

## Transcriptional regulation of the human soluble epoxide hydrolase gene *EPHX2*

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Received 2 December 2006; received in revised form 12 November 2007; accepted 12 November 2007

Available online 3 December 2007

### Abstract

Soluble epoxide hydrolase (sEH) is a multifunctional protein encoded by the *EPHX2* gene. The biological functions and enzyme kinetics of sEH have been extensively investigated, however, little is known about its transcriptional regulation and mechanisms of tissue specific expression. Here, a luciferase gene based reporter assay was used to identify the minimal promoter and *cis* regulatory elements of *EPHX2*. The minimal promoter was identified as a GC-rich region between nts –374 and +28 with respect to the putative transcriptional start site. A reporter plasmid carrying this minimal promoter showed higher or similar activities in comparison to a reporter plasmid carrying nts –5,974 to +28 of *EPHX2* in 9 human cell lines that were tested. Sp1 binding sites that are involved in augmenting the minimal promoter activity of *EPHX2* were identified by nested deletion analysis, site-specific mutation, electrophoretic mobility shift assay, and chromatin immunoprecipitation assay. © 2007 Elsevier B.V. All rights reserved.

**Keywords:** Epoxide hydrolase; *EPHX2*; Promoter; Transcription

### 1. Introduction

Soluble epoxide hydrolase (sEH; E.C.3.3.2.3) is encoded by a gene, *EPHX2*, that is found on human chromosome 8, region 8p21–12 [1]. *EPHX2* has 19 exons that encode a mature protein of 555 amino acid residues. In humans, sEH seems largely involved in the metabolism of epoxyfatty acids. An example is the hydration of epoxy-eicosatrienoic acids (EETs) to their dihydroxy eicosatrienoic acid derivatives (DHETs) [2,3]. EETs are important signaling molecules derived from arachidonic acid through metabolism by cytochrome P450s (including CYP2C8, CYP2C9, and/or CYP2J2) [4–7]. EETs have biological roles in cardiovascular biology, renal function, inflammation response and pain [6,8,9]. The stabilization of EETs through the inhibition of sEH has beneficial effects on blood pressure, reduction of inflammation, and reduction of organ

damage, and analgesia [10–16]. These results underline the importance of sEH in human health.

The biologically active form of sEH is a homodimer of two 62.5 kDa subunits [17]. sEH is a bifunctional enzyme with epoxide hydrolase activity (C-terminal region) that acts on epoxyfatty acids and a Mg<sup>2+</sup>-dependent phosphatase activity (N-terminal region) that acts on lipid phosphates [18,19]. However, there may be other endogenous substrates, and the biological role of the phosphatase activity is unknown [19]. sEH activity is highest in mammalian liver and kidney [20–23], but it has also been found in many other tissues. In humans and rodents sEH expression occurs in liver, kidney, lung, heart, gut, brain, placenta, bladder, prostate, testis, spleen, skin, ovary, vascular endothelium, and some smooth muscle [13,17,24–32]. Although the distribution and tissue-enriched expression of sEH have been studied, the molecular mechanisms of sEH regulation are poorly understood. Several studies have shown that sEH can be regulated by endogenous chemical mediators and by some xenobiotics. 1) The sEH is induced by the administration of agonists of peroxisome proliferator activated receptor alpha (PPAR-alpha) in rodents [23,31,33]. Most organisms appear to respond to these agonists with a 2- to 3-fold increase

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in hepatic sEH activity. 2) Male mice show 55% and 283% higher sEH activities in liver and kidney, respectively, in comparison to female mice [31] suggesting the involvement of sex hormones in sEH regulation. Additionally, ovariectomy increases sEH activities in both the liver and kidney of mice suggesting a suppressive role of female sex hormones such as estrogen [31]. 3) sEH activity appears closely tied to the renin–angiotensin system. Angiotensin II (Ang II) appears to increase sEH protein levels and catalytic activity in rat renal cortical tissue [10]. Furthermore, Ang II has recently been shown to directly upregulate *EPHX2* expression [34]. 4) In humans, cigarette smoke transiently reduces sEH activity in the lung [24]. 5) Exposure to gamma radiation (12 h) increases the levels of *EPHX2* mRNAs in human cell lines [35]. This increase appears to result from NF $\kappa$ B induction. Interestingly, a splice variant of sEH that is found in mouse ovary shows differences in epoxide hydrolase activity [32]. In this splice variant, the first 44 residues of the N-terminal region are encoded by sequences that form part of the 2nd intron of the mouse *EPHX2* gene. This splice variant seems to have a role in ovarian function in mice. To our knowledge, such a splice variant has not been found for the human *EPHX2* gene.

In this study, we characterize the transcriptional regulation of *EPHX2* by transient reporter assay, electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation (ChIP) assay. A luciferase-based reporter assay was developed and used to determine the minimal promoter of *EPHX2* as well as enhancing and inhibitory regions that are found upstream of *EPHX2* in 9 different human cell lines. The identification of responsive elements within the promoter of *EPHX2* or cellular transcription factors that specifically interact with these responsive elements may lead to novel drug targets for high blood pressure, inflammation, and other diseases that are associated with sEH.

## 2. Materials and methods

### 2.1. In silico analysis and 5'-prime rapid amplification of cDNA ends (5'-RACE)

Sequence information of the 5'-flanking region of the *EPHX2* gene was obtained from the human genome database at the National Center for

Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>). *EPHX2* is located on chromosome 8 of the assembled sequence NT\_023666. The computer programs Database of Transcriptional Start Sites (DBTSS, <http://dbtss.hgc.jp/>) and Dragon GC+ Promoter Finder (<http://research.i2r.a-star.edu.sg/DRAGON/>) were used to identify the putative transcriptional start site of *EPHX2*. The GC content of the 5'-flanking region (nts -6,000 to +500 with respect to the putative transcriptional start site) of *EPHX2* was calculated on the basis of a 200 nts-long, running window. The computer program CpG Island Searcher (<http://www.cpgislands.com/>) was used to identify CpG islands within the 5'-flanking region (nts -10,000 to +10,000) of *EPHX2*. The computer program Transfac (<http://www.gene-regulation.com/>) was used to identify potential binding sites of transcription factors.

One microgram of human liver total RNA (Stratagene) was used as a template to produce 5'-terminal enriched cDNAs using a Smart cDNA Synthesis kit (Clontech). Universal primer A Mix, supplied in the kit, and a gene specific reverse primer (5'-TCCGGTCACTTCTCCAGTTC-3') were used to amplify the 5'-terminus of *EPHX2*. The amplification was performed as follows: 94 °C for 1 min, 63 °C for 1 min, 68 °C for 1 min for 25 cycles with KOD Hot Start DNA Polymerase (Novagen). The PCR product was ligated into the pCR Blunt II-TOPO vector (Invitrogen), and the sequences of the cDNA inserts of 20 randomly isolated plasmids were determined (UC Davis Division of Biological Sciences Sequencing Facility).

