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Cloning and characterization of the rat glutathione peroxidase gene

Ye-Shih Ho and Adriann J. Howard

Laboratory of Molecular Biology, Division of Allergy, Critical Care and Respiratory Medicine, Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA

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The increased activity of glutathione peroxidase (GSHPx) in rat lungs is associated with the development of tolerance of the animals to hyperoxia. To understand further the regulation of expression of this enzyme, the molecular structure of the corresponding rat gene was characterized. The rat GSHPx gene consists of two exons interrupted by a single intron of 217 base pairs. The same initiation sites for transcription were found to be utilized in both lung and liver. The promoter of the GSHPx gene contains neither a 'TATA' box nor a 'CAAT' box. Instead, it comprises two copies of Sp1 binding motif and one copy of AP-2 binding motif. These features of the promoter may offer a clue to the mechanisms by which the expression of this gene is controlled.

Genomic cloning; S1 nuclease mapping; Primer extension; Regulatory sequence

1. INTRODUCTION

Glutathione peroxidase (GSHPx) plays an important role in cellular antioxidant defense by reducing hydrogen peroxidase or various hydroperoxides using glutathione as a reducing agent to form water or corresponding alcohols, respectively ($H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ or $ROOH + 2GSH \rightarrow GSSG + ROH + H_2O$) [1]. This enzyme, which contains selenocysteine at the active site, is present on both cytosol and mitochondria in eukaryotic cells [2–5]. Molecular cloning data have revealed that selenocysteine is encoded by an opal nonsense codon (UGA) in mouse, rat, bovine and human genes [6–13]. However, the mechanisms by which selenocysteine is incorporated into this enzyme are less conclusive [14–16]. The recent discovery of a unique species of opal suppressor selenocysteyl-$tRNA^{Ser}$ in rat mammary tumor cells suggests selenocysteine is inserted co-translationally during the synthesis of this enzyme [17]. It has also been demonstrated by Berry et al. [18] that the 3′ untranslated sequence of rat glutathione peroxidase cDNA can substitute the 3′ untranslated sequence of Type I iodothyronine 5′ deiodinase cDNA (which also contains an opal stop codon coding for selenocysteine) in directing insertion of selenocysteine in response to UGA codon [18]. Their results further substantiate the co-translational mechanism for usage of opal codon in selenium incorporation into this enzyme.

Exposure of mammals to hyperoxia can cause extensive lung injury [19]. This type of pulmonary damage is due to the overproduction of reduced oxygen species in the lung. Resistance to a lethal concentration of oxygen (100%) does occur in adult rats after exposing them to a sublethal concentration of oxygen (85%) for 5–7 days, and this resistance is associated with an increase in the activities of lung antioxidant enzymes including glutathione peroxidase [20,21]. To elucidate the molecular mechanisms that regulate the expression of this enzyme in rat lungs during hyperoxia, we chose to first understand the structure of the corresponding gene. In this report, we describe the isolation and characterization of the rat GSHPx gene, and the nature of its promoter sequence which may play a role in regulating its expression under normal physiologic and hyperoxic conditions.

Correspondence address: Y.-S. Ho, Room 4.000, Institute of Chemical Toxicology, Wayne State University, 2727 Second Avenue, Detroit, MI 48201-2654, USA. Fax: (1) (313) 577-0082.

2. MATERIALS AND METHODS

2.1. *Isolation of genomic clones and DNA sequence analysis*

A female Sprague–Dawley rat genomic DNA library, constructed in vector λ Charon 4A with *Hae*III partially digested genomic DNA (Clontech Laboratories Inc., Palo Alto, CA), was screened with the 3′ *Eco*R1 fragment (from bases 625 to 1161) of the rat glutathione peroxidase cDNA according to the procedures described by Benton and Davis [22]. Two positively hybridized clones, clones 12 and 18, were isolated and subsequently determined to be identical by restriction digestion analysis. The 3′ end genomic DNA fragment was excised from clone 12 by *Eco*R1 digestion, and then ligated into plasmid pKS (Stratagene, LaJolla, CA) for DNA sequence analysis [23]. The 5′ end genomic DNA fragment which was found not to be released by *Eco*R1 digestion, presumably due to loss of 5′ *Eco*R1 cloning site during the library construction, was excised from the phage arm by *Kpn*I and *Eco*R1 digestion. Single-stranded plasmid DNAs were generated by infecting bacteria harboring various plasmids with helper M13 bacteriophage VCS [24], and then sequenced by Sanger's dideoxy chain-termination method using universal and complementary oligonucleotide primers [25].

