Transcriptional Regulation of Rat Scavenger Receptor Class B Type I Gene*

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The scavenger receptor class B type I (SR-BI) mediates the selective transport of lipids from high density lipoprotein to cells and plays an important role in the reverse uptake of cholesterol to the liver and in the delivery of substrates for steroidogenesis in steroidogenic organs. We report here on the isolation and characterization of the upstream promoter region of the rat SR-BI gene. The transcription start site for rat SR-BI was mapped, and DNA sequence analysis revealed the presence of binding sites for the Sp1 family in the proximal 5'-flanking region. Analysis of deletion mutants with different 5' lengths revealed that the region between −121 and −90 base pairs from the transcription start site is essential for the efficient transcription of SR-BI. Both Sp1 and Sp3 bind to three GC boxes in the region (−141 to −1 base pairs) in a sequence-specific manner. Mutations in any of the GC boxes decreased efficient transcription from this promoter in MA-10 mouse Leydig tumor cells. The overexpression of Sp1 or Sp3 protein enhanced the rat SR-BI promoter activity. These results indicate that Sp1 family members of transcription factors are essential for transcription of the rat SR-BI gene.

Steroidogenic tissue cells require cholesterol to support the synthesis of steroid hormones. Cholesterol, for steroidogenesis purposes, is preferentially supplied from circulating lipoproteins. Both low density lipoproteins (LDL) and high density lipoproteins (HDL) are capable of delivering cholesterol to support steroidogenesis, and the relative contributions of these two lipoproteins differ among species. HDL is the major source of cholesterol for steroidogenesis in rodents, whereas the well known LDL receptor pathway is generally believed to be important in humans. The cellular metabolism of LDL particles occurs primarily via the LDL receptor, as well as other members of the LDL receptor family (1–3), which process LDL via endocytic uptake and lysosomal degradation (1, 2). It is also noteworthy that cholesterol uptake from HDL is considered to be selective in that the uptake of cholesterol ester is independent of HDL internalization (4, 5). Krieger and co-workers (6) demonstrated that the scavenger receptor class B type I (SR-BI) is the protein that mediates the selective uptake of lipids from HDL. SR-BI is a member of the CD36 family (7) and was found to bind a broad spectrum of ligands, including both modified and native lipoproteins, as well as anionic phospholipids (8). The binding of HDL to SR-BI has been shown to be mediated by the major apolipoproteins, apoA-I, apoA-II, and apoC-III (9). It has been shown that the selective uptake of HDL cholesterol ester is solely dependent on the expression of SR-BI (10). SR-BI is expressed in the steroidogenic organs and liver, which all display a selective uptake of HDL cholesterol ester (6, 11, 12). SR-BI expression is coordinately regulated with the steriodogenesis by adrenocorticotropic hormone (13). Studies on homozygous null SR-BI knockout mice showed that SR-BI is also required for maintaining normal development of the oocyte and for female fertility (14), suggesting that SR-BI plays a critical role in female reproduction. In addition, we and other investigators have shown that the expression of SR-BI mRNA in the immature rat ovary is rapidly induced in the theca interna cells by pregnant mare serum gonadotropin (PMSG) or human choricogonadotropin and that expression was also observed in the corpus luteum of the adult rat ovary (11, 15). These observations support the conclusion that SR-BI serves as a selective mediator of cholesterol uptake for steroid hormone synthesis and plays an important role in female reproduction.

The regulation of the tissue-specific expression of SR-BI as well as the mechanism by which trophic hormones up-regulate this expression remains largely unknown. In this study, in order to elucidate the molecular mechanism of transcriptional regulation of SR-BI gene, we isolated and characterized the promoter region of the rat SR-BI gene. Our results indicate that the region from −121 to −90 upstream of the SR-BI transcription start site is essential for the expression of the rat SR-BI gene. The DNA sequences within the region to which protein binding occurs were characterized and shown to have homology to the consensus binding site for the three-zinc finger transcription factor, Sp1, which binds to the GC-rich sequences (16). We demonstrate that Sp1 binds to three sites within the region of the rat SR-BI gene promoter. Mutations in these sequences abrogate Sp1 binding and blunt the activity of the SR-BI promoter. These data suggest that Sp1 plays an important role in conferring promoter activity of the rat SR-BI gene. Sp1 is ubiquitously expressed and required for the constitutive and inducible expression of a variety of genes. To date, three Sp1-related proteins have been identified, namely Sp2, Sp3, and Sp4. Among these, Sp1 and Sp3 are thought to be impor-
tant for the expression of various genes in mammalian tissues (17). In this study, relationships of Sp3 as well as Sp1 for the gene expression of SR-BI were also examined.

