Structure and Expression of the Orphan Nuclear Receptor SHP Gene*

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To determine the organization of the orphan nuclear receptor SHP gene (Seol, W., Choi, H.-S., and Moore, D.D. (1996) Science 272, 1336–1339), genomic clones were isolated from human and mouse genomic libraries. The SHP gene was composed of two exons interrupted by a single intron spanning approximately 1.8 kilobases in human and 1.2 kilobases in mouse. Genomic Southern blot analysis and fluorescence in situ hybridization of human metaphase chromosomes indicated that the SHP gene is located at the human chromosome 1p36.1 subband. The 5′-flanking regions of human and mouse SHP genes were highly conserved, showing 77% homology in the region of approximately 600 nucleotides upstream from the transcription start site. Primer extension analysis was carried out to determine the transcription start site of human SHP to 32 nucleotides downstream of a potential TATA box. The human SHP gene was specifically expressed in fetal liver, fetal adrenal gland, adult spleen, and adult small intestine. As expected from this expression pattern, the activity of the mouse SHP promoter measured by transient transfection was significantly higher in the adrenal-derived Y1 cells than HeLa cells.

The nuclear receptor superfamily is a group of transcription factors regulated by small hydrophobic hormones such as retinoic acid, thyroid hormone, and steroids and also includes a large number of related proteins that do not have known ligands, referred to as orphan nuclear receptors (for reviews see Refs. 1, 2). The nuclear receptors directly regulate transcription by binding to specific DNA sequences named hormone response elements, generally located in promoters of target genes. The nuclear hormone receptors share a common domain structure. The central DNA binding domain (DBD) includes two zinc binding modules, which consist of a series of invariant cysteine residues. A conserved helical region termed the P box separates DBD and the ligand binding domain but lacks the conserved DBD (8). This orphan receptor interacts, both in vitro and in the yeast two-hybrid system with several conventional and orphan members of the receptor superfamily, including retinoid receptors (RAR and RXR), thyroid hormone receptor, and the orphan receptor CAR. In mammalian cells, this receptor specifically inhibited transactivation by the superfamily members with which it interacted, suggesting that it functions as a negative regulator of receptor-dependent signaling pathways. On the basis of its small size and its ability to interact with several superfamily members, it was named SHP (small heterodimer partner) (8). The ability of SHP to interact with multiple superfamily members was independently confirmed by isolation of its rat homologue on the basis of its interaction with the PPAR (peroxisome proliferator-activated receptor) (9).

The ability of SHP to interact with a range of receptors with different affinities suggests that the level of SHP gene expression could play a pivotal role in regulation of receptor-dependent signaling pathways. Thus, information regarding the SHP promoter and its activity is crucial to understanding the function of this novel orphan receptor. In this report, we characterized the structure of the human and mouse SHP genes, the location of the human gene, the expression pattern of SHP in various tissues, and the basal SHP promoter.

EXPERIMENTAL PROCEDURES

Isolation of SHP Genes from Genomic Phage Libraries—Human and mouse genomic phage lambda libraries (CLONTECH, CA) were screened using the SHP cDNA as a probe. One human and three mouse SHP clones were independently isolated and confirmed by Southern blot analysis. Boundaries between exons and introns were determined by DNA sequencing and primer extension reaction analysis. The 5′-flanking region of the SHP gene was analyzed by restriction mapping, DNA sequencing, and polymerase chain reaction analysis. Sequence homology between human and mouse genes was analyzed by using the GCG program.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank (9) and EBI Data Bank with accession number(s) AF044315 (mouse) and AF044316 (human).

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A Genomic Structures

Mouse SHP gene

Mouse SHP cDNA

Human SHP gene

Human SHP cDNA

B Exon - Intron Boundaries

Mouse

Human

exon 1

exon 2

TTCAACCCAGgtgacctcc
ttagcactagTOTOCCAG

TTCAACCCGgtgacctcc
ttagcactagTOTOCCCA

FIG. 1. Genomic structures of the SHP gene. A, schematic description of the human and mouse SHP genomic structure. Hatched boxes represent the exons, and the sizes of exons and introns are as indicated. B, intron and exon sequences of the exon/intron boundaries. Uppercase indicates the exons, and lowercase represents the intron.