DNA blot analysis of rat genomic DNA, and RNA blot analysis were performed according to the methods described by Southern [26] and Thomas [27], respectively.

2.2. *S1 nuclease mapping and primer extension experiments*

One antisense oligonucleotide with sequence 5′ CAGCGGGCGC-GCGGAGAAGGCATACACGGT 3′ complementary to nucleotide residues +72 to +101 was used in these experiments. For S1 nuclease mapping experiment, the oligonucleotide was initially labeled at 5′ end with γ-[^{32}P]ATP and polynucleotide kinase, and then annealed to a sense, single-stranded plasmid containing the 5′ end *Bam*HI–*Eco*RI fragment of the rat GSHPx gene, followed by synthesis of the complementary strand of DNA using the Klenow fragment of *E. coli* DNA polymerase I. The resultant double-stranded DNA was digested with restriction enzyme *Bam*HI and then the labeled single-stranded DNA was purified after separation on a polyacrylamide–urea gel. Fifty micrograms of total rat lung and liver RNA were used for S1 nuclease mapping according to the procedures described by Greene and Strubl [28].

The 5′ end ^{32}P-labeled oligonucleotide was also used in primer extension experiments. Fifty micrograms of total lung and liver RNA were used in primer extension experiments following the methods described by Kingston [29].

3. RESULTS AND DISCUSSION

3.1. *Isolation and characterization of the rat GSHPx gene*

Two identical rat genomic clones were isolated from a Sprague–Dawley rat genomic library using the 3′ *Eco*RI rat GSHPx cDNA fragment as a probe [9], and the nucleotide sequence of the DNA containing the entire GSHPx gene was completely determined (Fig. 1). By comparing the genomic and cDNA sequences, the rat GSHPx gene is divided into two exons interrupted by a single intron of 217 base pairs. There is no sequence homology found between the first 316 base pairs of the cDNA sequence determined by our laboratory [9] and the genomic DNA. Furthermore, the sequence of the 3′ *Eco*RI cDNA fragment for rat GSHPx cloned by Reddy et al. [7] was also not found in the genomic clone. These portions of cDNAs isolated by us and Dr. Reddy and colleagues are presumably derived from cloning artifacts, since no potential splicing donor and acceptor sites capable of generating those non-homologous cDNA sequences are found at the 5′ and 3′ end of the genomic sequence.

3.2. *DNA and RNA blot analysis of the rat GSHPx gene*

In order to understand the complexity of the GSHPx gene in rat genome, DNA blot analysis of total rat genomic DNA digested with various restriction enzymes was performed using the cDNA (from bases 318 to 1161, generated by the polymerase chain reaction [30]) as a probe. Fig. 2a shows that two *Pst*I genomic DNA fragments of 1.2 and 0.8 kilobases (kb), which are identical to the sizes predicted from genomic cloning data, hybridized with the probe. The numbers of other hybridized restriction genomic DNA fragments were also consistent to those predicted from the cloning result. These observations strongly suggest that a single gene coding for this enzyme is present per haploid rat genome.

Expression of GSHPx in rat tissues was also examined by RNA blot analysis (Fig. 2b). A single species of 1.3 kb mRNA was found to hybridize with the probe. The rat GSHPx gene expresses at a higher level in the liver than does in the lung.

3.3. *Mapping of translational start site(s)*

The transcriptional initiation site(s) in rat lung and liver was determined by both S1 nuclease mapping and