**EXPERIMENTAL PROCEDURES**

**Materials**—The DNA walking kit and the GC-melt were purchased from CLONTECH (Palo Alto, CA). The dual luciferase reporter assay system, the pGEM-T Easy vector, the Sp1 oligonucleotide, and the pGL3-Basic and pRL-SV vectors were purchased from Promega (Madison, WI). The cytomegalovirus (CMV) promoter/enhancer-directed expression vector, pcDNA3, was purchased from Invitrogen (Carlsbad, CA). The QIAGEN plasmid kit was purchased from QIAGEN (Hilden, Germany). Fugene-6 was obtained from Roche Molecular Biochemicals, and the dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit was purchased from Applied Biosystems. (γ-32P-ATP (111Tbq/mmol) was obtained from NEN Life Science Products. The anti-Sp1 (SC-644X) and the anti-Sp3 (SC-420X) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The QuickChange site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). The Superscript II reverse transcriptase was from Life Technologies, Inc. The protein assay kit was purchased from Bio-Rad. FMSG was a product from Teikokuozuki, Inc. (Tokyo, Japan).

**Isolation and Characterization of 5′-Upstream of Rat SR-BI Gene—** The DNA walking kit (CLONTECH) was used for the isolation of the 5′-upstream DNA fragment of the rat SR-BI gene. Briefly, samples of rat genomic DNA were separately digested with five different restriction enzymes, which recognize six base pairs and produce blunt ends. The digested DNA fragments were then separately adapter-ligated to produce five sets of DNA fragments with adapters at their ends. Each set of DNA fragments was amplified using adapter-specific 5′/3′ primers (GTAATACGACTCACTATAGGGC and a rat SR-BI gene-specific 3′-primer (ACGGAGGCCACAGAGATGACAGAAA). Second PCR reaction was done using nested primers (the 5′-primer, ACTATAGGCGACGC- GTGGT; and the 3′-primer, AGCAGAAGGGTGTCCTGGCAGACT). In order to effectively amplify the GC-rich sequences in the upstream region of the rat SR-BI gene, GC-melt (CLONTECH) was added to the PCR reaction mixture. The PCR products from each set were analyzed on a 1% agarose gel. Two different sets showed major PCR products corresponding to 2.3 and 0.8 kilobase pairs, respectively. These products were cloned into the pGEM-T Easy vector (Promega). The nucleotide sequences of oligonucleotides used for PCR

| Luciferase vector | Upper primer | Lower primer |
|-------------------|--------------|--------------|
| SR2170luc         | 5′-gagagctagCGTCTCTGAGTGCATAGAA | 5′-gagagctagCGTCTCTGAGTGCATAGAA |
| SR978luc          | 5′-gagagctagCTATCTCTTTGTGTCTGAGT | 5′-gagagctagCTATCTCTTTGTGTCTGAGT |
| SR416luc          | 5′-gagagctagTCTTAGGAAAGGAGGATGGT | 5′-gagagctagTCTTAGGAAAGGAGGATGGT |
| SR114luc          | 5′-gagagctagACATAGGCGACGCGACGCCT | 5′-gagagctagACATAGGCGACGCGACGCCT |
| SR121luc          | 5′-gagagctagCTCACCTAGAGCACCCAGCCT | 5′-gagagctagCTCACCTAGAGCACCCAGCCT |
| SR50lue           | 5′-gagagctagACATAGGCGACGCGACGCCT | 5′-gagagctagACATAGGCGACGCGACGCCT |
| mut 1             | CACCATAGAGACCCACGCCTCCTCCAGATTTTC | 5′-gagagctagCGTCTCTGAGTGCATAGAA |
| mut 2             | CACCATAGAGACCCACGCCTCCTCCAGATTTTC | 5′-gagagctagCGTCTCTGAGTGCATAGAA |
| mut 3             | CACCATAGAGACCCACGCCTCCTCCAGATTTTC | 5′-gagagctagCGTCTCTGAGTGCATAGAA |
| mut 12            | CACCATAGAGACCCACGCCTCCTCCAGATTTTC | 5′-gagagctagCGTCTCTGAGTGCATAGAA |
| mut 13            | CACCATAGAGACCCACGCCTCCTCCAGATTTTC | 5′-gagagctagCGTCTCTGAGTGCATAGAA |

