A clofibrate-induced mouse liver cDNA library was prepared and used to isolate the coding sequence for soluble epoxide hydrolase. A 1668-base pair (bp) clone was isolated and found to contain a 1283-bp open reading frame coding for 423 amino acids. Subsequent RNA polymerase chain reaction resulted in the isolation of 396 bp of additional 5'-sequence. Translation of the resulting 1659-bp open reading frame produced a 553-residue protein (62,527 Da) containing deduced peptide segments that matched the amino acid sequences of six peptide fragments isolated previously from CNBr digests of pure murine soluble epoxide hydrolase. Neither the DNA nor the protein sequence showed significant similarity to other currently published sequences. Structural analysis of the soluble epoxide hydrolase coding region suggested at least one potential regulatory motif. Expression of the composite cDNA in COS-7 cells resulted in a 5-10-fold increase in soluble epoxide hydrolase activity and a similar increase in soluble epoxide hydrolase protein amount compared to mock-transfected or vector control-transfected cells. Treatment of C57BL/6J mice with clofibrate led to a 4-fold increase in soluble epoxide hydrolase enzyme activity and steady-state mRNA levels.

Epoxide hydrolases (EC 3.3.2.3) are a family of enzymes that hydrolyze a variety of exogenous and endogenous epoxides to their corresponding diols. Epoxide hydrolases have been found in tissues of all mammalian species tested, with the highest levels being found in the liver and kidney (Wixtrom and Hammock, 1985). Four principal epoxide hydrolases are known: leukotriene epoxide hydrolase, cholesterol epoxide hydrolase, microsomal epoxide hydrolase, and sEH (previously called cytosolic epoxide hydrolase), the latter two of which utilize a broad spectrum of substrates, suggesting toxicological relevance (Wixtrom and Hammock, 1985). The leukotriene epoxide hydrolase acts on leukotriene A4, whereas the cholesterol epoxide hydrolase hydrolyzes compounds related to the 5,6-epoxide of cholesterol (Nashed et al., 1985; Finley and Hammock, 1988). The microsomal epoxide hydrolase hydrolyzes monosubstituted, 1,1-disubstituted, and cis,1,2-disubstituted epoxides and epoxides in cyclic systems. The more abundant soluble epoxide hydrolase hydrolyzes a wide range of epoxides not in cyclic systems. sEH has been purified and partially characterized from many mammalian species (Silva and Hammock, 1987; Meijer and DePierre, 1988). The best substrates appear to be 1,2-disubstituted aliphatic epoxides (Wixtrom and Hammock, 1985). Current evidence suggests that sEH is induced only by compounds that cause peroxisome proliferation (Moody et al., 1992). Chronic administration of such compounds leads to the development of hepatocellular carcinomas in rodents (Rao and Reddy, 1991).

In addition to degradation of potential toxic epoxides, sEH also may play a role in the formation or degradation of endogenous chemical mediators (Fitzpatrick and Murphy, 1988; Carroll et al., 1987; Imai et al., 1980; Sugiyama et al., 1987). For instance, microsomal cytochromes P450 oxidize arachidonic acid to four optically active cis-epoxyeicosatrienoic acids (Capdevila et al., 1990; Needleman et al., 1986; Karara et al., 1989), some of which have potent physiological effects (Fitzpatrick and Murphy, 1988; Berridge, 1987) and have been shown to be excellent substrates for sEH (Gill and Hammock, 1979; Chacso et al., 1983). sEH may therefore play a role not only in xenobiotic metabolism, but also in determining steady-state levels of physiological mediators.

As a first step in understanding how exogenous and endogenous epoxides are metabolized in vivo and what (if any) role sEH plays in peroxisome proliferator-induced liver carcinogenesis in rodents, we have cloned a cDNA for the murine sEH. In this report, we provide the first characterization of this cDNA, the predicted coding sequence, and the transient expression of the full-length cDNA in cell culture.

