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The rat *enhancer of split- and hairy-related protein-2* gene: hepatic expression, genomic structure, and promoter analysis☆

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Abstract

The rat enhancer of split- and hairy-related protein-2 (SHARP-2) is a basic helix-loop-helix transcription factor. The hepatic expression of SHARP-2 mRNA was investigated under various conditions. The level was decreased in the regenerating rat liver and malignant hepatoma cells. In contrast, the expression of SHARP-*2* mRNA was induced in rat livers by feeding a high-carbohydrate diet. To analyze the molecular mechanism involved in the regulation of the rat *SHARP-2* gene expression, the gene was cloned. It was approximately 6-kb in length and consists of five exons and four introns. To investigate the transcriptional regulatory region of this gene, *SHARP-2*/firefly luciferase reporter plasmids were transfected into hepatoma cells. A functional analysis of 5′-deletion constructs revealed that two E box sequences between –160 and –144 are mainly responsible for promoter activity. Although upstream stimulatory factors (USFs) bound to the element in vitro, USF2 failed to stimulate promoter activity from the element using the co-transfection experiment. Therefore, other E box-binding transcription factors differing from USF proteins or USF-associated proteins are necessary for transcriptional stimulation of the rat *SHARP-2* gene.

Keywords: Enhancer of split- and hairy-related protein-2; Liver; Genomic cloning; Nucleotide sequence; Transcriptional regulation; Upstream stimulatory factor

Basic helix-loop-helix (bHLH)[1] proteins play important roles in the transcriptional regulation of cellular proliferation, differentiation, and oncogenesis [1]. The majority of bHLH proteins bind to the E box sequence (5′-CANNTG-3′) and regulate gene transcription either positively or negatively.

Rat enhancer of split- and hairy-related protein-2 (SHARP-2) belongs to the bHLH family [2]. Mouse and human orthologs have also been cloned and are referred to as the stimulation of retinoic acid 13 (Stra13) and differentiated embryo chondrocytes 1 (DEC1), respectively [3,4]. It has been reported that Stra13 and DEC1 function as transcriptional repressors [5,6]. In the case of Stra13, the mechanism for this repression involves a physical interaction with the components of the basal transcription machineries, such as TATA-binding protein and TFIIB, and the histone deacetylase 1-Sin3A-N-CoR corepressor complex is recruited through its carboxyl-terminal repression domain [5].

SHARP-2/Stra13/DEC1 mRNAs are expressed ubiquitously. However, their expressions are regulated in a cell type-specific manner [2–4]. For example, SHARP-2 mRNA levels are increased by nerve growth factor in pheochromocytoma PC12 cells, Stra13 mRNA is induced during neuronal differentiation by treatment with retinoic acid in P19 mouse embryonal carcinoma

☆ The nucleotide sequences presented in this paper have been submitted to the DNA Data Bank of Japan under Accession No. AB096137.

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[1] *Abbreviations used:* bHLH, basic helix-loop-helix; SHARP-2, split- and hairy-related protein-2; SD, Sprague–Dawley; EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factors; ChoRE, carbohydrate response element; ChREBP, ChoRE-binding protein.

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cells, and DEC1 mRNA is highly expressed in chondrocytes that have differentiated as the result of cyclic AMP treatment [3,4]. These mRNAs are also induced by other signals, such as serum starvation, transforming growth factor-β, hypoxia, and insulin [5–8].

SHARP-2/Stra13/DEC1 may be involved in the control of proliferation and/or the differentiation of chondrocytes, nerve cells, fibroblasts, T cells, and adipocytes [2–5,9]. Indeed, SHARP-2/Stra13/DEC1 plays an oncogenic role when its expression is ectopic and deregulated [10]. It has recently been reported that DEC1 and a closely related protein, DEC2, are expressed in the suprachiasmatic nucleus in a circadian fashion and are regulators of the mammalian molecular clock [11]. The production and analysis of *Stra13*-deficient mice revealed that Stra13 is a key regulator of lymphocyte activation, which is vital for the maintenance of self-tolerance and the constraint of autoimmunity [12]. Other phenotypic differences between Stra13−/− mutants and their wild-type littermates have not been reported to date [12].

In this study, to elucidate the physiological role of SHARP-2 in the rat liver, we examined the expression of rat SHARP-2 mRNA under various conditions. Using animals and hepatoma cells, we examined the issue of whether the expression of SHARP-2 mRNA is regulated by proliferation and/or differentiation. We also report on the genomic structure and an analysis of the promoter region, in an attempt to develop a better understanding of transcriptional mechanisms of the rat *SHARP-2* gene in the liver.

Materials and methods

Materials

The Trizol reagent, RNase inhibitor, Superscript II, and Lipofectamine PLUS reagent were purchased from Invitrogen (Groningen, The Netherlands). The ExpressHyb hybridization solution and Advantage-GC Genomic PCR kit were purchased from Clontech (Palo Alto, CA). The oligotex dT30-super, ExTaq DNA polymerase, BcaBest DNA labeling kit, and BcaBest dideoxy sequencing kit were obtained from Takara BIOMEDICALS (Kyoto, Japan). [α-^{32}P]dCTP (110 TBq/mmol), [γ-^{32}P]ATP (110 TBq/mmol), Hybond N, Hybond N+, and Colony/Plaque Screen filters were purchased from Amersham Biosciences (Cleveland, OH). The pGEM-T Easy, pGL3-Basic, pGL3-Control, pRL-CMV, pCI-neo, and dual-luciferase reporter assay system were obtained from Promega (Madison, WI). The Sprague–Dawley (SD) rat male kidney genomic library was obtained from Stratagene (La Jolla, CA). The Qiagen lambda kit was purchased from Qiagen (Hilden, Germany). The Big Dye terminator cycle sequencing kit FS was purchased from Applied Biosystems Japan (Tokyo, Japan). The Invisorb plasmid kit was purchased from Invitek (Berlin, Germany). The Bio-Rad protein assay kit was obtained from Bio-Rad (Hercules, CA). Anti-GST (SC-113X), anti-USF1 (SC-8983X), and anti-USF2 (SC-862X) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells, cell culture, and animals

MH_1C_1 cells were purchased from the American Type Culture Collection (Manassas, VA). dRLh-84 and HepG2 cells were obtained from the Japanese Collection of Research Bioresources. These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C in a 5% CO_2 incubator. Male SD rats (6 weeks of age, 170–190 g body weight) were used in the experiments. Hepatocytes were freshly isolated from male SD rat liver using a collagenase perfusion method [13]. Partial hepatectomies (70%) were performed under ether anesthesia. Rats were sacrificed at the indicated times after surgery and livers were collected. To examine the effects of a high-carbohydrate diet on SHARP-2 gene expression, rats were starved for 48 h and then re-fed a high-carbohydrate diet (81% dextrose, 10% casein) for 16 h. The rats were then killed and their livers were removed. Animal care and procedures followed the National Institute of Health guidelines.