**Mutated bases are underlined. Lowercase letters represent adaptor sequences, including restriction enzyme sites.**

**Plasmids—** Rat SR-BI promoters containing various 5′ ends were generated by PCR using the longest rat SR-BI promoter (2.3 kilobase pairs in size) as a template. Primers used for PCR are shown in Table I. Promoter DNA fragments containing substituted nucleotide sequences, namely mut 1, mut 2, mut 3, mut 12, and mut 13, were also generated by PCR using primers with the indicated nucleotide substitutions (Table I). All of the luciferase constructs contain pGL3-Basic Vector, which lacks both elements of the eukaryotic promoter and enhancer sequences, and mutant DNA fragments, which contain the same SR-BI promoter and enhancer region (~141/13) with different point mutations. The promoter DNA fragments of mut 23 and mut 123 were generated by means of the QuickChange site-directed mutagenesis kit. The numbering of the nucleotides is relative to the transcription start site (+1). All the 5′ primers contained the Nhel site. A HindIII site was involved in the 3′-primer for mut 2 and mut 3. As a result, the PCR products of mut 2 and mut 3 were digested with Nhel/HindIII and were cloned into the pGL3 Basic Vector. The transcriptional activity of the pGL3 Basic Vector is 1.1. In the generation of luciferase constructs of mut 1, mut 12, and mut 13, on the other hand, ~40/+13 SR-BI promoter region was initially removed from the PCR products of mut 1, mut 12, and mut 13 by using a SmaI site at ~40. Then the digested luciferase were ligated with the pGL3-Basic Vector containing ~40/+13 SR-BI promoter region, resulting in the luciferase constructs, which contain the same SR-BI enhancer and promoter regions as those of wild type, mut 2, or mut 3 luciferase constructs. The vector which contains the ~40/+13 SR-BI promoter region was generated by removing the Nhel/SmaI fragment from the wild type reporter containing ~141/+13 SR-BI promoter region. All reporter plasmids were authenticated by DNA sequencing. pCMV-Sp1, pCMV-Sp3, pPac-Sp1, and pPac-USp3 vectors were generous gifts from Dr. Guntram Suske (Philipps-Universitaet Marburg, Marburg, Germany).

**Rat adrenal medium supplemented with 15% horse serum and antibiotics.** HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Cells were
dispensed into 24-well plates and cultured until reaching 70% confluence. DNA samples which contained each reporter plasmid and pRL Renilla luciferase control vector (for normalization) with or without an expression plasmid (SF-1pDNA5, pCMV-Sp1, or pCMV-Sp3) were mixed with 1.5 μl of FuGENE 6 (Roche), and the resulting mixture was added to the cells. The total amount of DNA (μg) was adjusted by adding the pDNA3 plasmid if any. Cells were harvested 48 h later, and luciferase activity was determined using a Dual Luciferase Reporter Assay System. Measurements were made using a Lumat LB9501 luminometer (Berthold) in a single tube, with the first assay from firefly luciferase followed by the Renilla luciferase assay. Firefly luciferase activities (relative light units) were normalized by Renilla luciferase activities.

Schneider line 2 (SL2) cells, a Drosophila cell line, were a generous gift from Dr. Nagoya (Nagoya University, Nagoya, Japan). SL2 cells were grown in Schneider's medium supplemented with 10% bovine serum at 25 °C. DNA transfection to SL2 cells was carried out by a calcium-phosphate method (19). Cells were plated at 1 × 10^6 cells/60-mm dish on day 0. On day 1, the cells were transfected with 2 μg of luciferase reporter plasmid and indicated amount of pPac-Sp1 or pPac-USp3 expression plasmid. Total DNA was added by the addition of the pPac plasmid. The culture medium was not changed before or after the addition of DNA. Cells were harvested 48 h after the transfection, and cell lysates were assayed. Protein concentrations were determined before or after the addition of DNA. Cells were grown in Schneider line 2 (SL2) cells, a generous gift from Dr. Nagoya (Nagoya University, Nagoya, Japan).