Primer Extension Analysis—The transcription start site of the SHP gene was determined by primer extension assay with an end-labeled oligonucleotide primer complementary to positions +34 to +69 of the human SHP coding sequence. Total RNA was prepared from HepG2 and JEG3 cells using guanidinium isothiocyanate and CsCl density gradient centrifugation (10). The isolated total RNAs were further purified using the Poly(A)-Tract mRNA isolation Kit (Promega, WI). The primer extension assay was basically performed as described previously (10). Briefly, 32P-labeled primer was mixed with 0.5 µl of poly(A)+ RNA in 20 µl of hybridization buffer and denatured at 65 °C for 90 min. The mixture was slowly cooled down to room temperature. After the addition of 30 µl of reverse transcription buffer (75 mM Tris-Cl, pH 8.3, 37.5 mM KCl, 4.5 mM MgCl2, 2 µl of 0.1 M dithiothreitol, 1 µl of 10 mM dNTPs, and 0.5 µl of Superscript II reverse transcriptase (Life Technologies, Inc.), the reaction was incubated at 42 °C for 60 min and terminated by the addition of 1 µl of 0.5 M EDTA. The extended transcripts were recovered by ethanol precipitation after RNA digestion and phenol extraction and resolved on a 6% denaturing polyacrylamide gel. The start site was determined by comparison to sequencing reactions carried out on the genomic clone using the same primer in parallel.

Northern Blot Analysis—A human adult tissue blot was purchased from CLONTECH (Palo Alto, CA), 13-week-old human fetus tissue RNA was isolated, and Northern blot analysis was carried out as described previously (11).

Construction of Promoter-Luciferase Fusion Vectors—A fragment containing 2080 base pairs of 5'-flanking sequences from the mouse SHP gene was generated by polymerase chain reaction using the genomic clone as template and used to replace the thymidine kinase promoter sequence of the pSHP2080Luc. The 5' deletion constructs were prepared by digestion with BamHI and restriction enzymes as indicated in Fig. 6 followed by Klenow fill-in and T4 ligation.

Cell Culture and Transient Transfection—Y1 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. The day before transfection, cells were split into 12-well plates. On the next day, the medium was changed, and 4 h later, cells were transfected in triplicate using calcium phosphate co-precipitation as described previously (12). For each well, equal molar amounts (0.5 µg of pSHP2080Luc, with others adjusted accordingly) of the pSHPLuc constructs were co-transfected with 0.5 µg of pTKGH as an internal control. The medium was replaced 18 h after transfection, and cells were further grown for 36 h in culture medium. The method was collected for measuring growth hormone activity, and the cells were harvested in lysis buffer (Promega, WI) according to the manufacturer's protocol. GH and luciferase activities were determined as described previously (11).

Genomic Southern Blot Analysis—Monochromosomal somatic cell hybrid blot was obtained from Biosis Laboratories (New Haven, CN). A zoo blot containing EcoRI-digested genomic DNAs from nine eukaryotic species was purchased from CLONTECH (Palo Alto, CA). The blots were hybridized with human SHP cDNA as a probe and washed with 2× SSC, 0.1% SDS for 30 min at 65 °C. Anti-human DNA was hybridized with human SHP cDNA as a probe and washed with 3× SSC, 0.1% SDS for 30 min at 65 °C. Flourescence in Situ Hybridization—Phytohemagglutinin (PHA)-stimulated lymphocyte was synchronized with mitomycin, and human male metachromosomes were hybridized with a biotin-labeled SHP genomic DNA probe (13). The labeling probe was labeled with biotin dUTP by nick translation, and the labeled probe was combined with human Cot-1 DNA and hybridized to normal metaphase chromosomes in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating slides in fluorescein-conjugated avidin. The slides were counter-stained with propidium iodide and analyzed with a Zeiss fluorescent microscope. A total of 80 metaphase cells were examined, with 65 exhibiting specific signals.
RESULTS

Structure of the SHP Gene—To investigate the genomic organization of the SHP gene, we isolated three genomic clones for the murine SHP gene and one for the human SHP gene, respectively, from genomic phage lambda libraries by using SHP cDNA (8) as a probe. The genomic structures of human and mouse SHP genes were characterized by Southern blotting and polymerase chain reaction analysis, and sequences of the intron-exon boundaries and part of the coding regions were determined. As indicated in Fig. 1, A and B, both the human and mouse SHP genes consist of two exons interrupted by one intron, and the sequence of exon-intron boundaries conform to the consensus splicing signals. No evidence for the existence of the DBD of nuclear hormone receptors was found with neither the human nor the mouse genomic clones (data not shown). The intron in the human and mouse SHP gene lies between the first and second nucleotides of the codon for aspartic acid 181. This is quite reminiscent of the structure of the gene encoding the closest relative of SHP, DAX-1, which also consists of two exons with an intron located exactly at the same relative position (14).