Preparation of total and poly(A)$^+$-RNA and Northern blot analysis

Total RNA was prepared from livers and various cells using the Trizol reagent. Total RNA (10 μg/ lane) was subjected to 0.8% denaturing agarose gel electrophoresis, then transferred to a Biodyne membrane (ICN Biomedicals, Inc., Glen Cove, NY), and UV-crosslinked for fixation. The ExpressHyb hybridization solution was used for prehybridization and hybridization. Washing was performed twice at 50 °C for 30 min in 0.1 × SSC, 0.1% SDS. After drying the filter, it was exposed to a FUJIX imaging plate. Hybridization signals were detected with the FUJIX BAS-2000 image analyzing system. Poly(A)$^+$-RNA was purified from rat liver total RNA using oligotex dT30-super. Purification procedures were performed according to the protocol provided by the supplier.

Probe DNAs

For SHARP-2, 1.2 kb- and 0.5 kb-probes were prepared using the reverse transcription-polymerase chain reaction (PCR) [14]. One microgram of rat liver total RNA was employed as a starting material. Combinations of oligonucleotides, SHARP-2-320, 5′-ATGGA

GCGGA TCCCC AGCGC-3′, and SHARP-2-1555, 5′-TTAGT CTTTG GTTTC TAAGT TTAAA G-3′ for the 1.2 kb-probe, and SHARP-2-1807, 5′-GGTCT ATAGC ACATC TCTGG GC-3′, and SHARP-2-2279, 5′-GGGTC TGGTT CACCG TCATT G-3′, for the 0.5 kb-probe, respectively, were used as primers. The products were subcloned into the pGEM-T Easy vector. After confirmation of the nucleotide sequence, the inserts were used as the probes. The probe for 36B4 was described previously [8]. These probe DNAs were labeled with [α-^{32}P]dCTP using the BcaBest DNA labeling kit.

Genomic library screening

Eight $\times 10^5$ independent clones of the SD rat male kidney genomic library were screened by the plaque hybridization method using ^{32}P-labeled probes. Prehybridization and hybridization were carried out at 65 °C in 5 × Denhardt's, 5 × SSC, 0.5% SDS, and 200 μg/ml of heated salmon sperm DNA without or with the ^{32}P-labeled probe, respectively. Washing was performed twice at 65 °C for 30 min in 0.1 × SSC, 0.1% SDS. After drying the filter, it was exposed to a Kodak BIOMAX film at −80 °C with an intensifying screen. Four independent clones were isolated. Lambda DNA was prepared from these positive clones using the Qiagen lambda kit. Their nucleotide sequences were determined with a DNA sequencer (Applied Biosystems 3100).

Primer extension analysis

Primer extension analysis was performed according to standard protocols [15]. Briefly, an antisense oligonucleotide primer, 5′-GGGGG CGTGC ATGAC CGGGT-3′, was labeled with [γ-^{32}P]ATP by the T4 polynucleotide kinase reaction. The ^{32}P-labeled primer was then hybridized with 15 μg of liver poly(A)$^+$-RNA prepared from rat that had been starved for 48 h or a rat given a high-carbohydrate diet for 16 h after starvation for 48 h, or yeast tRNA, and then reverse transcribed using the Superscript II. The products were analyzed on an 8% polyacrylamide/7 M urea gel using a M13 mp18 sequence ladder as markers. The gel was dried and then exposed to a Kodak BIOMAX X-ray film at −80 °C with an intensifying screen.

Construction of plasmids

Various DNA fragments of the rat *SHARP-2* gene promoter were amplified by PCR. Using combinations of eight forward primers, Luc-985, 5′-ccggg ctagc ACC CG GACGC CTCTG CATCG G-3′; Luc-449, 5′-ccggg ctagc GGCTT GAGTC AGACG CGGGC G-3′; Luc-192, 5′-ccggg ctagc GGGCA CTTTG CAGCC GCCAG-3′; Luc-160, 5′-ccggg ctagc CACGT GAGAC TCATG TGATG AAGC-3′; Luc-143, 5′-ccggg ctagc ATGAA GCCGG GGGAG GGCGG-3′; Luc-99, 5′-ccggg ctagc CAGCG GCCAG ACGTG CTTGG A-3′; Luc-72, 5′-ccggg ctagc GGGTA GAACA CGTAG CTTCT ACC-3′; Luc-42, 5′-ccggg ctagc TCGCT CCCAT TTAAC CCAGC-3′, and a common reverse primer LucR + 110, 5′-ccgga agctt CCGTG CGAGC CAAGT GAATG AG-3′, PCRs were carried out using lambda clone #5 as a template and an Advantage-GC Genomic PCR kit. After digestion with *Nhe*I and *Hin*dIII, the PCR products were subcloned into the *Nhe*I/*Hin*dIII sites of the pGL3-Basic vector to give pSHARP-2/Luc985, pSHARP-2/Luc449, pSHARP-2/Luc192, pSHARP-2/Luc160, pSHARP-2/Luc143, pSHARP-2/Luc99, pSHARP-2/Luc72, and pSHARP-2/Luc42, respectively.

The tk/Luc, MLP/E box-tk/Luc, and pCI-neo/USF2 plasmids were described previously [16]. Oligonucleotides 5′-CTAGC CAACA CGTGA GACTC ATGTG ATGA-3′ and 5′-CTAGT CATCA CATGA GTCTC ACGTG TTGG-3′ were annealed, phosphorylated, and ligated into the *Nhe*I site of the tk/Luc plasmid to obtain SHARP-2/E box-tk/Luc.