The supernatant, and the resulting suspension was gently stirred on ice for 45 min, and then centrifuged at 27,000 g for 10 min at 4 °C to remove the insoluble materials. Protein concentrations of the supernatant were determined using Bio-Rad protein assay reagents. Bovine serum albumin was used as a standard.

Firefly luciferase activity was measured with cell lysates containing equal amount of proteins.

**Isolation of Nuclei and Preparation of Nuclear Extract from MA-10 Cells**—Nuclei from MA-10 cells were prepared by the method of Hagebuchi and Wellauer (21) with minor modifications (22). All operations were carried out at 4 °C. MA-10 cells were homogenized in a Dounce homogenizer in 5 volumes of buffer A (15 mM Hepes/NaOH, pH 7.8, 60 mM KCl, 15 mM NaCl, 14 mM 2-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 1 mM phenylmethylsulfonyl fluoride, and 0.2% Nonidet P40) containing 0.3 mM sucrose. The homogenate was layered on top of a cushion of 0.9 mM sucrose in buffer A and centrifuged at 2,500 × g for 10 min at 4 °C. The precipitated crude nuclei were resuspended in buffer A containing 0.3 mM sucrose and re-centrifuged.

The resulting pellet was resuspended in buffer A and centrifuged again at 2,500 × g for 10 min at 4 °C. The preparation of a nuclear extract from a sample of pure nuclei was performed by the method of Frain et al. with minor modifications. Briefly, pure nuclei were resuspended in 5 volumes of buffer B (20 mM Hepes/NaOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) containing 0.3 mM sucrose. The homogenate was layered on top of a cushion of 0.9 mM sucrose in buffer B and centrifuged at 2,500 × g for 10 min at 4 °C. The supernatant was removed by aspiration.

**EMS—A nuclear extract (2.5 μg of proteins) was incubated for 30 min with a 32P-labeled oligonucleotide (0.1 ng) and 1 μg of poly(dA-dT) in buffer C. In the competition experiments, a 200-fold molar excess of unlabeled competitor DNAs was added. A supershift assay was carried out by preincubating the nuclear extracts for 30 min with 1 μl of anti-Sp1 or anti-Sp3 antibodies. After the binding reaction, the mixture was subject to PAGE (4% gel) in 45 mM Tris, 45 mM boric acid, 1 mM EDTA at 200 V for 60 min and, following this, the gel was dried and exposed to Kodak XAR film.

**RESULTS**

The 5' upstream region of the rat SR-BI gene was isolated using the PCR-based DNA walking kit. Two PCR products (2.3 and 0.8 kilobase pairs in size) were obtained from rat genomic libraries. Since the 5' upstream region of the rat SR-BI gene is quite rich in GC, the GC-melt kit was used to isolate the PCR products. In the absence of the GC-melt, no PCR product was amplified by the DNA walking kit procedure. The two DNA fragments were ligated into the pGEM-T Easy vector for sequencing. Sequence analysis revealed that the two DNA fragments differ only in their length at their 5'-regions. Fig. 1 shows the nucleotide sequence of the longer fragment. To determine the transcription start site for rat SR-BI gene, a primer extension analysis was performed. An end-labeled oligonucleotide primer, which is complementary to the 5'-untranslated region of rat SR-BI mRNA, was used. Total RNA from immature rat ovaries primed with 30 IU of PMSG for 6 h was used for the extension reaction. A radiolabeled product was detected in samples containing the RNA from the ovary and adrenal gland, where strong SR-BI gene expression is observed, whereas no product was detected in samples containing yeast tRNA or RNA from lung. The primer produced an extension fragment, which contained 67 nucleotides (Fig. 2), indicating that the start site for the transcription is located 128 bp upstream of the translation start site (Fig. 1).

The sequence contains a number of putative transcription factor binding sites. A potential TATA box-like sequence was present about 30 bp upstream from the transcription start site. The sequence is particularly rich in guanine and cytosine residues and contains four Sp1/Sp3 transcription factor consensus elements.