### 2.2. Cell cultures

The human cell lines used in this study are described in Table 1. Most of these cell lines were generously provided as gifts. DU-145 [36] was obtained from Alan Epstein of the Keck School of Medicine, University of Southern California, Los Angeles, CA, USA and Gerald DeNardo of the University of California Health Center, Sacramento, CA, USA. SW480 [37]; Caco-2 [38]; 293T [39] and HeLa [40]; and HuH-7 [41], ACHN [42], and SN12C [43] were obtained from Ken Kaplan; Bo Lonnerdal; Martin L. Privalsky; and Robert Weiss respectively, all from the University of California, Davis, CA, USA. HepG2 [44] was purchased from ATCC (Manassas, VA, USA). All of the cell lines were cultured in RPMI1640 medium (Mediatech) supplemented with 10% fetal bovine serum (v/v) (BioWhittaker) under a 5% CO<sub>2</sub> atmosphere at 37 °C. The cells were passaged at 90–100% confluency by washing with PBS and trypsinization (0.05% trypsin, 0.53 mM EDTA) for 3–5 min at 37 °C prior to subculture. This trypsinization procedure was also used to harvest cells for the luciferase-based reporter assay and sEH activity assay. Following trypsinization, the cell suspension was centrifuged at 400 ×g for 5 min at 5 °C, and resuspended in fresh RPMI1640 medium.

### 2.3. Generation of reporter plasmid constructs containing the 5'-flanking region of *EPHX2*

In order to generate the reporter plasmids, nts -5,974 to +28 (-5974/+28 construct), nts -5,974 to -325 (-5974/-325 construct), and nts -374 to +28

Table 1  
sEH transcription and expression and transfection efficiency in human cell lines

Cell line	Ref.	Origin	Disease	sEH specific activity (pmol/min/mg) <sup>a</sup>	Relative <i>EPHX2</i> mRNA ( <i>EPHX2</i> / <i>GAPDH</i> ) <sup>a</sup>	Renilla luciferase activity <sup>a</sup> (RLU/1 × 10 <sup>4</sup> cells) <sup>b</sup>
HepG2	[44]	Liver	Carcinoma	310 ± 50	0.18 ± 0.03	600 ± 30
HuH-7	[41]			940 ± 40	0.99 ± 0.20	130 ± 30
293T	[39]	Kidney	None <sup>c</sup>	210 ± 10	0.54 ± 0.08	98000 ± 11000
ACHN	[42]		Adenocarcinoma	31 ± 15	0.38 ± 0.04	30 ± 20
SN12	[43]		Carcinoma	n.d. <sup>d</sup>	0.08 ± 0.02	240 ± 60
Caco-2	[38]	Colon	Adenocarcinoma	120 ± 10	0.33 ± 0.02	780 ± 70
SW480	[37]			130 ± 30	0.68 ± 0.08	7000 ± 700
DU 145	[36]	Prostate	Carcinoma	440 ± 10	0.39 ± 0.03	9500 ± 400
HeLa	[40]	Cervix	Adenocarcinoma	52 ± 15	0.14 ± 0.05	3400 ± 200

<sup>a</sup> The control pRL-CMV plasmid contains a luciferase gene from *Renilla reniformis* under the control of a CMV promoter.

<sup>b</sup> Values are mean ± SD from three different experiments.

<sup>c</sup> Human cell line, derived from embryo kidney, expressing SV40 large T antigen abundantly.

<sup>d</sup> n.d. indicates not detectable under the condition of our assay.

(–374/+28 construct) of the 5'-flanking region of *EPHX2* were amplified by PCR and cloned immediately upstream of the firefly luciferase gene in the pGL3-Basic reporter plasmid (Promega). PCR was performed with KOD Hot Start DNA Polymerase in the presence of 5% DMSO according to the manufacturer's protocol. Genomic template DNAs were isolated from  $1 \times 10^7$  HepG2 cells following standard procedures [45]. Forward and reverse primers (F1: 5'-CGCGGTACCCAAGGAGGGGAGAGAACAAGT-3', F2: 5'-CGCGGTACCGCATTCAGTCCAGCAAGT-3', R1: 5'-CGCCTCGA-GACGCAGCTAACCTGGGAGAT-3', and R2: 5'-CGCCTCGAGCTTCTG-GACGGACTGACA-3') were synthesized (Sigma Genosys) on the basis of the sequence of the *EPHX2* gene (GenBank accession no. NM\_001979). Restriction endonuclease recognition sites (underlined) for *KpnI* and *XhoI* were incorporated into the forward and reverse primers, respectively. Following PCR, the products were digested with *KpnI* and *XhoI* and inserted immediately upstream (at the *KpnI* and *XhoI* sites) of the firefly luciferase gene of pGL3-Basic. The –5974/+28, –5974/–325, and –374/+28 constructs were generated using the primer sets F1+R1, F1+R2, and F2+R1, respectively. Additional reporter plasmids, containing nested deletions or a point mutation of the –374 to +28 nts region of *EPHX2* were generated by PCR using the template DNAs and primers described in Table 2. The authenticity of each of the constructs was confirmed by nucleotide sequencing.

Table 2  
Primers and templates used to generate the reporter plasmids

Primer (5'–3')		
F3:	GGGCATAAGAGACTTTGGACTTT	
F4:	CGTTACATCCAGAGGCGGAGA	
F5:	TCTGACTCCCTTTCTGTGC	
F6:	TTTCCTGTGCCCTCCCC	
F7:	CCCCTCCCCTGCCTCTTTC	
F8:	TGCCTCTTCCCGGCCAGAG	
F9:	GCCAGAGTCCAGCCTTAACC	
F10:	CCGGGCAGAGGGCGGAGTC	
F11:	CCCCTTAAGGGGGTGTGGGG	
F12:	AGGCGGGGCCAGGGCAGG	
F13:	GGGCAGAGCCGGGCCAAG	
F14:	GCTGGGCGGGTTCATGCGC	
F15:	TGGCCTTCGCGCATCTCCC	
F16:	GGGGGTGTGGGGAGGGGGCCAGAGCCGGGCCAAG	
F17:	TCGGGCAGAGCCGGGCC	
R3:	GGTACCTATCGATAGAGAAATGTTCTGGC	
R4:	GTTAAGGCTGGACTCTGGCC	
R5:	CCTCCCCACACCCCTTAAC	
R6:	TTAACGGGGTAAAGGCTGGACTCTGGCC	
R7:	ACCTGCCCTGGCCCCGCC	
Construct	Primer set	Template used
–272/+28	F3+R3	–374/+28
–230/+28	F4+R3	–374/+28
–165/+28	F5+R3	–374/+28
–155/+28	F6+R3	–374/+28
–146/+28	F7+R3	–374/+28
–136/+28	F8+R3	–374/+28
–123/+28	F9+R3	–374/+28
–104/+28	F10+R3	–374/+28
–86/+28	F11+R3	–374/+28
–63/+28	F12+R3	–374/+28
–41/+28	F13+R3	–374/+28
–24/+28	F14+R3	–374/+28
–4/+28	F15+R3	–374/+28
–374/+28 $\Delta$ (–104/–87)	F11+R4	–374/+28
–374/+28 $\Delta$ (–63/–42)	F13+R5	–374/+28
–374/+28 $\Delta$ (–104/–87, –63/–42)	F16+R6	–374/+28
–374/+28 $\Delta$ (–104/–42)	F13+R4	–374/+28
–63/+28TT	F17+R7	–63/+28
–374/+28TT	F17+R7	–374/+28

## 2.4. Luciferase activity assay

The human cell lines used in these assays are described above and in Table 1. Cells from each of the human cell lines were seeded into 24-well tissue culture plates (Falcon) at a density of  $5 \times 10^4$  cells/well, allowed to attach and recover for 24 h, and then transfected with 200 ng of each plasmid using FuGENE6 transfection reagent (Roche). All of the transfections were carried out according to the protocol provided by the manufacturer using 0.6  $\mu$ l of the transfection reagent per well. For each transfection, 4 ng of a control plasmid, pRL-CMV (Promega), expressing luciferase of *Renilla reniformis* (renilla luciferase) under control of a cytomegalovirus (CMV) promoter, was added as an internal control reporter. After a 48 h-long incubation at 37 °C, the cells were lysed with 100  $\mu$ l/well (500  $\mu$ l/well for 293T) of Triton Lysis Buffer (TLB: 100 mM potassium phosphate, pH 7.8; containing 0.2% Triton X-100). The luciferase assay was carried out by means of the Dual-Glo Luciferase Reporter Assay System (Promega) using 20  $\mu$ l of the cell lysate. Luciferase activities were determined using a Spectra Fluor Plus microplate reader (TECAN). Firefly luciferase activity was normalized with respect to renilla luciferase activity, and shown as relative luciferase activity (relative luciferase activity=firefly luciferase activity/renilla luciferase activity). The transfection efficiency (using pRL-CMV) of each cell line is shown as relative light units (RLU) of renilla luciferase per  $1 \times 10^4$  cells (Table 1). The Pearson correlation coefficient was used to determine statistically significant correlations. This coefficient was determined using the computer program R (<http://www.r-project.org/>).