The nucleotide sequences of all inserts were confirmed.

Transient DNA transfections and luciferase reporter assays

All plasmids used for transfection were prepared using an Invisorb plasmid kit, followed by CsCl density gradient ultracentrifugation. DNA transfections were carried out using the Lipofectamine PLUS reagent, as described previously [17]. Briefly, MH_1C_1 or HepG2 cells were plated in a 24-well plate at a density of 5×10^4 cells/well 24 h before transfection. For analysis of deletion constructs, the cells were transfected with 300 ng of the indicated reporter plasmid and 2 ng of the pRL-CMV plasmid. For determination of the transcriptional role of USF2, the luciferase reporter plasmid (200 ng), pRL-CMV (2 ng), and the expression plasmids (100 ng) were used for transfection. Three hours after transfection, the medium was changed. After 48 h, the cells were harvested and firefly and sea pansy luciferase activities were determined using the dual-luciferase reporter assay system and a Berthold Lumat model LB9501 luminometer (Wildbad, Germany). Firefly luciferase activities were normalized by sea pansy luciferase activities.

Electrophoretic mobility shift assays

Nuclear extracts were prepared as described previously [18]. The nucleotide sequences of oligonucleotides used in electrophoretic mobility shift assay (EMSA) are listed in Table 1. EMSAs were performed as described previously [19]. For a competition analysis, a 200-fold

Table 1
Nucleotide sequences of oligonucleotides used in EMSAs

Oligonucleotides	Nucleotide sequences
SHARP-2	5′-gatcCCAACACGTGAGACTCATGTGATGA-3′ 3′-GGTTGTGCACTCTGAGTACACTACTctag-5′
E box	5′-gatcTCCGGTCACGTGACCGGA-3′ 3′-AGGCCAGTGCACTGGCCTctag-5′
CRE	5′-gatcCGGCCCCTTACGTCAGAGGCGAG-3′ 3′-GCCGGGGAATGCAGTCTCCGCTCctag-5′

molar excess of competitor DNAs was added to the binding mixture. For supershift assays, antibodies were first mixed with the nuclear extracts on ice for 30 min and a ^{32}P-labeled probe was then added to the mixture, followed by a 30 min incubation. After completion of the binding, the mixture was subjected to electrophoresis on a native polyacrylamide gel (19:1 = acrylamide: bisacrylamide) in 44.5 mM Tris–HCl, pH 8.0, 44.5 mM boric acid, and 1 mM EDTA at 200 V for 1 h. The gels were dried and exposed to a FUJIX imaging plate (Kanagawa, Japan). Signals were detected with the FUJIX BAS-2000 image analyzing system.

Results

Analysis of hepatic expression of rat SHARP-2 mRNA

To analyze the possible roles of SHARP-2 in the rat liver, the mRNA level of SHARP-2 was determined by Northern blot analysis. We first determined the level of SHARP-2 mRNA under various proliferative conditions such as a regenerating liver, isolated hepatocytes, and two rat hepatoma cell lines. As shown in Fig. 1, the expression of SHARP-2 mRNA was observed in all tissues and cells examined. However, the levels varied with the proliferative conditions used. In the case of regenerating rat livers, the level of SHARP-2 mRNA decreased to 66.4% at 8 h, 40.9% at 24 h, and 28.8% at 48 h and then restored to 48.7% at 96 h after the partial hepatectomy (Fig. 1A). We then examined the expression of SHARP-2 mRNA in isolated hepatocytes and hepatoma cell lines including MH_1C_1 and dRLh-84 cells. MH_1C_1 cells are a well-differentiated hepatoma cell line, which expresses some liver-specific genes and dRLh-84 cells are a poorly differentiated malignant hepatoma cell line, which does not express any liver-specific genes [20]. The levels of SHARP-2 mRNA in isolated hepatocytes and MH_1C_1 cells were slightly higher than that of malignant dRLh-84 cells (Fig. 1B).

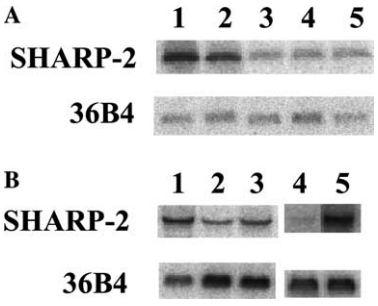

Fig. 1. Northern blot analysis of SHARP-2 mRNA. Total RNAs (10 μg) were loaded onto 0.8% denatured agarose gels. (A) Expression of SHARP-2 mRNA in the regenerating rat liver. Total RNAs were isolated from the pooled livers of two rats in each condition. Lane 1, 0 time; lanes 2 through 5, rat livers at 8, 24, 48, and 96 h after partial hepatoectomy, respectively. (B) Expression of SHARP-2 mRNA in isolated hepatocytes, hepatoma cell lines, and rat liver under various conditions. Lane 1, isolated hepatocytes; lane 2, dRLh-84 cells; lane 3, MH_1C_1 cells; lane 4, liver starved for 48 h; and lane 5, liver from rats fed a high-carbohydrate diet for 16 h after starvation for 48 h. Probe DNAs were depicted on *left*, SHARP-2, enhancer of split- and hairy-related protein-2; 36B4, ribosomal protein 36B4.

A major function of the liver is to regulate blood glucose levels. Therefore, the effect of a high-carbohydrate diet on the expression of SHARP-2 mRNA was investigated. Rats were starved for 48 h and then re-fed a high-carbohydrate diet for 16 h. The level of SHARP-2 mRNA in the rat liver was induced by the administration of a high-carbohydrate diet (Fig. 1B).

These results suggest that the level of expression of SHARP-2 mRNA reflects the differentiation status of the liver rather than its proliferative status.