Chromosomal Location of the SHP Gene—To determine the chromosomal location of the SHP gene, a monochromosomal somatic cell hybrid Southern blot was hybridized with human SHP cDNA as a probe. The monochromosomal somatic cell hybrid panel consists of 24 hybrid cell lines, each carrying 1 or 2 human chromosomes within the context of mouse or hamster background, as shown in Fig. 2A. Somatic cell hybrid 0A1AR that contained human chromosome 1 showed a 3.8-kilobase band hybridized to the human SHP cDNA probe. The identical band also showed up in control lanes of human and mixtures of human-mouse genomes. These results indicate that the SHP gene is located in human chromosome 1. To further locate the SHP gene on the human chromosome 1, fluorescence in situ hybridization was carried out (Fig. 3). To confirm the precise location of the gene, the fluorescent signals were visualized directly on R-banded metaphase spreads, and the identical spreads were separately stained with Giemsa (data not shown). These analyses demonstrated that the SHP gene resides in a single locus on human chromosome 1 at position 1p36.1. A genomic Southern blot containing EcoRI-digested DNAs from various species was hybridized with human SHP cDNA as a probe (Fig. 2B). Two genomic EcoRI fragments (approximately 7 and 2.8 kilobases) from human DNA, as well as one or two bands from various species were hybridized to the probe, indicating that the SHP genes are well conserved throughout different species (Fig. 2B). Since the human SHP genomic clone has three EcoRI sites in its intron, those two bands are likely to originate from an identical SHP genome. This result is consistent with an idea that the SHP gene is a single-copy gene.

Expression of the SHP Gene—Northern blot analysis was

![Fluorescence in situ hybridization](image-url)
used to identify the expression pattern of the SHP gene in various human tissues. SHP mRNA was detected in spleen and small intestine among human adult tissues examined (Fig. 4A). In addition, SHP gene expression was also detected in fetal liver and fetal adrenal gland (Fig. 4B). These results confirm and extend previous results with the mouse (8) and rat (9) genes, which indicated a relatively tissue specific pattern of expression. To identify the transcription start site for the SHP gene, primer extension analysis was used with HepG2 mRNA as a template (Fig. 5A). The apparent start site of transcription identified by these studies lies 32 nucleotides downstream from a consensus TATA box. This TATA motif is present within both the human and murine sequences, which are relatively well conserved over the 600 base pairs of 5'-flanking region (Fig. 5B) and match the 5'-end of the longest cDNA clone isolated previously (8). To confirm that the 5'-flanking sequences of the SHP gene can confer promoter activity, an approximately 2-kilobase fragment of the mouse sequences was inserted into a luciferase reporter construct. As expected from the SHP expression in the adrenal gland, this construct showed significantly higher basal activity in the adrenal-derived Y1 cells than HeLa cells (Fig. 6). Finally, a series of 5'-deletion mutants were constructed to locate sequences required for the promoter activity in Y1 cells. As indicated in Fig. 6, full activity was observed with rather short fragment from −139 to +22, with a further deletion to −68 showing significantly decreased expression. Thus, these results map the minimal SHP sequences necessary for the promoter activity in adrenal cells to approximately −139 to +22. The specific transcription factors involved in this activity remain unclear.

**DISCUSSION**

Here, we characterized the structure and expression of the SHP gene. As it was the case with DAX-1 (14), the SHP gene consists of two exons with a single intron located near the C terminus. Since this position lies within a relatively well conserved region of the ligand binding domain, it will be interesting to examine whether other receptor genes also have an
FIG. 6. SHP promoter activities in Y1 and HeLa cells. A series of reporter constructs was generated containing 2080 base pairs of 5′-flanking sequences from the mouse SHP gene. Smaller fragments generated using the indicated restriction sites (Bx, Bs, M, and N = BstXI, BsoI, MseI, and NdeI) were linked to a luciferase reporter. These constructs were transfected into the SHP-expressing Y1 cells or the SHP-nonexpressing HeLa cells. The luciferase activities from three separate transfections were normalized to the GH value.

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