**MATERIALS AND METHODS**

cDNA Library Construction and Screening—Total RNA was isolated (as described by Chomczynski and Sacchi, 1987) from the livers of male Swiss Webster mice (Charles River Breeding Laboratories, Inc.) after treatment of the mice for 14 days with 0.5% (w/v) clofibrate (2,4-chlorophenoxy)-2-methylpropanoic acid ethyl ester) as described (Dietze et al., 1990). Poly(A)+ RNA was selected by oligo(dT)-cellulose chromatography using an Invitrogen mRNA isolation kit. A cDNA library from the RNA of one individual was constructed using the 5'-EcoRI and 3'-XhoI cloning sites of the Uni-Zap vector system (Stratagene) according to the supplied protocol. A total of 1.5 X 106 primary plaques were obtained with 5% nonrecombinants. We used PCR to prepare a homologous murine sEH probe that could be used for library screening. We constructed 5'-degenerate (5'-GGAAGCTTACTGATCT/AAAGC-CT/CA/AA/AGC/AGC) and 3'-degenerate (5'-GCGAATTCCAGAAAGA/TCCAG/TTG/AA/G) primers that bracketed 30 residues of a 34-residue mouse sEH CNBr fragment (peptide 10) (Dietze et al., 1993). These primers contained flanking restriction sites and were used for low stringency PCR (the annealing temperature for the first three cy

---

* This work was supported in part by National Institute of Environmental Health Sciences Grant RO1 ES02710 (to B. D. H.). The University of California at Davis is a National Institute of Environmental Health Sciences Center for Environmental Health supported by Grant P30 ES05707 and an Environmental Protection Agency Center for Ecotoxicology supported by Grant CR169658. The costs of publication of this article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L05791.

‡ Recipient of National Research Service Award A107662-02 from the National Institutes of Health.

§ Recipient of a Burroughs Wellcome toxicology award. To whom correspondence should be addressed. Tel.: 916-752-7519; Fax: 916-752-1537.

1 The abbreviations used are: sEH, soluble epoxide hydrolase; PCR, polymerase chain reaction; t-ENPG, trans-2(S,3S)-epoxy-3-(4-nitrophe nyl)glycidol; bp, base pair(s).
Clones was 37 °C for 45 s, an additional 30 cycles were at 55 °C, and the extension temperature was 72 °C for 1 min) to amplify. The cycle numbers were 40 for the 89-bp product from the cDNA library. Sequencing reactions showed that two of the three independent clones contained identical internal sequences that coded for the 30-amino acid peptide. One of the two 89-bp clones was labeled using PCR (Jansen and Ledley, 1989) and was used to screen the unamplified cDNA library as described (Sambrook et al., 1989).

Sequence Analysis—DNA from two positive plaques was isolated and purified as described (Sambrook et al., 1989). Restriction analysis showed that both clones were the same size. One of the two clones (pEH-1) was subcloned as a series of nested deletions using the Erase-A-Base system (Promega Biotech, Madison, WI) and was sequenced by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase 11 (United States Biochemical Corp.).

Construction of Additional Clones—Northern blot analysis suggested that our psEH-1 clone was ~400 bases shorter than the sEH mRNA. RNA PCR (Sambrook et al., 1989) was used to isolate additional sEH 5′-sequence. A 3′-primer (primer 382, 5′-AAATTTGACAGGGCCGGCGCCCTCGAGGTTATGCAAGGAAGCT- (TCAGCAGGAAACG) was a degenerate primer that matched the first 5′-end of psEH-1. This clone added an additional 234 bp and 78 amino acids, which still did not equal the total length of the sEH mRNA. We therefore repeated our RNA PCR analysis of Swiss Webster mouse poly(A)+ RNA to determine if additional sequence could be isolated. We used primer 382 as described above for first-strand synthesis and for the 3′-primer and a 5′-primer (5′-GCGTCCTAGGAGGAGCCGGCTGGGCCTGGTCCGTG- (GTAGCC) matching the first 17 bp of psEH-2) and primer 382 as the 5′-primer for reverse transcription and as a 3′-primer for PCR. The unique 3′-Spel site and the unique 5′-BclI site at the 3′-end of the 500-bp PCR clones and near the 5′-end of psEH-1 were ligated into the 1.5-kb EcoRI-HindIII fragment of pBR322. The 822-bp product was then digested with SpeI and BclI to verify identity to psEH-2 before digesting with PstI and subcloning into XhoI/Sacl-digested pJ311 to give psEH-pJ311. Plasmid DNA used for transfections was purified by centrifugation on a cesium chloride/ethidium bromide equilibrium gradient (Sambrook et al., 1989).