Structure of the rat SHARP-2 gene

As the first step in examining the mechanism of hepatic gene transcription in the rat *SHARP-2* gene, we screened a SD rat kidney genomic library. Four positive clones, #1, #5, #21, and #22, were isolated from 8×10^5 independent clones. The nucleotide sequences of the rat *SHARP-2* gene were determined and have been submitted to the DNA Data Bank of Japan (Accession No. AB096137). As shown in Fig. 2, the rat *SHARP-2* gene spans approximately 6-kb in length and consists of five exons and four introns. Exon 1 through 5 consists of

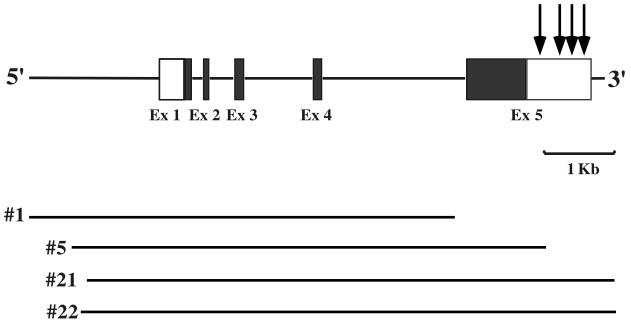

Fig. 2. Structure of the rat *SHARP-2* gene. Exons are depicted by boxes. Open and closed boxes represent noncoding and coding sequences, respectively. The scale for 1 kb is indicated. Clones, #1, #5, #21, and #22 are depicted by bars at the bottom. Arrows indicate putative polyadenylation signals.

Table 2
Nucleotide sequences of exon–intron boundaries of the rat *SHARP-2* gene

Exon		Exon/intron junction 5′ Donor/ 3′ Acceptor				Intron
No.	Size (bp)					Size (bp)
1	512	CCTGTCAGG	gtaagt.	tctccctgcag	GATGGATTT	168
2	70	GACAGCAAG	gtaggt.	attccttgcag	GAAACTTAC	278
3	108	AAACTTACT	gtaagt.	tgtttcctcag	ACTTTGGGT	904
4	124	TACAAGCTG	gtgagt.	tttttctccag	GTGATCTGT	1946
5	2316					
Consensus sequence		CAG A	gtaagt/ g	ttttttttncag ccccccc t	G	

The sizes of the exons and introns, along with the exon–intron boundary sequences, are shown. Exon sequences are given in capitals and introns in lower case letters. Each intron begins with a GT and ends with an AG. The consensus sequence was obtained from Sharp [21] and Mount [22].

512, 70, 108, 124, and 2,316 bp, respectively. As shown in Table 2, the nucleotide sequences of all the exon/intron boundaries are consistent with the GT–AG rule [21,22]. The nucleotide sequences of these exons are almost identical to those reported for SHARP-2 cDNA [2], except for six bases in the non-coding region in exon 1 and two bases in the coding region in exon 5 (replacement "ga" with "ag"). The latter differences lead to the replacement of the lysine residue with an arginine residue.

The fifth exon contains a 851 bp coding sequence, the TAA translation termination site, and the 3′-untranslated region. Although multiple polyadenylation signals are present, a major signal was at position 1,465 downstream the termination codon was predicted from the size of the SHARP-2 mRNA.

Determination of the transcription initiation site

To determine the transcription initiation site of the rat *SHARP-2* gene, a primer extension analysis was performed. Two major products of 197 and 193 bases were observed using rat liver poly(A)$^+$-RNA but not yeast tRNA. In addition, the intensity of these two bands in the high-carbohydrate diet-fed rat liver was higher than that in the starved rat liver. This finding is consistent with the Northern blot analysis (Fig. 1B). These nucleotides were G and C residues, corresponding to 377 and 373 nucleotides upstream from the ATG translation initiation codon, respectively (Fig. 3). The G residue was then numbered as position +1. The nucleotide sequences of the promoter region encompassing approximately 1-kb from the ATG initiation codon were determined (Fig. 4).

Identification of transcriptional regulatory region of the rat SHARP-2 gene

To investigate the transcriptional regulatory region of the rat *SHARP-2* gene, we constructed the pSHARP-2/Luc985, in which a DNA fragment of −985 to +110 relative to the major transcription initiation site of the rat *SHARP-2* gene was linked upstream of the firefly luciferase reporter gene. The pGL3-Basic, a promoterless luciferase reporter vector, pGL3-Control, a simian virus 40 enhancer/promoter-driven luciferase reporter vector, and the pSHARP-2/Luc985 were transiently transfected into MH_1C_1 cells using a lipofection method and their luciferase activities were then determined. As shown in Fig. 5, when the pGL3-Basic was transfected, luciferase activity was quite low. In contrast, the pSHARP-2/Luc985 showed a 50-fold higher luciferase activity than that of the pGL3-Basic and approximately 65% that of the pGL3-Control. The effects of successive 5′-deletion mutants of the rat *SHARP-2* gene promoter fused to the luciferase reporter plasmid on the luciferase activity were then evaluated (Fig. 5). While deletion up to −160 (pSHARP-2/Luc160) resulted in a nearly 2-fold higher value than that of the pSHARP-2/Luc985, the luciferase activity of a construct deletion up to −143 (pSHARP-2/Luc143) dramatically decreased to 34% that of the pSHARP-2/Luc160. Further deletions up to −42 (pSHARP-2/Luc42) gradually decreased to 11% that of the pSHARP-2/Luc160. These results indicate that an inhibitory region exists from −192 to −161, the nucleotide sequence between −160 and +110 of the rat *SHARP-2* gene is required for promoter activity, and that a major positive regulatory region exists in the nucleotide sequence between −160 and −144.

Upstream stimulatory factor proteins bind to the E box sequences of the rat SHARP-2 gene

The region corresponding to the nucleotide sequence between −160 and −144 contains two E box sequences, 5′-CACGTG-3′ and 5′-CATGTG-3′ (Fig. 4). We then carried out EMSAs to identify which proteins in the nuclear extracts of MH_1C_1 cells interact with the region. When the ^{32}P-labeled SHARP-2 oligonucleotide corresponding to the region was mixed with nuclear extracts of MH_1C_1 cells, DNA–protein binding complexes were observed (Fig. 6A). The complex decreased on the

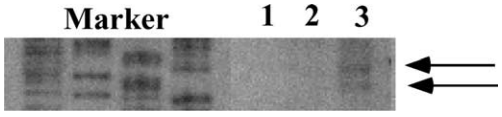

Fig. 3. Determination of the transcription initiation site by primer extension analysis. Fifteen micrograms of rat liver poly(A)$^+$-RNAs or yeast tRNA was hybridized with a ^{32}P-labeled primer, and then extended by reverse transcriptase reaction. After denaturation, the products were electrophoresed on an 8% polyacrylamide/urea gel using sequence ladders of M13 mp18 DNA as size markers. Transcription initiation sites are indicated by arrows. Lane 1, yeast tRNA, lane 2, rat liver starved for 48 h; and lane 3, liver from rats fed a high-carbohydrate diet for 16 h after starvation for 48 h.