## 2.5. sEH activity assay

sEH was released from  $1 \times 10^6$  cells by sonication (Sonic Dismembrator Model 100, Fisher Scientific) for 3 s in 500  $\mu$ l of cell lysis buffer (20 mM sodium phosphate, pH 7.4; 5 mM EDTA; 1 mM DTT; 1 mM PMSF; and 0.01% Tween-20). Cell debris was removed by centrifugation (10,000  $\times$ g) for 15 min at 4 °C and the supernatant was stored at –80 °C until used. sEH activity was measured in a 100  $\mu$ l reaction volume containing 0.05 mM of racemic [ $^3$ H]-*trans*-1,3-diphenylpropene oxide (t-DPPO) as substrate as described previously [46]. The reaction mixture was incubated at 30 °C for 30 min. The reaction was then quenched by the addition of 60  $\mu$ l of methanol, and 200  $\mu$ l of isoctane was added in order to extract the remaining epoxide from the aqueous phase. Parallel reactions were performed in which 1-hexanol (200  $\mu$ l) was used for extraction (instead of the isoctane) in order to measure potential glutathione S-transferase activity which can also transform the substrate [46]. Diol (or glutathione conjugate) in the aqueous phase was quantified using a scintillation counter (Wallac Model 1409, Wellesley, MA).

## 2.6. Semi-quantitative RT-PCR

The cells were released from the washed culture dish by use of trypsin (0.05% trypsin, 0.53 mM EDTA) and subsequently washed three times with PBS (centrifugation at 1000  $\times$ g for 5 min at 5 °C). Total RNA was extracted from the cells using an RNeasy Mini kit (Qiagen). Total RNA (1  $\mu$ g) was converted to cDNA using Superscript II reverse transcriptase (Invitrogen). The primers and the PCR conditions employed are as follows: GAPDH: 5'-CAGCCTCAAGATCAT-CAGCA-3' (sense) and 5'-TTCTAGACGGCAGGTCAGGT-3' (antisense), *EPHX2*: 5'-GTGTTTCATTGGCCATGACTG-3' (sense) and 5'-CTCAGTGAC-CATCCTGCTGA-3' (antisense); 28 cycles composed of steps at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The products of the PCR were separated on a 2% agarose gel containing ethidium bromide and visualized with UV light. Relative mRNA abundance was quantified by densitometry using ImageJ software (<http://rsb.info.nih.gov/ij/>) with normalization to GAPDH (*EPHX2/GAPDH*).

## 2.7. Electrophoretic mobility shift assay (EMSA)

EMSA was used to measure the ability of nuclear extracts to bind to nts –54 to –33 (Oligo 1: 5'-CAGGGCAGGGGCGGGCCAGAGC-3'), –74 to –47 (Oligo 2: 5'-GTGTGGGGAGGAGGCGGGCCAGGGCAG-3'), –86 to –64 (Oligo 3: 5'-CCCCTTAAGGGGGTGTGGGGAGG-3'), or –102 to –80 (Oligo

4: 5'-GGGCAGAGGGCGGAGTCCCGTTA-3') of the 5'-flanking region of *EPHX2*. The single-stranded oligonucleotides (Oligos 1 to 4 above) were labeled at the 3'-end with biotin (3' End Labeling kit, Pierce), then annealed to their respective antisense strands to form labeled double-stranded DNAs. The binding reaction (20 min at room temperature) was carried out in a final volume of 20  $\mu$ l of binding buffer (10 mM Hepes, pH 7.5, 2.5 mM MgCl<sub>2</sub>, 50 mM NaCl, 0.5 mM DTT, 4% glycerol, 1  $\mu$ g of poly dI–dC, 1  $\mu$ g of BSA) containing the biotin-labeled probe (20 fmol), with or without nuclear extract from the 293T cell line. The nuclear extract was prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer's protocol. In the supershift experiments, the nuclear extract was preincubated with anti-Sp1 antibody (Santa Cruz Biotechnology) for 30 min on ice. DNA-nuclear extracts and/or anti-Sp1 antibody complexes were separated on 6% nondenaturing polyacrylamide gels and visualized by the LightShift Chemiluminescent EMSA kit (Pierce) following the manufacturer's protocol.

### 2.8. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were carried out using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. 293T cells ( $1 \times 10^6$  cells) were seeded into 10 cm-diameter tissue culture plates (Falcon) then incubated for 3 days after which the cells were crosslinked with 1% formaldehyde at 37 °C for 10 min. Following fixation the cells were rinsed with ice-cold PBS containing 1x complete, EDTA-free, protease inhibitor cocktail (Roche), then detached from culture surface using a Teflon policeman, and then centrifuged (400  $\times$ g for 4 min at 4 °C) to pellet the cells. SDS lysis buffer (400  $\mu$ l) was added to the cell pellet and the cells were lysed by incubation for 10 min on ice. The lysate was sonicated 4 times for 10 s (Sonic Dismembrator Model 100, Fisher Scientific) in order break the chromosomal DNA into fragments of less than 1 kb. This solution was then centrifuged using a microcentrifuge (10,000  $\times$ g for 10 min at 4 °C) and the supernatant was diluted 10-fold in ChIP dilution buffer. The diluted supernatant (20  $\mu$ l) was used as the "Input" for immunoprecipitation and subsequent PCR.

Immunoprecipitation was performed overnight at 4 °C with 1  $\mu$ l of anti-Sp1 antibody (2  $\mu$ g/ $\mu$ l) or 2  $\mu$ l of control rabbit IgG (1  $\mu$ g/ $\mu$ l). Immuno-complexes were isolated from the overnight immunoprecipitation reaction by the addition of 30  $\mu$ l of a salmon sperm DNA/protein A agarose slurry (supplied in the kit), and incubation for 1 h at 4 °C. The precipitates were washed sequentially with 1 ml each of low-salt wash buffer, high-salt wash buffer, and LiCl wash buffer, then twice with TE buffer. The immuno-complexes were eluted by adding 500  $\mu$ l of elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>) and incubation for 15 min at room temperature. In order to remove the DNA crosslinks, 20  $\mu$ l of 5 M NaCl was added to the eluates and the solution was heated at 65 °C for 6 h. Following heating 20  $\mu$ l of 1 M Tris–HCl (pH 6.5) and 2  $\mu$ l of 10 mg/ml proteinase K, were added and the solution was incubated at 45 °C for 1 h. The released DNA fragments were purified with a Gel Extraction kit (Qiagen) in a final volume of 50  $\mu$ l. The DNA fragments were used as a template for PCR using forward (5'-GAGATTCTAGCCTGGGGTCC-3') and reverse (5'-ACGCAGCTAACCTGGGAGAT-3') primers that amplified the –214 to +28 nts region of the 5'-flanking region of *EPHX2*. PCR was performed following the semi-quantitative RT-PCR method described above for 28, 33 or 38 cycles of amplification. The resulting DNA was resolved on a 1% agarose gel and stained with ethidium bromide.