As a first step in determining the sequence required for transcriptional activity of the rat SR-BI gene, a series of plasmids was prepared. These plasmids contained upstream fragments of −2170/+13 (SR2170luc), −978/+13 (SR978luc), −416/+13 (SR416luc), −141/+13 (SR141luc), −121/+13 (SR121luc), −90/+13 (SR90luc), −90/−13 (SR40luc), respectively, which were placed upstream of the luciferase reporter gene. The plasmids were transiently transfected into MA-10 cells, and the reporter activity determined. As expected in Fig. 3, the SR2170luc plasmid has activity about 50% lower than SR978luc, SR416luc, SR141luc, and SR121luc constructs. This data strongly suggest that the region between −121 and −90 is important for the positive regulation of rat SR-BI expression. This region contains two
GC boxes, which are compatible with putative Sp1 binding sites (GC box 2 and GC box 3). Removal of the most proximal Sp1 site (GC box 1), between −90 and −40, essentially abolished promoter activity. Although the 5′-deletion analysis suggests that Sp1 family transcription factors regulate SR-BI promoter activity, this does not directly address the roles of the GC boxes relative to SR-BI expression. As a result, we individually and combinatorially mutated the three GC boxes within the SR141luc reporter construct.

As shown in Fig. 4, mutations in any one of the GC boxes resulted in only 20–35% of the activity relative to the wild type promoter SR141luc. mut 13 (mutated in both GC boxes 1 and 3) caused a further decrease in activity to 8% of the control value. mut 12 (mutated in both GC boxes 1 and 2) and mut 23 (mutated in both GC boxes 2 and 3) did not result in any further decrease compared with those of mut 1 (mutated in GC box 1) and mut 3 (mutated in GC box 3), respectively. These results suggest that each GC box is required for a high level of SR-BI expression.

To determine if nuclear proteins are capable of specifically binding to these sites, EMSAs were performed using nuclear extracts from MA-10 cells. For these experiments, radiolabeled oligonucleotides corresponding to −121/−86 (containing GC boxes 2 and 3) and −61/−40 (containing GC box 1) of the rat SR-BI promoter were used with or without an added 200-fold excess of unlabeled competitor DNA. The results of these experiments are shown in Fig. 5.
the use of radiolabeled $-121/-86$ probe, excess amounts of neither mut 3 nor mut 23 (Table II) were effective in preventing complex formation, whereas mut 2 was partially effective. This suggests that the GC box 3 plays a more important role in terms of complex formation than GC box 2. With the use of radiolabeled $-61/-40$ probe, the excess unlabeled mut1 had no effect on complex formation (lane 14).

To identify the proteins in the MA-10 nuclear extract that bind to these positions in the native rat SR-BI promoter, EMSA supershift experiments were performed by using the radiolabeled $-121/-86$ and $-61/-40$ oligonucleotides and antibodies that are specific for Sp1 and Sp3 (Fig. 6). Initial complex bands with the radiolabeled $-121/-86$ probe were supershifted by the addition of either the Sp1 (lane 2) or Sp3 (lane 3) antibodies, although the initial bands were still observed faintly. The simultaneous addition of anti-Sp1 and Sp3 antibodies caused the supershift and the complete disappearance of the initial

![Figure 5](image)

**FIG. 5.** EMSA analyses of the GC boxes within the 141 bp upstream of the rat SR-BI gene. An end-labeled $-121/-86$ or $-61/-40$ oligonucleotide was incubated with 2.5 μg of nuclear extract from MA-10 cells. Unlabeled oligonucleotides (200-fold molar excess) were used as competitor DNAs. Protein-DNA complexes were separated by a 4% PAGE and subjected to autoradiography. The arrows on the left indicate the positions of the protein-DNA complexes.

![Figure 6](image)

**FIG. 6.** Supershift analyses for Sp1 and Sp3 using labeled $-121/-86$ or $-61/-40$ oligonucleotide. An end-labeled $-121/-86$ or $-61/-40$ oligonucleotide was incubated with 2.5 μg of nuclear extract from MA-10 cells. The nuclear extract was preincubated with antisera, directed against Sp1 and/or Sp3 for 30 min prior to the addition of the probe. Protein-DNA complexes were separated by a 4% PAGE and subjected to autoradiography. Arrows on the left represent the positions of complexes initially formed, and those on the right represent the positions of supershifted bands (SS).