addition of a 200-fold molar excess of unlabeled SHARP-2 oligonucleotide to the binding mixture but not the unrelated CRE oligonucleotide (Fig. 6A). As expected, the addition of a 200-fold molar excess of unlabeled E box oligonucleotide led to the disappearance of the binding complex. These results suggest that E box-binding proteins bind to the SHARP-2 oligonucleotide. When the labeled E box oligonucleotide was incubated with the same extract, DNA–protein binding complexes were observed. The DNA–protein complex disappeared when a 200-fold molar excess of unlabeled E box but not CRE oligonucleotide was added. The addition of a 200-fold molar excess of unlabeled SHARP-2 oligonucleotide led to a decreased level of the binding complex (Fig. 6A). These results suggest that the binding affinity of these proteins to SHARP-2 oligonucleotide is lower than that for the E box oligonucleotide.

We then examined the issue of whether upstream stimulatory factor (USF) proteins, ubiquitous E box-binding proteins, are capable of binding to the SHARP-2 oligonucleotide using specific antisera against either USF1 or USF2 or both. As shown in Fig. 6B, when the SHARP-2 oligonucleotide was used as the probe, supershifted bands and a parallel decrease in the intensity of the DNA–protein complex band were detected on the incubation of nuclear extracts of MH_1C_1 cells with anti-USF antibodies. In contrast, a specific band was not

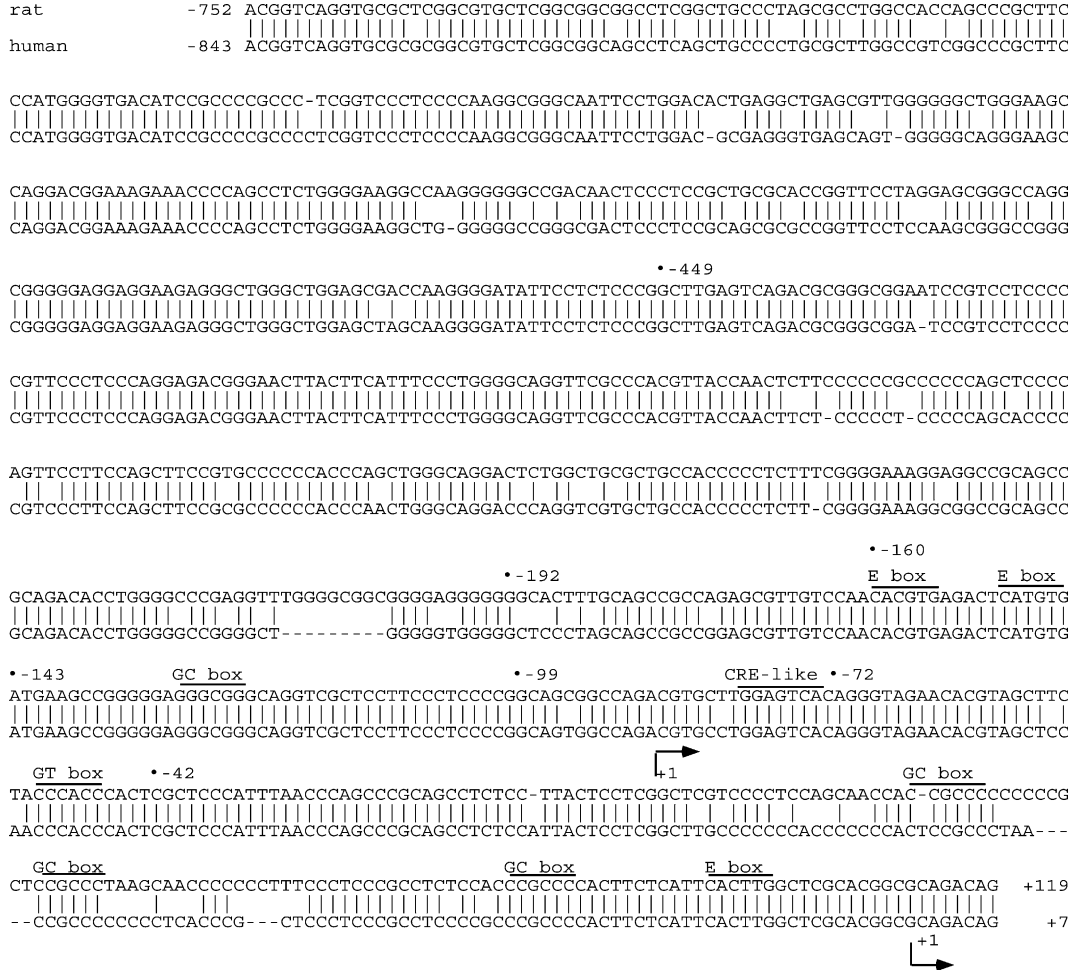

Fig. 4. Sequence comparison of the promoter region of rat *SHARP-2* and human *DEC1* genes. Nucleotide sequences of rat *SHARP-2* (top line) and human *DEC1* (bottom line) genes are aligned. The transcription initiation sites for these two genes are indicated by arrows and assigned as +1. Putative *cis*-acting elements are underlined. End points of the 5′-deletion constructs are indicated by dots.