## 3. Results

### 3.1. Sequencing of the –5974/+28 construct

The nucleotide sequence of the 5'-flanking region of *EPHX2* from the HepG2 cells (in the –5974/+28 construct) was nearly identical to that found on human chromosome 8 nts 5,716,979 to 5,722,980 (GenBank accession no. NT\_023666). There were three differences between our sequence and the NT\_023666 sequence. Our sequence had an extra CTCTT repeat at nt

5,719,222, and lacked T and CACACACA sequences at nts 5,721,164 and 5,722,557, respectively.

### 3.2. Putative transcriptional start site of *EPHX2*

The transcriptional start site of *EPHX2* was predicted by two methods: *in silico* analysis of the 5'-flanking region (to 5974 nts upstream) and 5'-RACE. The Dragon GC+ Promoter Finder program predicted transcription initiation at the first T in the sequence CTTCGC, i.e., nt 5,722,954 of the NT\_023666 sequence. In contrast, DBTSS analysis of 210 human sEH-encoding ESTs showed that transcription initiates at the first C (nt 5,722,953) in the CTTCGC sequence in 40 of these ESTs (~20%); whereas, transcription initiates at the first T in CTTCGC in only 2 of the 210 ESTs (<1%). In comparison, sequencing of 5'-RACE products identified numerous 5'-prime ends, which corresponded to nts 5,722,945, 5,722,957, 5,722,968, 5,722,973, 5,722,977, 5,722,981, 5,722,997, and 5,722,999 of the NT\_023666 sequence. Consistent with these findings, multiple transcriptional start sites have been shown to be associated with a TATA-less, GC-rich sequences [47,48]. In this study, transcription initiation was predicted to occur at the first C of the CTTCGC sequence (nt 5,722,953) of the 5'-flanking region of *EPHX2*.

### 3.3. Relative transcriptional activity of the 5'-flanking region of *EPHX2*

The *in silico* analysis of the 5'-flanking region (to 5,974 nts) of *EPHX2* did not identify a consensus TATA box or CAAT sequences. However, the *in silico* analysis did identify a 751 nts-long region (nts –344 to +407) with high GC content (~64%) near the predicted transcriptional start site (Fig. 1). A CpG island was also identified around nts –291 to +507 (Fig. 1A). In order to test the *in silico* predictions, a transient expression assay based on a reporter firefly luciferase gene was established. In the human liver cancer-derived HepG2 cell line, the reporter plasmid carrying nts –374 to +28 of *EPHX2* (–374/+28 construct) gave the same relative luciferase activity as a plasmid carrying nts –5,974 to +28 (–5974/+28 construct) indicating that the –374 to +28 sequence was the minimal essential promoter (Fig. 1B). However, the relatively low transfection efficiency of HepG2 cells (i.e., only 1/160th the efficiency of 293T cells, see below) made it difficult to measure relative luciferase activity when reporter plasmids carrying DNA fragments shorter than the –374 to +28 region were tested.

In order to identify a cell line which supported higher transfection efficiency and transcriptional activity, 9 human cell lines (Table 1) were screened. Initially, endogenous sEH activity and relative mRNA abundance of *EPHX2* (Table 1) were measured in these cell lines under the assumption that high sEH activity would directly correlate to high levels of *EPHX2* mRNAs. A good correlation ( $r=0.73$ ,  $p<0.05$ ) was found between sEH activity and relative mRNA abundance. All of the cell lines tested showed lower sEH activities in comparison to that reported from a S9 fraction prepared from human kidney

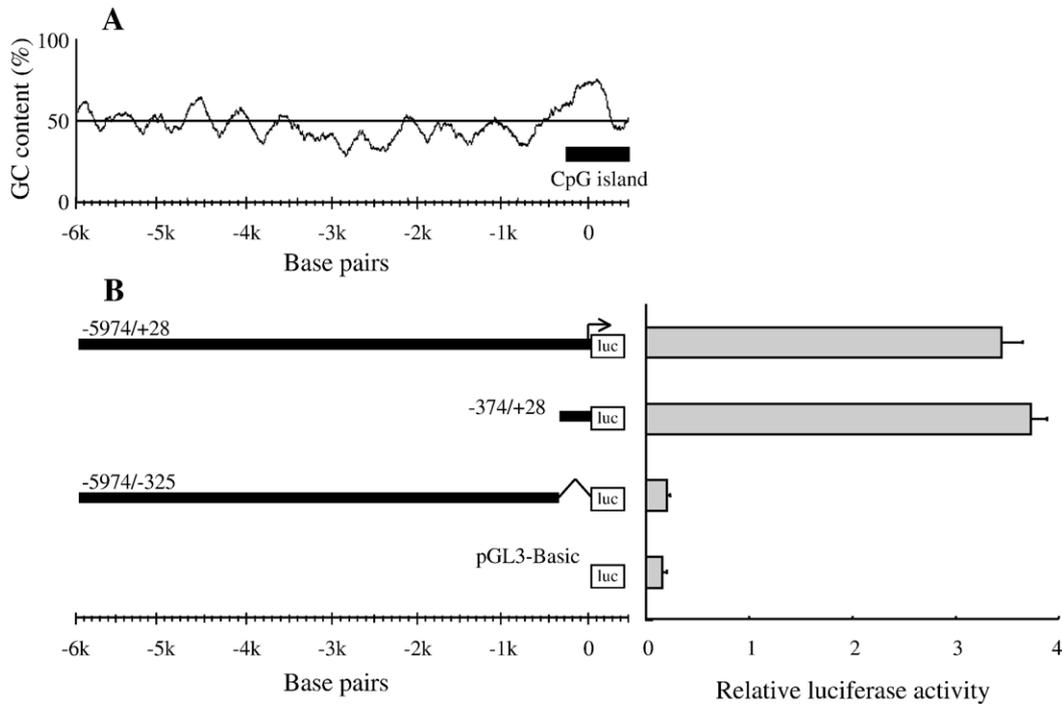


Fig. 1. GC content and putative minimal essential promoter analysis of the 5'-flanking region of *EPHX2*. (A) GC content (%) of the 5'-flanking region of *EPHX2* from 6000 nts upstream to 500 nts downstream of the putative transcriptional start site. A moving window of 200 nts was used to calculate the GC content. The bar indicates a putative CpG island (−291 to +507). (B) The minimal promoter sequence for expression of a reporter firefly luciferase (*luc*) gene in HepG2 cells was found from 374 nts upstream to 28 nts downstream (−374/+28 construct) of the putative transcriptional start site of *EPHX2*. The −5974/−325 construct lacks 352 nts (from 324 nts upstream to 28 nts downstream) of the −5974/+28 construct. The pGL3-Basic construct contains no *EPHX2*-derived sequence. The transcriptional activity of these constructs is shown in terms of relative luciferase activity (i.e., firefly luciferase activity/control renilla luciferase activity). Error bars indicate the standard deviation of three independent experiments. The arrow indicates the putative transcriptional start site of *EPHX2*.

using the same t-DPPO substrate (1200 to 5500 pmol/min/mg) [21]. Of the 9 cell lines tested, HuH-7 showed the highest sEH activity (940 pmol/min/mg) and relative mRNA abundance ( $EPHX2/GAPDH=0.99$ ). sEH activity was not detected in the SN12C cell line under the conditions used in this study, even though the cell line showed low levels of relative mRNA abundance of *EPHX2* (Table 1).