| Mutated bases are underlined. |
|------------------------------|
| **Table II**                  |
| **Nucleotide sequences of oligonucleotides used in EMSAs** |
| Oligonucleotides | Nucleotide sequences |
| $-61/-40$ | 5’-tcgaAACAGGGGGCCGGCTGCC-3’ |
| mut 1 | 3’-TTTGGCCCGCCGACGGGagct-5’ |
| $-121/-86$ | 5’-AACACGGGGCTTGCTGCC-3’ |
| mut 2 | 3’-TTTGGCCCGGACGACGG-5’ |
| mut 3 | 5’-tcgattcACAGAGCCCGGACGGGCTGCC-3’ |
| mut 23 | 3’-AGTGGTATCGTCGGGCGGGGAAAGGagct-5’ |
| Sp1 | 5’-TACCATCGACCGAGTCCCGCGCCGGGAGG-3’ |
| NF-Y | 3’-TTTGGCCCGCCGACGGG-5’ |
| $-61/-40$ | 5’-ATTCGATCGGGGGGCAGG-3’ |
| $-86$ | 3’-TAAGCTAGCCCGCGCCG-5’ |
| mut 1 | 5’-gattcGCGGTCGGCCGCG-3’ |
| mut 2 | 3’-CAGCAGTAAACAAAGGCGC-5’ |
bands (lane 4). Similar results were obtained with the use of radiolabeled −61/−40 probe (lanes 6–8). These results indicate that Sp1 and Sp3 proteins in the MA-10 cell nuclear extracts are actually binding to −121/−86 and −61/−40 regions of the rat SR-BI promoter.

We next determined whether Sp1 and Sp3 proteins are involved in SR-BI promoter activity. MA-10 cells were transiently co-transfected with luciferase reporter plasmids and expression vectors for Sp1 or Sp3. Two different SR-BI promoter constructs (SR978luc and SR141luc) were used as luciferase reporter plasmids. As shown in Fig. 7 (A and B), co-transfection of the Sp1 expression vector caused an increase in the SR-BI promoter activity of both SR978luc and SR141luc reporters in a dose-dependent manner. However, co-transfection of the Sp3 expression vector failed to enhance the activity of the SR-BI promoter. One possible explanation is that effects of transfection of the Sp3 expression vector are masked by endogenous Sp3 proteins in the MA-10 cells. Therefore, in order to confirm the effect of Sp3 on transcriptional activity of the SR-BI gene promoter, the SL2 cell line, which lacks endogenous Sp1 families, was employed. As shown in Fig. 8A, co-transfection of either the Sp1 or the Sp3 expression vector caused an increase in the SR-BI promoter activity of both SR978luc and SR141luc reporters in the SL2 cells. However, the increment of the promoter activity by Sp3 was about 30% of that caused by Sp1 but statistically significant (p < 0.01, pPac versus pPac-USp3). Furthermore, the increment of promoter activity by Sp1 was only marginal when mut123 (mutated in all of the three GC boxes) reporter construct was used (Fig. 8B). These results clearly indicate that Sp1 and Sp3 directly interact with the three GC boxes within the SR141luc reporter construct and positively regulate SR-BI promoter activity.
HeLa cells were transiently transfected with 0.1 gene. essential for the rat SR-BI gene expression. To date three the reason for this discrepancy is not clear.

beyond 1517 bp upstream of the translation start site, although reported by Lopez (25). Recently, the nucleotide sequence of the rat SR-BI gene a single site for each SF-1 and NF-Y at the proximal region for the human gene, there are five Sp1 binding sites and a GC-rich region. A similar GC-rich sequence was also found in the case of the human gene, there are five Sp1 binding sites and a GC-rich region. Four Sp1 binding sites are present in the proximal -flanking region of the human SR-BI gene.

It has been reported that SF-1, an orphan member of the nuclear hormone receptor gene family, binds to the human SR-BI gene promoter and is an important regulator of SR-BI expression in steroidogenic cells, in which SF-1 is expressed at high levels. In this study, we examined the effects of SF-1 on the regulation of the rat SR-BI gene expression. Regulation of rat StAR gene promoter activity by SF-1 was also examined for the sake of comparison. StAR is the protein that delivers cholesterol from the outer to the inner mitochondrial membrane, and expression of this protein is positively regulated by SF-1. HeLa cells, which express no SF-1 protein, were transiently transfected with either SR978luc, SR141luc, or StAR1597luc along with 0.001 g of pRL Renilla luciferase control vector. The cells were simultaneously transfected with 0.3 g of SF1-pcDNA3 expression vector or with 0.3 g of pcDNA3 control vector. Results were presented as -fold induction, by comparing the relative luciferase activities obtained with the transfection of SF1-pcDNA3 vector versus those obtained with the transfection of pcDNA3 control vector. Each value represents the mean and standard error of four independent transfection experiments.