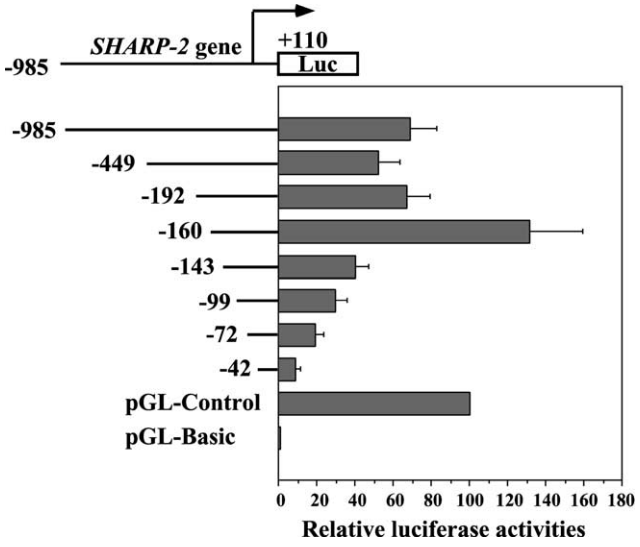

Fig. 5. Deletion analysis of promoter activity of the rat *SHARP-2* gene. Schematic diagrams of the 5′-deletion reporter constructs are depicted on the left. MH_1C_1 cells were transiently transfected with 300 ng luciferase reporter plasmid and 2 ng of the pRL-CMV. Firefly luciferase activities were normalized by sea pansy luciferase activities. The value of the pGL3-Control is defined as 100. The relative luciferase activities of the deletion constructs are expressed as percentages of that of the pGL3-Control shown on the right. All values shown represent means ± standard error of four independent experiments.

affected with anti-GST antiserum. When the E box oligonucleotide was used as the probe, the same results were observed. These results indicate that USF proteins bind to the SHARP-2 oligonucleotide.

USF2 fails to stimulate transcription from the E box element of the rat SHARP-2 gene promoter

Finally, to determine the transcriptional role of USF, co-transfection experiments with USF2 expression vector were performed. Three reporter plasmids, the tk/Luc, MLP/E box-tk/Luc, and SHARP-2/E box-tk/Luc, were used as the reporter plasmids. The tk/Luc contains a thymidine kinase promoter linked to the firefly luciferase gene. The MLP/E box-tk/Luc and SHARP-2/E box-tk/Luc harbored three copies of the E box sequences of the adenovirus major late promoter and four copies of the E box sequences of the *SHARP-2* gene just upstream of the tk promoter, respectively. When the tk/Luc reporter plasmid was co-transfected with the pCI-neo/USF2 into HepG2 cells, luciferase activity showed 1.3-fold activation (Fig. 7). In contrast, when the MLP/E box-tk/Luc was co-transfected with the pCI-neo/USF2, the luciferase activity increased to 2.0-fold. When the SHARP-2/E box-tk/Luc was co-transfected with the

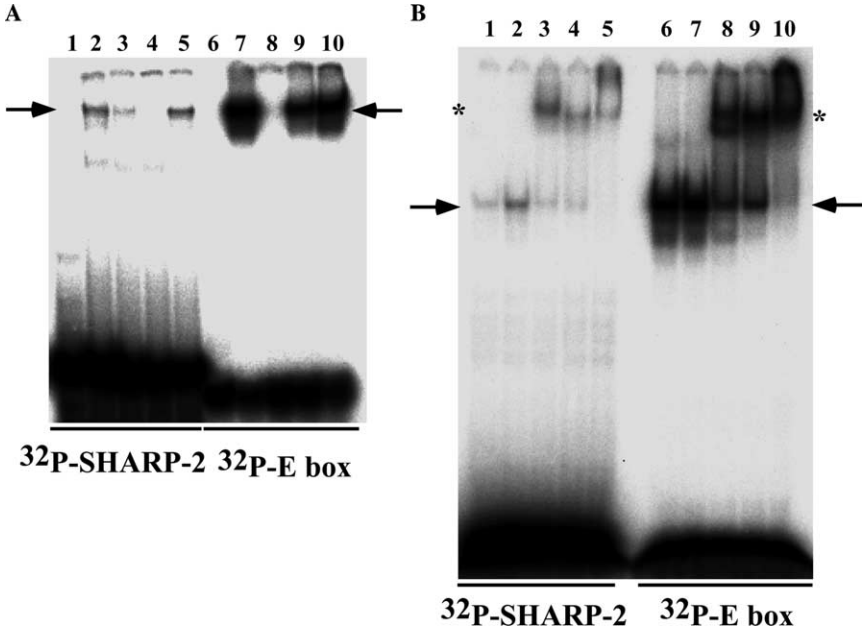

Fig. 6. USF proteins bind to a regulatory region of the rat *SHARP-2* gene. (A) A ^{32}P-labeled SHARP-2 (lanes 1–5) or E box oligonucleotide (lanes 6–10) was incubated with 10 μg of nuclear extracts of MH_1C_1 cells. The competitor DNAs were used at a 200-fold molar excess (lanes 3–5 and 8–10). Arrows indicate the position of a specific protein/DNA complex. Lanes 1 and 6, probe DNA alone; lanes 2 and 7, no competitor DNA; lane 3, SHARP-2; lane 4, E box; lanes 5 and 10, CRE; lane 8, E box; and lane 9, SHARP-2. (B) The nuclear extracts were preincubated with or without antiserum directed against GST, USF1, or USF2 for 30 min prior to the addition of the ^{32}P-labeled SHARP-2 (lanes 1–5) or E box probe (lanes 6–10). The arrows indicate the USFs/DNA complex. Asterisks indicate a USF/DNA complex-supershifted complex (SS) with antibody. Lanes 1 and 6, no antiserum; lanes 2 and 7, anti-GST antiserum; lanes 3 and 8, anti-USF1 antiserum; lanes 4 and 9, anti-USF2 antiserum; and lanes 5 and 10, anti-USF1 and anti-USF2 antisera. The protein/DNA complex was separated by a 6% (A) or 4% (B) PAGE and examined using a BAS 2000 image analyzing system.