The ability of the −5974/+28 and −374/+28 constructs to induce relative luciferase activity was tested in the 9 human cell lines in which sEH activity was screened (Fig. 2). No correlation was found between sEH activity and relative luciferase activity of the −5974/+28 and −374/+28 constructs ( $r=0.32$  ( $p=0.4$ ) and  $0.04$  ( $p=0.9$ ), respectively). There was, however, a weak correlation between sEH activity and the ratio of the relative luciferase activity of the −5974/+28 construct with respect to the −374/+28 construct ( $(-5974/+28)/(-374/+28)$ ) ( $r=0.66$ ,  $p=0.05$ ). The shorter −374/+28 construct showed the same or higher relative luciferase activity in comparison to the longer −5974/+28 construct in 8 of the 9 cell lines tested (Fig. 2). This suggested that the −5974 to −375 region may contain “inhibitory sequences”. In contrast, the −5974/+28 construct showed 1.7-fold higher relative luciferase activity in comparison to the −374/+28 construct in the HuH-7 cells.

The transfection efficiency (shown as RLU of renilla luciferase per  $1 \times 10^4$  cells in Table 1) of the pRL-CMV

plasmid was more than 10-fold higher in 293T cells than in the 8 other cell lines tested. Transfection efficiency in the 293T cell line was about 160-fold higher than that of HepG2 cells.

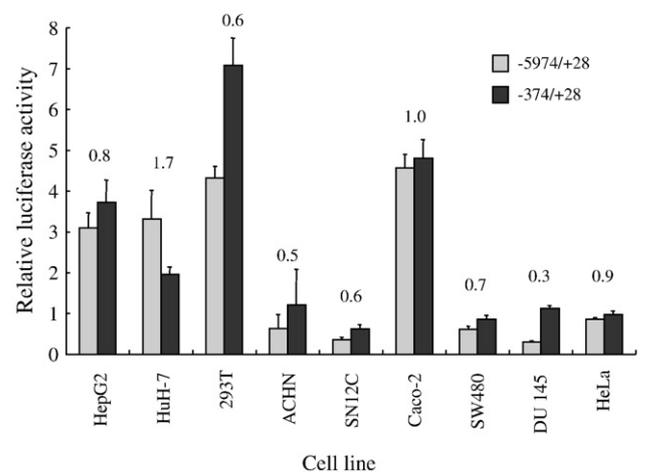


Fig. 2. Relative luciferase activity of two reporter constructs carrying 6.0 kb (−5974/+28 construct) or 0.4 kb (−374/+28 construct) of the 5'-flanking region of *EPHX2* in 9 human cell lines (described in Table 1). The results are expressed as relative luciferase activity (i.e., firefly luciferase activity/control renilla luciferase activity). The numbers above the bars indicate the ratio of the relative luciferase activity of the −5974/+28 construct divided by that of the −374/+28 construct. Error bars indicate the standard deviation of three independent experiments.

### 3.4. Transcriptional analysis of the GC-rich region of the 5'-flanking region of EPHX2

The *in silico* analysis identified numerous potential *cis*-elements within the GC-rich region (nts -400 to +20) of the 5'-flanking region of *EPHX2* (Fig. 3). These potential regulatory elements include c-Rel/NFκB, AP-1, and cMyc/Max1 elements, as well as 20 potential Sp1 binding sites. Sp1 sites are also referred to as GC boxes [49–51]. In order to identify functional regulatory elements within this region, nested deletions were generated and the ability of the resulting fragments to activate the firefly luciferase gene in pGL3-Basic was determined in 293T cells (Fig. 4). Reporter plasmids carrying nts -374 to -165 of the 5'-flanking region of *EPHX2* showed identical relative luciferase activities. Relative luciferase activity gradually declined in reporter plasmids

carrying shorter lengths of the 5'-flanking region (from nts -165 to -63). Relative luciferase activity was not detected in reporter plasmids containing less than 63 bp of the 5'-flanking region. Statistically significant ( $p < 0.01$ ) differences were found in reporter plasmids carrying deletions of nts -165 to -155 (AP-1), -104 to -86 (Sp1), -86 to -63 (Sp1), and -63 to -41 (Sp1s and cMyc/Max1). Potential regulatory elements found in these regions are indicated within the parentheses.

### 3.5. Transcriptional regulation of the GC-rich region of the 5'-flanking region of EPHX2

Reporter plasmids carrying nested deletions of the 5'-flanking region of *EPHX2* indicated that the -165 to +28 nts region is important for transcriptional activity (Fig. 4). In order