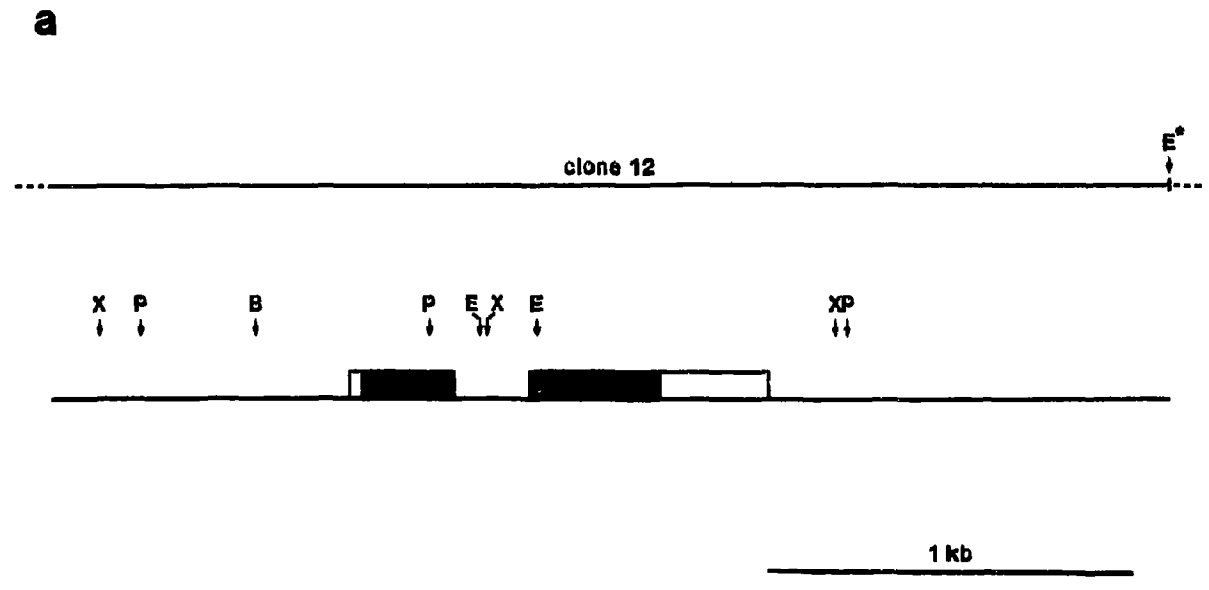

Fig. 1. (a) Restriction map and organization of the rat GSHPx gene. E, B, H, P and X represent restriction sites for enzymes *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I and *Xho*I, respectively. Solid and open boxes represent translated and untranslated regions of the mRNA, respectively. The map of recombinant phage clone 12 is shown at the top. E* denotes *Eco*RI restriction site artificially generated during library construction.