COS-7 cells were grown as a monolayer culture in Dulbecco’s modified Eagle’s medium (ICN) containing 10% fetal calf serum (ICN) plus penicillin (G 100 units/ml) and streptomycin sulfate (100 μg/ml). Cell transfactions were mediated by lipofectin (Life Technologies, Inc.) according to standard protocol. Approximately 1 x 10⁶ cells were transfected in 100-mm dishes with 5 μg of psEH-pJ311, 5 μg of pJ311 vector control, or a mock transfection control containing lipofectin only (60 μl). All transfections except the mock transfection contained 2.5 μg of the β-galactosidase plasmid pSV-β-Gal (Promega Biotech) as an internal positive control. After 36 h, cells were washed five times with phosphate-buffered saline and then collected by scraping into 1.5-ML centrifuge tubes. The cells were pelleted (12,000 x g, 4 °C, 30 s), resuspended in 150 μl of lysis buffer (40 mM Tris, pH 8.0, 150 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA), and lysed by three cycles of freezing and thawing. The cells were again centrifuged, and the supernatant was used for enzyme assays and protein determination.

β-Galactosidase was assayed as described (Sambrook et al., 1989). sEH activity was assayed using trans-[3H]stilbene oxide as de- scribed above. In addition to measuring sEH enzyme activity in transiently transfected COS-7 cells, we analyzed transfected COS-7 cell soluble protein for expression of sEH using immunoblotting analysis. We used antibodies developed in rabbits against both the murine sEH and the rhesus sEH (Silva and Hammock, 1987). SDS electrophoresis was performed according to Laemmli (1970) using 3.5% stacking and 10% resolving gels. Molecular mass standards were from Bio-Rad (immunoblotting was performed according to Burnette (1981) using 0.2-μm pore size nitrocellulose in a Bio-Rad mini Trans-Blot cell. After overnight transfer (35 V, room temperature), blots were blocked by shaking for 30 min in 50% calf serum (ICN), 50% blotting buffer (350 mM NaCl, 0.25% Tween 20, pH 7.4). Primary antiserum (1:5000) was added for 2 h with shaking at room temperature. Bound IgG was detected as described by Blake et al. (1984) using alkaline phosphatase-labeled goat anti-rabbit-IgG (1:2000 in blocking buffer; Sigma). Protein concentrations were determined using the biuret method (Girardier et al., 1996) according to protocol directions using bovine serum albumin as standard.

RESULTS

Isolation of Composite sEH cDNA—Screening of the clofibrate-induced mouse liver cDNA library with the PCR-generated probe resulted in the isolation of two hybridizing clones with inserts of similar size. One (psEH-1) was isolated, labeled, and used for Northern blot analysis of mRNA isolated from a clofibrate-induced Swiss Webster mouse (data not shown). The results showed that the sEH mRNA was ~2.1 kilobases, 400 bp larger than psEH-1. In addition, the sequence of psEH-1 did not contain the 9-residue sEH peptide that had been previously isolated (Dietze et al., 1993). We therefore used a series of PCR reactions on clofibrate-induced Swiss Webster mouse liver mRNA to isolate the remaining 400 bp upstream from the 5′-end of psEH-1. For reverse transcription and as a 3′-primer for PCR, we used a 36-base primer that was complementary to the 5′-end of psEH-1 and overlapped a unique BclI site. For the 5′-primer, we used a degenerate primer matching the 9-residue peptide not found in the original clone and, in a separate experiment, a 5′-primer that was complementary to the 5′-end of the rat sEH cDNA clone. Both PCR amplifications yielded clones that were identical in their region of overlap and that had a 90-bp sequence overlap identical to the 5′-end of psEH-1. Three independent 500-bp PCR clones were sequenced and found to be identical, except for a 1-bp change that was most likely due to polymerase misincorporation. One of the two 500-bp PCR clones was digested with SpeI and BclI and ligated into SpeI/BclI-digested psEH-1 to give the putative full-length cDNA psEH-2 (Fig. 1). This composite clone was slightly larger than 2000 bp and included a 90-bp poly(A) tail

2 M. Arand, personal communication.
The size of the sEH message in control and clofibrate-induced mice was the same as in clofibrate-induced Swiss Webster mice (data not shown).

Cellular localization of sEH-The predicted molecular mass of the mature sEH protein is 62,527 Da (Fig. 2). All six previously identified sEH peptide fragments were identified, confirming the identity of the cDNA as sEH. An additional peptide fragment that was isolated by trypsin digestion of pure sEH was also identified. A putative polyadenylation signal is located 19 bases upstream from the start of the poly(A) tail. The 3'-terminus of the mouse sEH contains a Ser-Lys-Ile sequence postulated to be an ambiguous peroxisomal targeting signal (Arand et al., 1991).