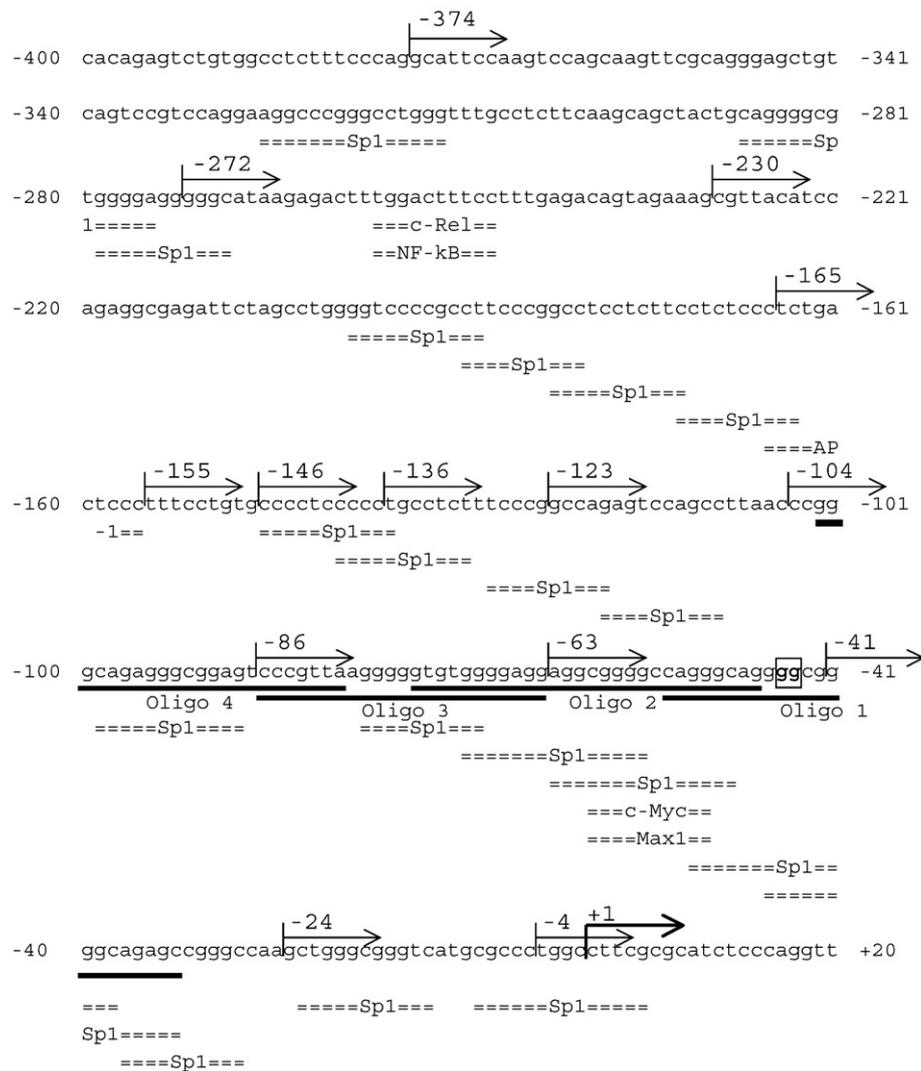


Fig. 3. Nucleotide sequence of the 5'-flanking region of *EPHX2* from -400 nts upstream to 20 nts downstream of the putative transcriptional start site. The sequence corresponds to nts 5,722,553 to 5,722,972 of human chromosome 8 (GenBank accession no. NT\_023666). The putative transcriptional start site C is marked by +1 and indicated by a bold arrow. Numbers to the left and right indicate nucleotides upstream (-) and downstream (+) of the putative transcriptional start site. Putative transcription factor binding sites (Sp1, c-Rel/NFκB, AP-1, and cMyc/Max1) are indicated below the sequence. The 5' ends of the nested deletions (-272 to -3) used in the promoter analysis (see Fig. 4) are indicated by arrows above the sequence. The underlined text indicates the oligonucleotides (Oligos 1 to 4) used for EMSA in Fig. 7A. The GG to TT mutation site of the -374/+28TT and -63/+28TT constructs is boxed and indicated by bold.

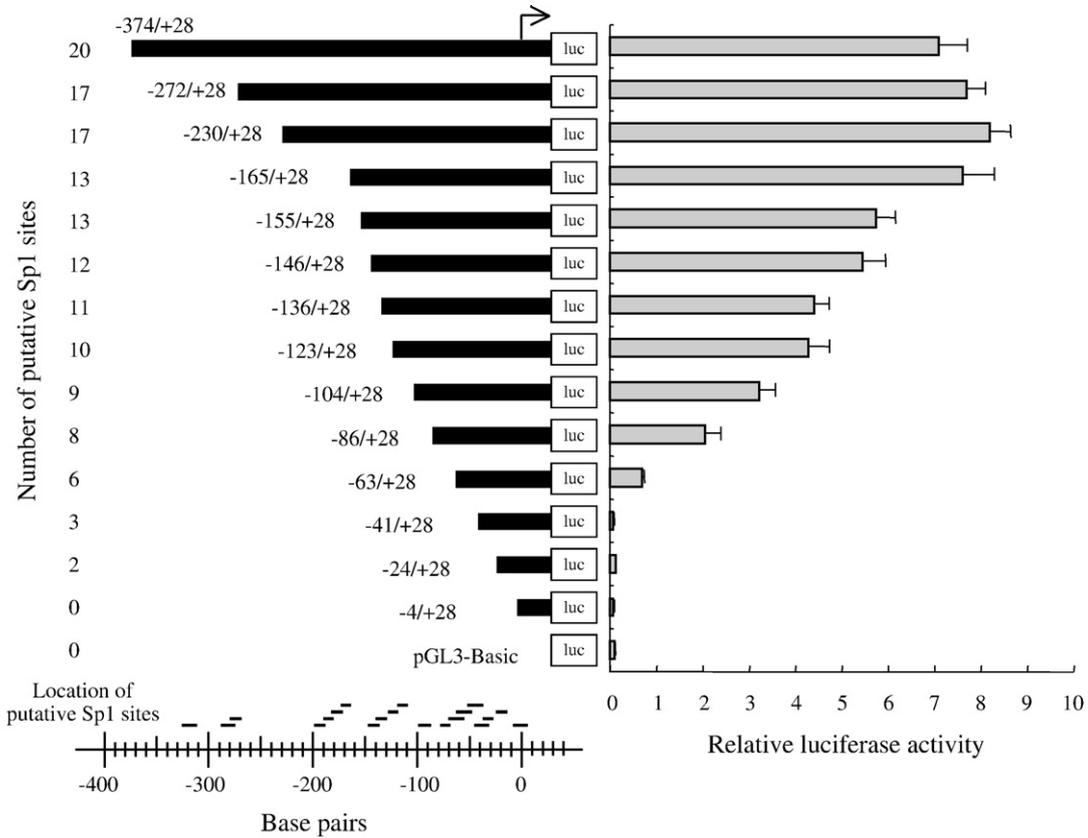


Fig. 4. Relative luciferase activity following transfection of human 293T cells with reporter plasmids carrying nested deletions of the 5'-flanking region (from 374 nts upstream to 28 nts downstream) of the putative transcriptional start site of *EPHX2*. The nucleotide sequence of the 5' ends of the nested deletions are shown in Fig. 3. The results are expressed as the relative luciferase activities (i.e., firefly luciferase activity/control renilla luciferase activity). Error bars indicate the standard deviation of three independent experiments. The number and relative locations of the putative Sp1 binding sites within are shown to the left and bottom, respectively. The arrow indicates the putative transcriptional start site.

to test whether specific regions within the -165 to +28 nts region were important for full promoter activity, deletion of nts -104 to -87 or -63 to -42 from the -374/+28 construct were generated (Fig. 5). The -374/+28 Δ(-104/-87) and -374/

+28 Δ(-63/-42) constructs resulted in 20% and 40% reductions, respectively, of relative luciferase activity in comparison to the -374/+28 construct. Deletions of both regions, the -374/+28 Δ(-104/-87, -63/-42) construct, resulted in an 80% drop

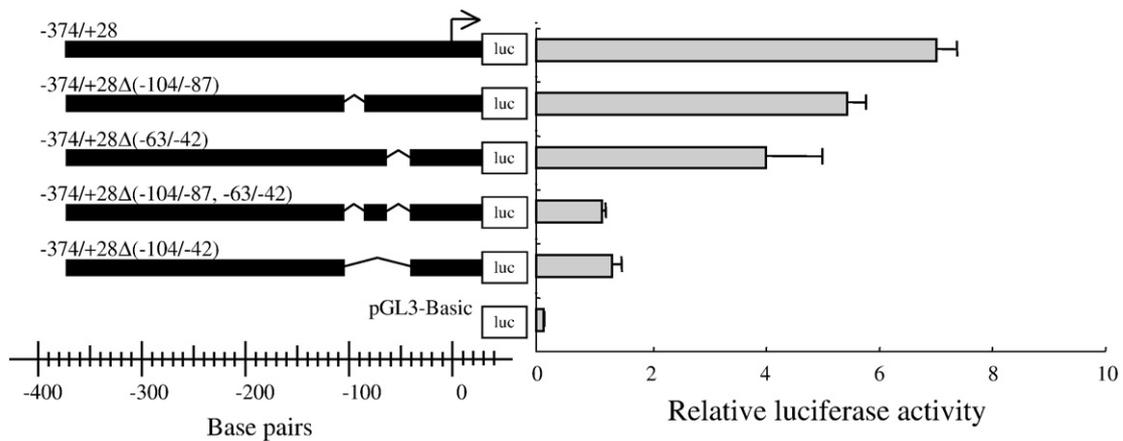


Fig. 5. Relative luciferase activity was measured following transfection of human 293T with reporter plasmids carrying various deletions of the -374/+28 construct {-374/+28 Δ(-104/-87), -374/+28 Δ(-63/-42), -374/+28 Δ(-104/-87, -63/-42), and -374/+28 Δ(-104/-42)}. The results are expressed as relative luciferase activity (i.e., firefly luciferase activity/control renilla luciferase activity). Error bars indicate the standard deviation of three independent experiments. The arrow indicates the putative transcriptional start site.

in relative luciferase activity. Deletion of the entire  $-104$  to  $-42$  region  $-374/+28 \Delta(-104/-42)$  construct, also showed an approximately 80% drop in relative luciferase activity (Fig. 5).