b

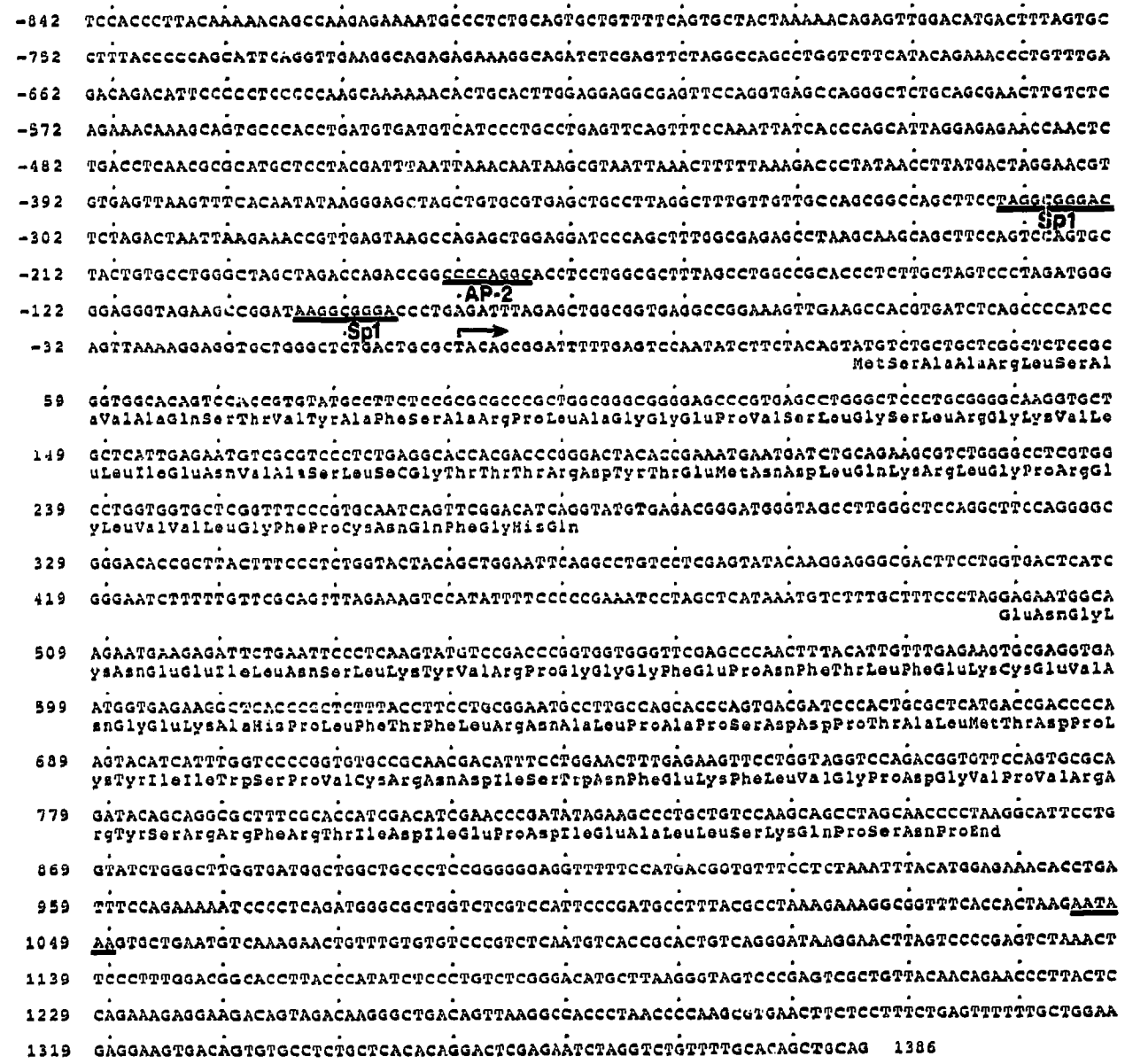

Fig. 1. (b) Nucleotide sequence and deduced amino acid sequence of the rat GSHPx gene. The transcriptional start site is numbered as nucleotide residue 1 of the gene. The sequences homologous to the known eukaryotic regulatory elements and polyadenylation site are underlined.

primer extension experiments (Fig. 3). A single-stranded DNA probe encompassing nucleotides −260 to +101 of the genomic sequence was used in S1 nuclease analysis. As shown in Fig. 3, multiple S1 nuclease-resistant fragments approximately 100 nucleotides long were observed. The amounts of the protected fragments derived from the protection of RNA isolated from lung or liver were proportional to the levels of the GSHPx RNA in each tissue revealed by RNA blot analysis. The same size, multiple primer- extended DNA fragments were also obtained from primer extension experiments. Furthermore, identical S1 nuclease protected and primer-extended fragments were derived from lung and liver RNAs. These results indicated that the same multiple transcriptional initiation sites were used in rat lung and liver. The most prominent band revealed by both mapping experiments corresponds to the transcript initiated at 35 base pairs upstream from the first AUG codon of the gene. This transcriptional start site was then designated as nucleotide residue 1 of the rat GSHPx gene.

3.4. *Characterization of 5' flanking region of the rat GSHPx gene*

The nucleotide sequence immediately upstream from the transcriptional start site contains neither a 'TATA' nor a 'CAAT' box. However, several potential regulatory elements were found within the promoter region