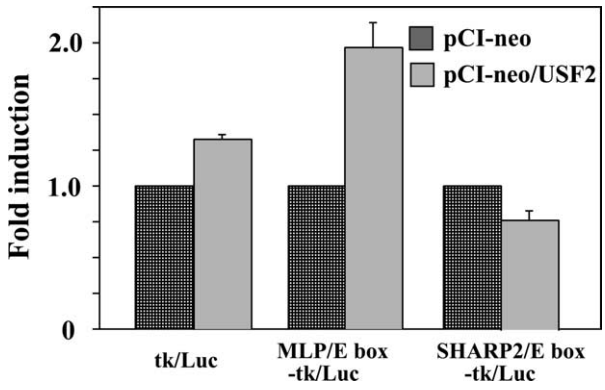

Fig. 7. USF2 fails to stimulate transcription from the E box of the rat *SHARP-2* gene promoter. Reporter plasmid (200 ng), pRL-CMV (2 ng), and the cytomegalovirus enhancer/promoter-directed expression vector (100 ng) were transfected into HepG2 cells. Forty eight hours after transfection, both firefly and sea pansy luciferase activities were determined. The pCI-neo is an empty vector and pCI-neo-USF2 expresses the entire coding sequence of USF2. The normalized firefly luciferase activity from each reporter plasmid co-transfected with the pCI-neo was defined as 1. Each column and bar represent the mean and standard error of at least three independent transfection experiments.

pCI-neo/USF2, the luciferase activity was slightly decreased to 0.7-fold. These results suggest that USF2 fails to stimulate transcription from the E box element of the *SHARP-2* gene.

Discussion

SHARP-2 gene expression was examined in the rat liver and hepatoma cell lines. We also report herein on molecular cloning of the rat *SHARP-2* gene and the characterization of the 5′-flanking region. This is the first demonstration of the determination of genomic organization and promoter activity of the rat *SHARP-2* gene.

The level of SHARP-2 mRNA was determined by Northern blot analysis using total RNA extracted from the rat liver under various conditions (Fig. 1). Generally, after a partial hepatoectomy, differentiated phenotypes rapidly disappear from the liver and the resulting cells begin to divide and grow. This is a useful model system for reproducing cell proliferation and the differentiation of hepatocytes. In regenerating livers, the expression of SHARP-2 mRNA rapidly decreased. In addition, SHARP-2 mRNA levels in isolated hepatocytes and well-differentiated hepatoma MH_1C_1 cells were slightly higher than that of poorly differentiated malignant hepatoma dRLh-84 cells. Moreover, the level of SHARP-2 mRNA in the normal rat liver was induced by a high-carbohydrate diet. These observations from in vivo and in vitro experiments indicate that the expression of SHARP-2 mRNA may be involved in a differentiation function rather than a proliferative function in the liver.

To understand the mechanisms involved in the regulation of *SHARP-2* gene expression in the rat liver, we cloned and characterized the rat *SHARP-2* gene. The rat *SHARP-2* gene is approximately 6-kb in size and consists of five exons and four introns (Fig. 2). All the exon–intron boundaries are consistent with the GT–AG rule (Table 1). Primer extension analysis demonstrated two transcription initiation sites in the rat *SHARP-2* gene (Fig. 3). The nucleotide sequences of the promoter region of the gene were GC-rich and no classical promoters such as a CCAAT box or a TATA box were present. The multiple transcription initiation sites can be explained by the lack of TATA box in the region upstream of the gene, since the TATA box is essential for an accurate transcription initiation site and the transcription initiation site is variable in most TATA-less genes [23].

We then examined the promoter activity of the rat *SHARP-2* gene using a luciferase reporter assay. When the pSHARP-2/Luc985 was transfected into MH_1C_1 cells, it showed a high luciferase activity, comparable to that of the pGL3-Control. A successive deletion analysis indicated that the region of the nucleotide sequence −160 to +110 is required for promoter activity and that at least a positive transcriptional regulatory region exists in the nucleotide sequence between −160 and −144 (Fig. 5). The nucleotide sequences of the promoter region were submitted to the TFSEARCH program in a search for putative transcription factor-binding sites. Potential binding sites for transcription factors were found (Fig. 4). The nucleotide sequences of the human *DEC1* gene promoter have recently been reported [24,25]. We compared the promoter region of the rat *SHARP-2* gene to that of human *DEC1* (Fig. 4). The nucleotide sequence of the promoter region of the rat *SHARP-2* gene was highly similar to that of the human gene. In particular, several potential binding sites for transcription factors are completely conserved between the human and rat. No binding sequences for liver-enriched transcription factors including hepatocyte nuclear factors (HNF) 1, 3, 4, and 6, and CCAAT/Enhancer-binding proteins were identified in either gene promoter. Instead, E boxes, a cyclic AMP response element-like sequence, a GT box, and GC boxes are present. A positive regulatory region corresponding to nucleotide sequence between −160 and −144 contains two E box sequences. Interestingly, these E boxes are separated by 5 bp. The hepatic transcription of the rat *L-type pyruvate kinase*, *Spot 14*, and *fatty acid synthase* gene is regulated by a high-carbohydrate diet [26]. It has been reported that a common carbohydrate response element (ChoRE) involving in the transcriptional stimulation of these genes by carbohydrates is mapped on their promoters and that the ChoRE consists of two copies of E box sequence separated by 5 bp [27]. As shown in Fig. 1B, the level of SHARP-2 mRNA in the

liver is increased by feeding a high-carbohydrate diet. Therefore, it is possible that the region functions as the ChoRE of the rat *SHARP-2* gene promoter. It has been reported that the ChoRE is bound by a ChoRE-binding protein (ChREBP), which is expressed in the liver, kidney, and small intestine [28]. The binding of the regulatory region of the *SHARP-2* gene promoter with ChREBP explains the hepatic expression and regulation by a high-carbohydrate diet. However, EMSAs revealed that proteins binding to the region are USF1 and USF2 proteins, which are ubiquitous E box-binding proteins (Fig. 6). We then examined transcriptional role of USF2 on the *SHARP-2* gene promoter. When the SHARP-2/E box-tk/Luc was co-transfected into MH_1C_1 cells with pCI-neo/USF2, the luciferase activity decreased to 60% (data now shown). However, under same conditions, when the MLP/E box-tk/Luc as a positive control was co-transfected with pCI-neo/USF2, the luciferase activity remains unchanged (data now shown). Therefore, it suggests that exogenously introduced USF2 into MH_1C_1 cells is not suitable to analyze transcriptional role of USF2. When the level of expression of endogenous transcription factors is high, activity of exogenously introduced transcription factor cannot be determined. Then, we switched MH_1C_1 cells to HepG2 cells, which are well-employed cells for transcriptional analysis of USF proteins. Interestingly, co-transfection experiments showed that USF2 failed to activate and somewhat repressed transcription via the E box sequence of the rat *SHARP-2* gene promoter but not that of the adenovirus major late promoter (Fig. 7). It has been reported that USF proteins repress transcriptional stimulation by interleukin-1β of the inducible nitric oxide synthase gene [29]. Therefore, USF proteins may repress transcription of the rat *SHARP-2* gene. However, in any cases, these results suggested that other E box-binding transcription factor(s) differing from USF proteins or USF-associated proteins are necessary for transcriptional stimulation of the rat *SHARP-2* gene. This putative protein including ChREBP should bind to the E box element of the rat *SHARP-2* gene in a high affinity manner since amounts of USF proteins shown in EMSA are very high. At this time, the issue of whether ChREBP contains in the binding complex cannot be determined since ChREBP antiserum is not available. Of course, the possibility that other regulatory regions are required for the liver-specific regulation of the rat *SHARP-2* gene cannot be ruled out. In addition to these E boxes, there are several GC boxes and a GT box in the proximal promoter region, which are located in the nucleotide sequence between −143 and +110. Azmi et al. have very recently reported that Sp1 may stimulate the transcription from this region of the mouse *Stra13* gene [30]. Further studies will be required to address the issues of whether ChREBP is involved in the transcription of the rat *SHARP-2* gene promoter or not, and precise mapping of *cis*-acting elements in the proximal promoter region and identification of their binding proteins.