The results of luciferase reporter assay (Figs. 4 and 5) indicated that the  $-63$  to  $-42$  nts region is essential for *EPHX2* transcription. This 22 nts-long region contained or overlapped six potential regulatory elements including four Sp1, one cMyc, and one Max1 sites (Fig. 3). Only the putative Sp1 site that starts at nt  $-47$  showed complete identity to the Sp1 consensus sequence GGGGCGGGGC [52]. Thus, reporter constructs,  $-63/+28$ TT and  $-374/+28$ TT, in which the conserved Sp1 site was mutated from GGGGCGGGGC to GGTTTCGGGGC were tested in the luciferase reporter assay. No relative luciferase activity ( $p < 0.01$ ) was observed with the  $-63/+28$ TT construct, whereas, the  $-374/+28$ TT construct showed a 23% reduction in relative luciferase activity ( $p < 0.01$ ) (Fig. 6). The transcriptional activity of the  $-374/+28$ TT construct ( $5.4 \pm 0.4$ ) was slightly higher than that of the  $-374/+28 \Delta(-63/-42)$  construct ( $4.3 \pm 0.7$ ), in which the Sp1 consensus sequence was completely removed (Fig. 5).

### 3.6. EMSA and ChIP assay

Electrophoretic mobility assays were performed in order to test whether the putative Sp1 sites that are located within nts  $-102$  to  $-33$  of the 5'-flanking region of *EPHX2* can bind to proteins in the nuclear extract of 293T cells (Fig. 7A). On the basis of the supershift with anti-Sp1 antibody, Sp1 in the nuclear extract of 293T cells bound to nts  $-54$  to  $-33$  (Oligo 1),  $-74$  to  $-47$  (Oligo 2), and  $-102$  to  $-80$  (Oligo 4). However, Sp1 appeared not to bind to a DNA fragment containing nts  $-86$  to  $-64$  (Oligo 3). Additionally, ChIP assays were performed to further test whether Sp1 binds to the *EPHX2* flanking region (Fig. 7B). The chromosomal DNA–protein complexes from 293T cells were specifically recognized by the anti-Sp1 antibody, and the subsequent PCR using a pair of oligonucleotide primers that targeted the  $-214$  to  $+28$  region of the *EPHX2* confirmed that Sp1 was binding to the Sp1 binding site within this region.

## 4. Discussion

Soluble epoxide hydrolase is a key enzyme in the arachidonic acid pathway in mammals [3]. Inhibition of sEH has been associated with improvements in various disease models such as high blood pressure, atherosclerosis, and kidney failure presumably by increasing EETs and possibly other fatty acid epoxides [10,13,15,53,54]. On the other hand, our observations also suggest that increased expression of *EPHX2* could lead to cardiovascular diseases. In contrast to the large body of work that has been done on the enzymatic properties and biological roles of sEH, the transcriptional regulation and tissue-enriched expression of *EPHX2* are poorly defined. Here we characterize the essential and enhancing elements of the promoter of *EPHX2*. Transcriptional repressors of *EPHX2*, analogous to compounds that inhibit enzymatic activity, could be potential targets for therapeutic application. Alternatively, enhancement of *EPHX2* transcription may offer beneficial effects in certain cases [55]. For example, increased *EPHX2* transcription could reduce or inhibit angiogenesis that is caused by EETs in cancerous tissues [56]. In agreement with this, sEH expression is generally reduced in human malignant tissues [57]. Furthermore, a variety of lipid epoxides including both fatty acids and terpenes [58] are excellent substrates for sEH. This suggests that sEH may catalyze other important chemical mediators that are involved in disease induction.

The *in silico* analysis of the 5'-flanking region of the *EPHX2* did not identify typical TATA or CAAT box motifs. However, a GC-rich region was found between nts  $-374$  and  $+28$  of *EPHX2* (Fig. 1A). This approximately 0.4 kb-long region was found to contain the minimal essential sequence for full relative luciferase activity (Fig. 1B). Androgen {e.g., GG(A/T)ACANNNTGTTCT [59]} and PPAR-alpha {e.g., AGGTCA(A/T)AGGTCA [60]} responsive elements were not found in the 5'-flanking region (nts  $-5,974$  to  $+28$ ) of *EPHX2*. This was somewhat surprising since PPAR-alpha agonists such as clofibrate [23,31,33] and testosterone [31] have been shown to increase hepatic sEH activity in rodents. These response elements may, however, be found in other regions or they may

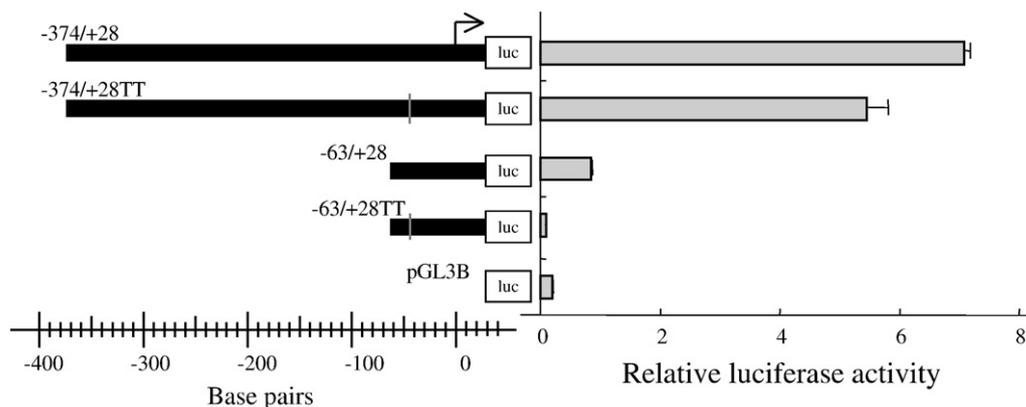


Fig. 6. Mutational analysis of the  $-374$  to  $+28$  and  $-63$  to  $+28$  nts regions of the 5'-flanking region of *EPHX2*. Relative luciferase activity following transfection of human 293T with reporter plasmids carrying GG to TT mutations of the  $-374/+28$  ( $-374/+28$ TT) and  $-63/+28$  ( $-63/+28$ TT) constructs. The mutations are described in the text and shown in Fig. 3. The results are expressed as relative luciferase activity (i.e., firefly luciferase activity/control renilla luciferase activity). Error bars indicate the standard deviation of three independent experiments. The arrow indicates the putative transcriptional start site.