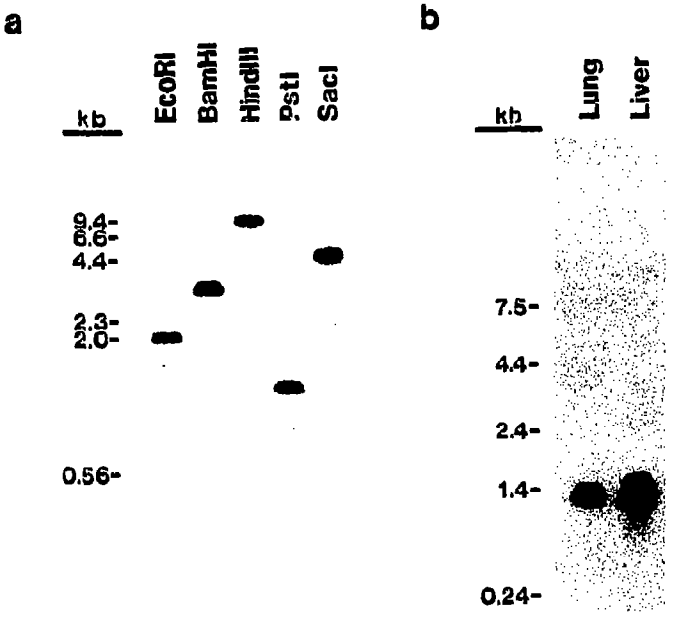

Fig. 2. DNA and RNA blot analyses of the rat GSHPx gene. (a) DNA blot analysis of the rat GSHPx gene. Ten micrograms of genomic DNA isolated from Sprague–Dawley rat liver were digested with various restriction enzymes, and used for blot analysis. The restriction enzymes used are shown at the top of the autoradiograph. DNA size markers are *Hin*dIII-digested λ DNA. (b) RNA blot analysis of GSHPx mRNA in rat lung and liver. Fifty micrograms of total rat lung and liver RNAs were used in blot analysis. The positions of RNA size markers are shown on the left.

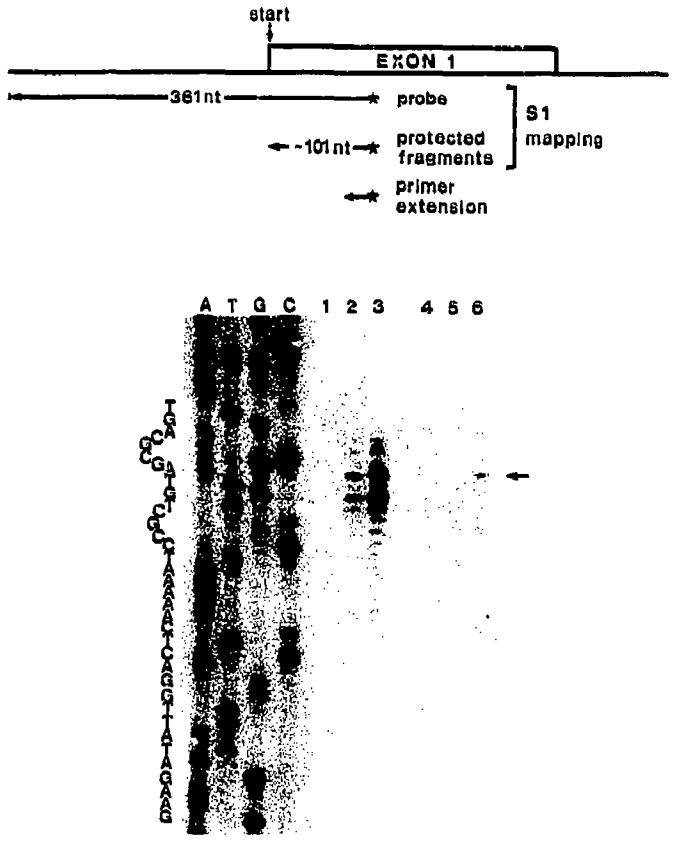

Fig. 3. Mapping of the transcriptional initiation site(s) of the rat GSHPx gene. The transcriptional initiation site(s) was determined by both S1 nuclease mapping (lanes 1–3) and primer extension (lanes 4–6) experiments. Fifty micrograms of yeast tRNA (lanes 1 and 4), total rat lung RNA (lanes 2 and 5) and total rat liver RNA (lanes 3 and 6) were used in each experiment. A dideoxynucleotide sequencing ladder, obtained with the same primer and single-stranded DNA template used to prepare the probe for S1 nuclease mapping, was generated and used for estimation of the sizes of the resultant DNA fragments. The identical primer extended fragments derived from lung RNA to those from liver RNA can be seen after a longer exposure of the autoradiograph. The arrow indicates the major S1 nuclease-resistant and primer-extended fragments.

