF is a member of family 1, possibly in a distinct subfamily.

Induction of P450CMEF by PAHs. Fig. 5 contains information on the time courses of the induction of the mRNAs and AHH activities. Fig. 5*A* shows Northern blots of the ≈4.2- and ≈5.3-kb mRNAs and β-actin mRNA and the locations of 18S and 28S rRNAs. Although basal levels of AHH activity are dependent on cell growth (ref. 10 and unpublished data), within the first 4 h these levels were substantially unchanged (Fig. 5*B*) as are the basal mRNA levels (Fig. 5*C*). Accordingly, the data for the relative amounts of AHH or mRNA activity were normalized to the average values of the controls during the first 4 h.

The results in Fig. 5*B* show that, for both DMBA and BA, AHH activity increased rapidly. However, in DMBA-treated cells, activity maximized from 6 to 10 h and then decreased (data not shown) presumably because of a negative feedback (36) and/or the toxic effects of DMBA metabolites. For BA, which is nontoxic in 10T½ cells (14), AHH activity continued to increase up to 24 h but at a progressively decreasing rate.

Fig. 5*C* shows the combined relative amounts of mRNA in the 5.3- and 4.2-kb bands normalized to β-actin. Although the data have more scatter than those for AHH activities (Fig. 5*B*), a comparison of the two sets of results suggests several points. (*i*) DMBA and BA clearly induced amounts of mRNA and AHH activity that were appreciably greater than was induced by acetone treatment alone. (*ii*) By 1 h, mRNA levels were already elevated by DMBA or BA treatment (Fig. 5*C*), inductions that preceded the induction of AHH activity (Fig. 5*B*). The latter point suggests that AHH activity followed

mRNA induction. (*iii*) Induction by DMBA or BA increased mRNA levels by ≈3-fold, whereas AHH activity increased continuously during the same period.

CONCLUSION AND DISCUSSION

Although only part of the P450CMEF cDNA has been sequenced, the results support the likelihood that it is a P450 gene in 10T½ cells that is induced by DMBA and BA and illustrate the application of a method of applying PCR technology and highly degenerate primers to identify additional genes of the P450 superfamily. A similar approach was used to clone a cDNA after its partial amino acid sequence had been determined (25). In this report, we have demonstrated that one gene of a gene family can be identified without knowledge of its amino acid sequence.

Critical to the interpretation and, therefore, the success of the approach was the ability to extract information about known genes from GenBank and to identify relatively conserved regions of genes of the same family. Comparisons were made possible by the large amount of information on P450 genes and their products in GenBank and were facilitated by the search/comparison programs that were available. Where applicable, the method described could supplant the more conventional procedures for identifying additional genes.

The feature that a cDNA probe hybridized to two sizes of mRNA, ≈4.2 and ≈5.3 kb, warrants comment. These lengths appear to be longer than all of the known P450 mRNAs. Although the relationship between the two bands is unknown, the phenomena of a probe for a single P450 gene hybridizing to more than one mRNA band has been reported (37–39). The bands might come from two genes with significant homologies, from more than one poly(A) addition site (37), or be due to an alternatively spliced or initiated transcript.

The gene P450CMEF was found to be inducible by DMBA and BA in C3H 10T½ cells. From a separate study, a role for P450CMEF in neoplastic transformation and cell killing was suggested by the observations that conditions that increase these cellular effects increase AHH activity and the induction of P450CMEF mRNA (data not presented). In addition, the sequence information (Fig. 3) and the kinetics of induction (Fig. 5) suggest that P450CMEF is a member of P450 family 1 whose products are known to be involved in PAH metabolism and carcinogenesis. A peptide sequence of 31 amino acids—encoded by a gene designated P450-RAP from rat adrenal tissue that is involved in DMBA and BA metabolism (12, 13)—was deduced from protein analysis (C. R. Jefcoate, personal communication). Thirty amino acids of the latter peptide are identical with part of the sequence in Fig. 3. A 0.8-kb cDNA probe for P450-RAP (kindly supplied by C. R. Jefcoate, University of Wisconsin) hybridized to the same two major RNA bands as in Fig. 5*A* (data not presented). Antibodies to the product of rat P450-RAP cross-reacted (13) with a protein that was extracted from 10T½ cells by Pottenger *et al.* (18); the gene for the latter material was designated P450EF (18). However, the relationship between P450CMEF and P450EF is not clear because base-sequence data for P450EF are not available.

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