Structural Analysis of Mouse sEH cDNA—The DNA and deduced amino acid sequences for sEH show no significant similarity to any of the other epoxide hydrolase enzymes so far isolated or to DNA sequences deposited in GeneBank (release 71.0). The sEH sequence was analyzed using the PEST-FIND algorithm of Rogers et al. (1986) to look for potential PEST sequences. PEST regions are enriched in proline, glutamic acid, serine, threonine, and, to a lesser extent, aspartic acid and are thought to be involved in protein stability and regulation (Rogers et al., 1986; Rechsteiner, 1990). One potential PEST sequence was identified (PEST score of −0.30) in a hydrophilic sequence 291–312) (Fig. 3). This region has 3 serines, 2 prolines, 1 aspartic acid, and 3 glutamic acids in a 10-residue sequence. This hydrophilic region is preceded and followed by strong hydrophobic regions, a characteristic of many PEST sequences (Rogers et al., 1986).

Enzyme Assays and Northern Blot Analysis—We analyzed the in vivo expression of sEH in the inbred C57BL/6J mouse strain with and without induction by the peroxisome proliferator clofibrate. The results showed that induction of enzyme activity is similar to the induction of steady-state mRNA levels (Fig. 4). This is the first comparison of the relative steady-state levels of sEH mRNA and sEH enzyme activity and supports a role for transcriptional regulation of sEH expression in mice. The size of the sEH message in control and clofibrate-induced C57BL/6J mice (Fig. 4D) was the same as in clofibrate-induced Swiss Webster mice (data not shown).

Functional Analysis of psEH-2—To determine if our composite cDNA coded for a functional enzyme, we cloned psEH-2 into the SV40 eukaryotic expression vector pJ30 and transiently transfected COS-7 cells. The results (Table I) show that after subtracting sEH activity not inhibited by the sEH inhibitor t-ENPG, cells transfected with psEH-2-pJ30 expressed 5 to 10-fold more sEH activity than did pJ30 vector-transfected or mock-transfected cells. Transfected cells expressed approximately the same level of sEH activity as did mock-transfected cells when 1 mM t-ENPG was included in the enzyme assay. These results suggest that COS-7 cells have low but measurable basal sEH activity. The differences in specific activity among the experiments in Table I are most likely due to differences in cell density during transfections.

We used immunoblotting to confirm the expression of murine sEH in the transfected COS-7 cells from Experiments 2 and 3 in Table I. The results show that the anti-mouse sEH antiserum recognizes a band that migrates identically to purified mouse sEH and is only expressed in cells transfected with psEH-pJ30 (Fig. 5A). These results support the conclusion that psEH-2 is a functional epoxide hydrolase. If, however, we used the anti-rhesus sEH antiserum (Fig. 5B), cells from all the treatments expressed detectable levels of a protein that migrated identically to mouse sEH. Because COS-7 cells are monkey kidney cells, these results are consistent with the enzyme activity results and suggest that COS-7 cells express basal levels of sEH.

DISCUSSION

The composite nucleotide sequence of psEH-2 was 2059 nucleotides in length and contained an open reading frame coding for 553 amino acids. The molecular mass and amino acid composition of the protein predicted from the nucleotide sequence agreed with previous reports of sEH purified from mouse liver (Prestwich and Hammock, 1985; Dietze et al., 1990). Previous amino acid sequencing of peptide fragments purified from mouse liver sEH (Dietze et al., 1993) provided a total of 135 amino acid residues, comprising six CNBr fragments and one trypsin fragment. psEH-2 contains 133 of these residues in the predicted order. In addition, all three of the 500-bp PCR clones we sequenced showed no significant intra- or intersequence differences in regions of overlap, and they all contained a shared 9-residue peptide fragment isolated previously from CNBr digests of pure sEH. These results strongly support the conclusion that the composite psEH-2 is the correct cDNA for murine liver sEH.

Because the sEH protein contains a blocked NH$_2$ terminus, it is not known which residue is at the 5'-terminus of the mature protein. The most upstream peptide isolated from CNBr digests starts at Met-54, which is the second Met found in the cDNA.

The lack of sequence similarity of the murine sEH to the microsomal epoxide hydrolase is rather surprising since their substrate specificities overlap to some extent (Wixtrom and Dietze and B. D. Hammock, unpublished data.)
to 312 shows a possible PEST sequence (Hammock, 1985).

amino acid sequence not preceded by sequences preceded by a Met were predicted by cyanogen bromide digests of pure protein (as previously reported by Dietze 1985).