We have cloned and characterized the upstream DNA fragment of the rat SR-BI gene. Only a single SR-BI transcription start site was identified in the immature rat ovary and adrenal gland using the primer extension assay. Nucleotide sequence analysis revealed that the proximal 5'-flanking region involves a GC-rich sequence. Four Sp1 binding sites are present in the GC-rich region. A similar GC-rich sequence was also found in the proximal 5'-flanking region of the human SR-BI gene. In the case of the human gene, there are five Sp1 binding sites and a single site for each SF-1 and NF-Y at the proximal region (25). Recently, the nucleotide sequence of the rat SR-BI gene has been reported by Lopez et al. (26). However, the sequence reported by Lopez et al. is different from ours in the region beyond 1517 bp upstream of the translation start site, although the reason for this discrepancy is not clear.

We have shown that Sp1 family transcription factor(s) is essential for the rat SR-BI gene expression. To date three Sp1-related proteins have been identified, namely Sp2, Sp3, and Sp4. Sp1 and Sp3 are thought to be important for the expression of various genes in mammalian tissues. In this study we have shown that both Sp1 and Sp3 are involved in complex formation with the GC boxes in the rat SR-BI promoter. In recent years it has been shown that Sp1 and/or Sp3 are indeed involved in the hormone-regulated induction of a variety of genes, although these proteins are ubiquitously expressed. We and others have shown that rat SR-BI gene expression is positively regulated by trophic hormone human chorionic gonadotropin in the ovary (11, 15). Gonadotropins exert their actions by increasing intracellular cAMP levels via the activation of adenylyl cyclase. Several reports have shown that Sp1-binding sites are required for cAMP-mediated induction of genes. For example, the genes for cholesterol side-chain cleavage cytochrome P450 (27, 28), the rhesus growth hormone variant (29), serum/glucocorticoid-inducible kinase (30), and the luteinizing hormone β-subunit (31) have been shown to require Sp1-binding sites for cAMP-mediated induction. Sp1 is regulated by phosphorylation and dephosphorylation. Cyclic AMP-dependent protein kinase also catalyzes the phosphorylation of Sp1 and enhances its binding to the cognate sequence. Transfection of Sp1 stimulates the cAMP-dependent transcriptional activity of cholesterol side-chain cleavage cytochrome P450 gene promoter in SL2 cells which lack the endogenous Sp1 protein family (32). Similar coordinated regulation by the Sp1 family and cAMP signal transduction pathway may function in the expression of the rat SR-BI gene. However, the overexpression of the cyclic AMP-dependent protein kinase catalytic subunit in MA-10 cells could not enhance rat SR-BI promoter activity, whereas the rat StAR gene promoter activity was shown to be enhanced (5-fold) in a similar experiment (data not shown). Further study is clearly needed in order to clarify the relationship between the Sp1 family and the cAMP-dependent activation of the rat SR-BI gene. Lopez et al. (26) reported that the sterol regulatory element-binding protein-1a binds to GC box 3 and activates transcription of SR-BI gene (26). However, our EMSA supershift experiments showed that the antibodies against Sp1 and Sp3 caused complete disappearance of the complex bands that had been initially formed with the GC box 3 probe. At least in MA-10 cells, Sp1 and Sp3 may be the major proteins that interact with SR-BI GC box 3, although SREBP is another member that interacts with the box.