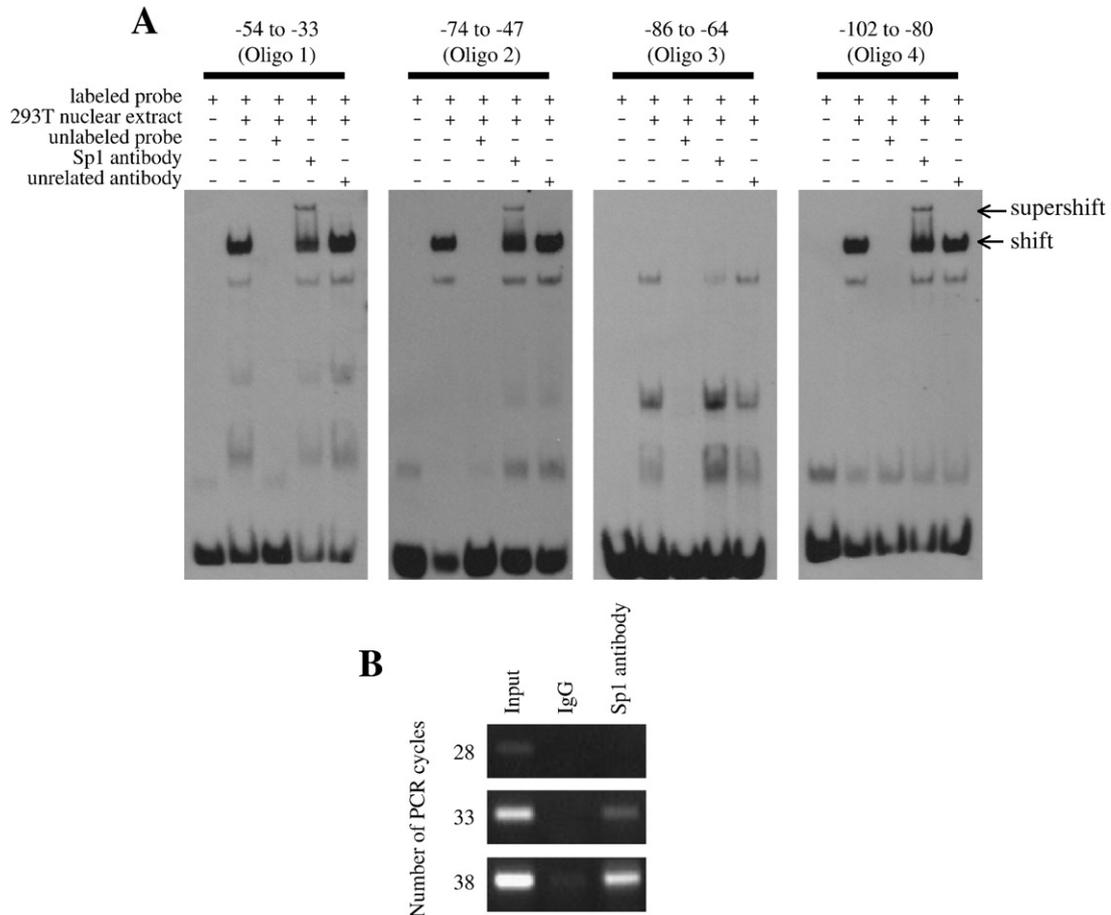


Fig. 7. EMSA and ChIP assay of the 5'-flanking region of *EPHX2*. (A) The electrophoretic mobility shift assay was performed using biotin-labeled probes (Oligo 1: -54 to -33, Oligo 2: -74 to -47, Oligo 3: -86 to -64, and Oligo 4: -102 to -80). Following incubation with nuclear extract and/or anti-Sp1 antibody, the reaction was separated by 6% native PAGE. A 100-fold molar excess of unlabeled probe was used in the competition assays. Arrows at the right indicate DNA–nuclear extract complex (shift) or DNA–nuclear extract–anti-Sp1 antibody complex (supershift). (B) The chromatin immunoprecipitation assay was performed using chromosomal DNA fragments (Input) from 293T cells. Immunoprecipitation was performed with control IgG or anti-Sp1 antibody. Following heat and proteinase K treatment, the released chromosomal DNA fragments were used as template for PCR using primers that specifically amplified the -214 to +28 region of the 5'-flanking region of *EPHX2*. The number of PCR cycles is shown to the left.

be different from the response elements in rodents. Furthermore, there may be inherent differences between human and rodent models such that the extrapolation of potential enzyme induction effects observed with PPAR ligands in the rodent model to humans should be done with caution. These observations also illustrate the need for similar reporter analysis systems for *EPHX2* in rodents and other non-human model species.

Our analysis of the 5'-flanking region of *EPHX2* indicated that the GC-rich region (-374 to +28) of *EPHX2* is involved in the basic transcriptional regulation of *EPHX2*. To further evaluate the role of this region as a core promoter, we evaluated the ability of the -374/+28 construct to induce luciferase activity in 9 human cell lines. We found that the transfection efficiency of these cell lines varied by more than 1000-fold. Furthermore, correlations between sEH activity and relative luciferase activity for each construct (-5974/+28 and -374/+28) were not found. There was, however, a weak correlation ( $r=0.66$ ,  $p=0.05$ ) between sEH activity and a ratio of the relative luciferase activity of the -5974/+28 construct with

respect to the -374/+28 construct  $\{(-5974/+28)/(-374/+28)\}$  (Fig. 2). These findings suggested that in addition to promoter effects, tissue-enriched factors are also important. Additionally, these findings suggested that the mechanisms of transcriptional regulation among these cells could be different. Using the cell line (293T) with the highest transfection efficiency, we found that the -165 to +28 nts region is the minimal sequence necessary for full luciferase activity (Fig. 4). Furthermore, luciferase activity gradually declined in reporter plasmids carrying shorter lengths of the 5'-flanking region (from nts -165 to -63). This suggested that one or more of the four regulatory elements located in this region  $\{-165$  to -155 (AP-1), -104 to -86 (Sp1), -86 to -63 (Sp1), and -63 to -41 (Sp1s and cMyc/Max1) $\}$  are functionally active.

Transcriptional regulation of genes that are expressed under TATA-less promoters often involves sequences that are found within GC-rich regions [48]. Sp1 binding sites are also commonly located within CpG islands in order to maintain the appropriate methylation pattern of downstream genes [61–63]. Through analysis of the minimal essential sequence (nts

–165 to +28) required for full expression, we found numerous potential regulatory elements, especially Sp1 sites in this –165 to +28 region (Fig. 3). We showed that nts –63 to –42 are more important for *EPHX2* transcription than nts –104 to –87 (Fig. 5). Electrophoretic mobility assays also showed that Sp1 binds to nts –54 to –33, nts –74 to –47, and nts –102 to –80 but not nts –86 to –64 (Fig. 7A). Furthermore, the ChIP assay showed that Sp1 binds to the –214 to +28 region of the *EPHX2* (Fig. 7B). Only one completely conserved Sp1 consensus sequence motif, GGGGCGGGC (nts –47 to –38), was found within the –165 to +28 nts region. Mutation of this sequence to GGTTCGGGGC resulted in a loss of transcriptional activity (Fig. 6). This suggested that in *EPHX2*, the Sp1 site immediately upstream of the putative transcriptional start site is essential for expression. Because Sp1 expression is generally ubiquitous [64] such a promoter system could explain the ubiquitous expression of sEH in numerous tissues in mammals [17,20,21,23,25–30,65–67]. However, it does not explain the regulation of *EPHX2* by PPAR- $\alpha$  agonists and testosterone as predicted from *in vivo* induction studies in rodents. As discussed earlier, this discrepancy could be due to the effects of clofibrate and testosterone being indirect rather than direct, but it also could illustrate a difference between man and rodents.

Ang II is known to increase sEH activity and sEH protein levels in kidney [10] and vascular endothelium [34]. Ang II induces the promoter activity of *EPHX2* in transient transfection assays, through the binding of c-Jun to the AP-1 binding site of the promoter. These data tie the regulation of sEH and EETs to the renin–angiotensin dependent regulation of blood pressure and inflammation [34]. The molecular basis of the high basal levels of sEH expression in renal, hepatic and a few other tissues as well as its regulation by endogenous compounds and xenobiotics awaits further work.

## Acknowledgments

Support for this work was provided by NIEHS R37 ES02710, NIEHS P42 ES04699, NIH/NHLBI R01 HL59699-06A1, and NIEHS P30 ES05707. We thank Drs. Michael L. Goodson and Michael S. Denison from the University of California at Davis for their technical advice.

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