(Fig. 1b). Two stretches of DNA sequence homologous to the Sp1 binding consensus sequence, (GT)(GA)GGCG(GT)(GA)(GA)(CT), are present at positions −312 and −104 [31]. One copy of AP-2 binding motif conferring activation of gene expression in response to phorbol esters of cAMP locates at position −181 [32,33]. Since the regulatory sequences for transcription of a particular gene are often conserved across species during the evolution of mammals, we further compared the promoter sequences of the GSHPx genes among rat, mouse and human [6,13]. The promoters of rat and mouse GSHPx genes share a high degree of homology (Fig. 4). Nonetheless, there is virtually no homology found between the rodent and the published, short sequence of human promoter (134 base pairs) [13]. The human GSHPx promoter was originally defined by comparing the cloned genomic and cDNA sequences, rather than by S1 nuclease mapping and primer extension experiments. It is possible that the cDNA for human enzyme might be derived from a rare species of transcript initiated 5′ to the normal transcriptional start site. We, therefore, further aligned the human sequence upstream from the first AUG to the promoters of rat and mouse genes (Fig. 4). Interestingly, several regions of homologous sequence were found among these species. The Sp1 binding motif present at position −104 of the rat promoter apparently is conserved in all species. The AP-2 binding site was found to be unique in rat promoter. Additional three regions of highly homologous DNA sequence between the second Sp1 binding site and mRNA start site were also evident. Further experiments are required to dissect the function of each of the conserved DNA elements in regulating the expression of the GSHPx gene.

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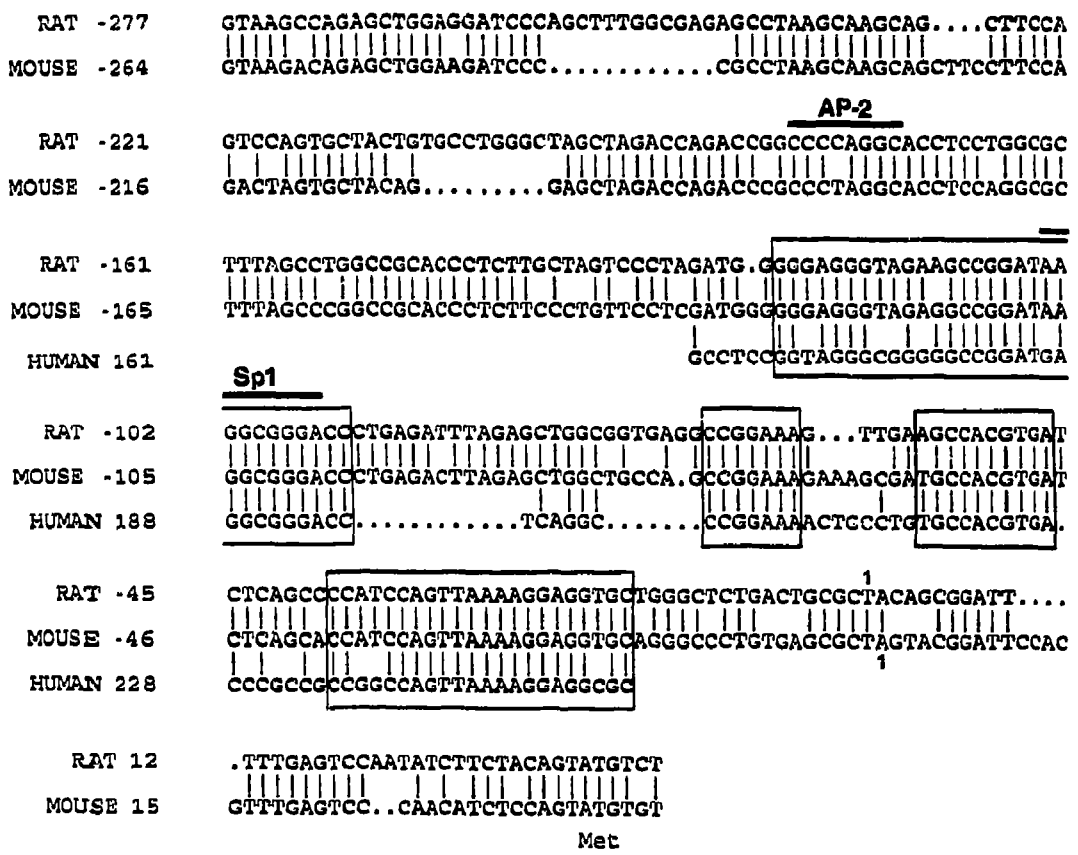

Fig. 4. Comparison of promoter sequences of rat, mouse and human GSHPx genes. Solid line boxes indicate positions at which homologous sequences are present. The positions of Sp1 binding and AP-2 motifs are also overlined.

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