Only a few studies have been directed at the characterization of SR-BI gene promoters. In human, Cao et al. (25) reported that SF-1, an orphan member of nuclear hormone receptor family, binds to the proximal site of human SR-BI gene promoter region and that efficient transcription from this promoter in adrenocortical Y1 cells is dependent on the intact SF-1 site. SF-1 mRNA is constitutively expressed in adrenocortical cells, the Leydig cells in the testis, and the thecal and corpus luteal cells in the ovary (33). SF-1 activates the expression of a number of components of the steroidogenic complex, including the P450 side-chain cleavage enzyme gene (34), the 17α-hydroxylase/c17–20 lyase gene (35), the aromatase cytochrome P450 gene (36–38), and StAR (39–42). The finding by Cao et al. supports the view that SF-BI also plays an important role in delivering lipids for steroidogenesis. However, Cao et al. also concluded that transcription factors other than SF-1 participate in the regulation of the human SR-BI gene expression, even in steroidogenic tissues. In addition, SF-1 is not expressed in a number of tissues that express SR-BI including the liver, mammary gland, and human placenta. These facts indicate that other transcription factors must regulate SR-BI expression in these tissues.

**Fig. 9. Effect of SF-1 on the promoter activity of the rat SR-BI gene.** HeLa cells were transiently transfected with 0.1 µg of reporter vectors (SR978luc, SR141luc or StAR1597luc) along with 0.001 µg of pRL Renilla luciferase control vector. The cells were simultaneously transfected with 0.3 µg of SF1-pcDNA3 expression vector or with 0.3 µg of pcDNA3 control vector. Results were presented as -fold induction, by comparing the relative luciferase activities obtained with the transfection of SF1-pcDNA3 vector versus those obtained with the transfection of pcDNA3 control vector. Each value represents the mean and standard error of four independent transfection experiments.

**Discussion**

We have cloned and characterized the upstream DNA fragment of the rat SR-BI gene. Only a single SR-BI transcription start site was identified in the immature rat ovary and adrenal gland using the primer extension assay. Nucleotide sequence analysis revealed that the proximal 5'-flanking region involves a GC-rich sequence. Four Sp1 binding sites are present in the GC-rich region. A similar GC-rich sequence was also found in the proximal 5'-flanking region of the human SR-BI gene. In the case of the human gene, there are five Sp1 binding sites and a single site for each SF-1 and NF-Y at the proximal region (25). Recently, the nucleotide sequence of the rat SR-BI gene has been reported by Lopez et al. (26). However, the sequence reported by Lopez et al. is different from ours in the region beyond 1517 bp upstream of the translation start site, although the reason for this discrepancy is not clear.

We have shown that Sp1 family transcription factor(s) is essential for the rat SR-BI gene expression. To date three
In contrast to the human SR-BI gene, there is no SF-1 binding site consensus sequence in the 5’-upstream region proximal to the transcription start site of rat SR-BI. Instead, there is an SF-1 site (87% identity to the SF-1 site reported for the rat StAR gene) 28 bp downstream of the transcription start site. Another SF-1 consensus site is found at a very distant site (516 bp upstream) from the transcription start site. However, the removal of the region containing the distal SF-1 site had no effect on SR-BI promoter activity. Lopez et al. have reported, however, that the two SF-1 sites (the distal site and the downstream site in the first exon) are important for the rat SR-BI promoter activity in human HTB9 bladder carcinoma and mouse Y1 tumor cells (43). The reason for the discrepancy is presently unknown, but might be due to differences in the promoter constructs used. All promoter constructs used in this study did not contain the downstream SF-1 site, which is present in the first exon. In addition, even when the reporter construct containing the distal SF-1 site was transfected into HeLa cells, which do not express endogenous SF-1 protein, the promoter activity was not affected by the co-transfection of SF-1 expression vector. On the other hand, rat StAR promoter activity was actually enhanced by the co-transfection of SF-1 expression vector in the same culture system (Fig. 9). The findings suggest that SF-1 may not be essential for the expression of rat SR-BI gene. Unlike StAR or other steroidogenic enzymes, SR-BI is expressed in the liver, where SR-BI is believed to play a crucial role for the reverse uptake of cholesterol. Since SF-1 is not expressed in the liver, a different combination of transcription factors including the Sp1 family may function to express SR-BI gene at least in the liver.

In the present study, we demonstrated that the Sp1 family transcription factors, rather than SF-1, are important for rat SR-BI gene expression. However, these Sp1 family proteins are ubiquitously expressed. Tissue-specific factors that have not yet been characterized must participate in the regulation of SR-BI gene expression in coordination with Sp1 family proteins. In order to define the regulation of SR-BI gene expression, further studies will be required for characterizing such factors.

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