FIG. 3. Composite cDNA sequence and deduced amino acid sequence of murine soluble epoxide hydrolase. Underlined amino acid sequences preceded by a Met were predicted by cyanogen bromide digests of pure protein (as previously reported by Dietze et al. (1993)). Underlined amino acid sequence not preceded by a Met (Asp-494 to Lys-503) was predicted from a trypsin digest. Underlined DNA sequence is the polyadenylation signal. ***, stop codon. The molecular mass predicted by the open reading frame is 62,927 Da. In the original peptide sequencing, Ile-302 was read as a Thr, and Arg-498 was read as a blank.

Fig. 2. Hydrophobicity plot of murine sEH. Positive values are hydrophobic, and negative values are hydrophilic. The number of residues is given along the bottom. The sequence from residues 291 to 312 shows a possible PEST sequence and phosphorylatable serines.
not significantly homologous to this epoxide hydrolase. The cholesteryl epoxide hydrolase has not yet been cloned.

Another interesting feature that is revealed by the cDNA is the Ser-Lys-Ile carboxyl terminus. This has been shown by Gould et al. (1989) to be a cytosolic targeting motif in COS cells using a chloramphenicol acetyltransferase reporter system. Arand et al. (1991) also have found this tripeptide at the 3'-terminus of a partial cDNA sequence from rat and have postulated that this motif is an ambiguous peroxisomal targeting motif in the rat. This conclusion is based on previous studies that clearly show sEH in mice to be both a cytosolic and a peroxisomal protein (Chang and Gill, 1991). The partial cDNA for the rat sEH is nearly identical to the mouse cDNA, including the Ser-Lys-Ile 3'-terminus. The data suggest therefore that if only one functional gene exists for sEH in both rats and mice, then the Ser-Lys-Ile terminus is apparently targeted differently in rodent liver than it is in COS cells. An Ala-Lys-Ile 3'-terminus has been shown to be a peroxisomal targeting sequence in Candida tropicalis (Nuttley et al., 1988).

A comparison of the hydrophobicity and structural analyses shows one hydrophilic region containing an unusually large percentage of serines, glutamic acids, and prolines. Peptide residues of this type are typical of PEST sequences (Rogers et al., 1986; Rechsteiner, 1990); and indeed, this region of sEH has a fairly high PEST score of -0.30, which is slightly below the value that delineates a strong (0-35) PEST sequence. However, the absolute value of the PEST-FIND score is determined by strict requirements of the algorithm, some of which may not always be justified (Wang et al., 1989). This region is most likely on the surface of the sEH and is followed by a hydrophobic region, also characteristic of PEST sequences. The function of this region is not known, but may be involved in sEH turnover or phosphorylation.

Several studies have shown that mouse sEH is induced only
by compounds that cause peroxisomal proliferation in the liver (see review by Moody et al. (1992)). The mechanism or significance of this induction is not known; however, long-term administration of peroxisome proliferators results in hepatocarcinogenesis in mice and rats (Rao and Reddy, 1991). We measured levels of sEH enzyme activity and sEH mRNA in control and clofibrate-treated C57BL/6J mice. Our results show that the relative induction of sEH enzyme activity is similar to the relative induction of sEH mRNA and are consistent with the hypothesis that induction by clofibrate is mediated at least in part at the mRNA level. There is no apparent difference in the size of the sEH message in control and induced mice.

We expressed the mouse sEH cDNA in COS-7 cells to verify structural and functional integrity of the psEH-2 clone. The sEH activity in transfected COS-7 cells was inhibited by t-ENPG, a selective sEH inhibitor (Dietze et al., 1993), and was 5–10-fold greater than in vector- or mock-transfected cells. These results suggest that psEH-2 is a functional epoxide hydrolase. COS-7 cells express low but detectable sEH activity and sEH protein and thus may be able to down-regulate sEH expression in transfected cells. It would be of interest to determine if clofibrate or other peroxisome proliferators can induce sEH expression in transfected COS-7 cells.

The availability of the mouse cDNA clone will allow many additional studies of the molecular biology of sEH to be performed. These could include identification of upstream regulatory regions of the sEH gene and experimental manipulation of potential peroxisomal targeting sequences and catalytic sites using site-directed mutagenesis and expression.

Acknowledgments—We thank Michael Arand for the sequence of the rat sEH cDNA prior to publication; Aaron Dubberley, Wen-Wen Lin, and Tongyan Tian for help with DNA sequencing; Martin Privalski for the COS-7 cells; Eric Dietze for the sequence of the sEH trypsin fragment; and Suresh Subramani for the pJ3n vector.

REFERENCES