Acknowledgments

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References

[1] M.E. Massari, C. Murre, Mol. Cell. Biol. 20 (2000) 429–440.
[2] M.J. Rossner, J. Dorr, P. Gass, M.H. Schwab, K.-A. Nave, Mol. Cell. Neurosci. 9 (1997) 460–475.
[3] M. Boudjelal, R. Taneja, S. Matsubara, P. Bouillet, P. Dolle, P. Chambon, Genes Dev. 11 (1997) 2052–2065.
[4] M. Shen, T. Kawamoto, W. Yan, K. Nakamasu, M. Tamagami, Y. Koyano, M. Noshiro, Y. Kato, Biochem. Biophys. Res. Commun. 236 (1997) 294–298.
[5] H. Sun, R. Taneja, Proc. Natl. Acad. Sci. USA 97 (2000) 4058–4063.
[6] L. Zawel, J. Yu, C.J. Torrance, S. Markowitz, K.W. Kinzler, B. Vogelstein, S. Zhou, Proc. Natl. Acad. Sci. USA 99 (2002) 2848–2853.
[7] A.V. Ivanova, S.V. Ivanov, A. Danilkovitch-Miagkova, M.I. Lerman, J. Biol. Chem. 276 (2001) 15306–15315.
[8] K. Yamada, H. Kawata, Z. Shou, T. Mizutani, T. Noguchi, K. Miyamoto, J. Biol. Chem. 278 (2003) 30714–30724.
[9] Z. Yun, H.L. Maecker, R.S. Johnson, A.J. Giaccia, Dev. Cell 2 (2002) 331–341.
[10] Y. Li, H. Zhang, M. Xie, M. Hu, S. Ge, D. Yang, Y. Wan, B. Yan, Biochem. J. 367 (2002) 413–422.
[11] S. Honma, T. Kawamoto, Y. Takagi, K. Fujimoto, F. Sato, M. Noshiro, Y. Kato, K. Honma, Nature (London) 419 (2002) 841–844.
[12] H. Sun, B. Lu, R.-Q. Li, R.A. Flavell, R. Taneja, Nat. Immunol. 2 (2001) 1040–1047.
[13] P.O. Seglen, Methods Cell Biol. 13 (1976) 29–83.
[14] Z. Shou, K. Yamada, T. Inazu, H. Kawata, S. Hirano, T. Mizutani, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Kajitani, K. Okada, K. Miyamoto, Gene 302 (2002) 83–94.
[15] J. Sambrook, D.W. Russell, Molecular Cloning, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001.
[16] K. Yamada, T. Mizutani, Z. Shou, T. Yazawa, T. Sekiguchi, M. Yoshino, T. Inazu, K. Miyamoto, Biol. Reprod. 64 (2001) 1315–1319.
[17] N. Shimada, K. Yamada, T. Tanaka, H. Kawata, T. Mizutani, K. Miyamoto, T. Matsuzawa, Mol. Reprod. Dev. 60 (2001) 165–170.
[18] K. Yamada, T. Tanaka, T. Noguchi, Biochem. J. 324 (1997) 917–925.
[19] K. Yamada, T. Tanaka, K. Miyamoto, T. Noguchi, J. Biol. Chem. 275 (2000) 18129–18137.
[20] K. Imamura, T. Noguchi, T. Tanaka, in: G.E.J. Staal, C.W.M. van Veelen (Eds.), Markers of Human Neuroectodermal Tumors, CRC Press, Boca Raton, 1986, pp. 191–222.
[21] P.A. Sharp, Cell 23 (1981) 643–646.
[22] S.M. Mount, Nucleic Acid Res. 10 (1982) 459–472.

[23] W.S. Dynan, S. Sazer, R. Tjian, R.T. Schimke, Nature (London) 319 (1986) 246–248.
[24] M. Teramoto, K. Nakamasu, M. Noshiro, Y. Matsuda, O. Gotoh, M. Shen, S. Tsutsumi, T. Kawamoto, Y. Iwamoto, Y. Kato, J. Biochem. (Tokyo) 129 (2001) 391–396.
[25] K. Miyazaki, T. Kawamoto, K. Tanimoto, M. Nishiyama, H. Honda, Y. Kato, J. Biol. Chem. 277 (2002) 47014–47021.
[26] K. Yamada, T. Noguchi, Biochem. J. 337 (1999) 1–11.
[27] H.C. Towle, E.N. Kaytor, H.-M. Shih, Annu. Rev. Nutr. 17 (1997) 405–433.
[28] H. Yamashita, M. Takenoshita, M. Sakurai, R.K. Bruick, W.J. Henzel, W. Shillinglaw, D. Arnot, K. Uyeda, Proc. Natl. Acad. Sci. USA 98 (2001) 9116–9121.
[29] A.K. Gupta, B.C. Kone, Am. J. Physiol. Cell Physiol. 283 (2002) C1065–C1072.
[30] S. Azmi, H. Sun, A. Ozog, R. Taneja, J. Biol. Chem. 278 (2003) 20098–20109.