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Characterization of regulatory intronic and exonic sequences involved in alternative splicing of scavenger receptor class B gene

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ABSTRACT

Scavenger receptor class B type I (SR-BI) is a major receptor of the high-density lipoprotein that mediates cholesterol efflux and reverse cholesterol transport. Alternative splicing of the scavenger receptor class B (SR-B) gene is observed and different splice forms, SR-BI and scavenger receptor class B type II (SR-BII), have been shown to function and localize in distinct ways. We have previously shown that SR-B alternative splicing regulation is associated with splicing factor ASF/SF2. In this study, using a SR-B minigene as a model, we determined the critical regulatory regions in the upstream intron, intron 11, by serial deletion and mutation analyses. We also further characterized the regulatory elements in intron 11 as well as in the skipped exon, exon 12. Moreover, we studied the interactions of these elements with the splicing factor ASF/SF2. This study provides new insights into the mechanism of SR-B splicing and it is important for further study on the mechanism of SR-B alternative splicing regulation, such as its regulation by estrogen.

Scavenger receptor B type I (SR-BI), a physiological high-density lipoprotein (HDL) receptor, mediates cholesterol efflux to lipoproteins from atheromatous arteries [1] and reverse cholesterol transport from lipoprotein to liver [2]. It is encoded by the SR-B gene which is composed of 13 exons [3]. An alternative splice variant of SR-BI, scavenger receptor class B type II (SR-BII), has been identified [4]. SR-BII differs from SR-BI due to exon skipping in which a 129-nucleotide exon, exon 12, is included in SR-BI but not in SR-BII (reviewed in [5]). This exclusion causes an open reading frame shift in the mRNA and part of the 3′-untranslated region of SR-BI becomes the coding sequence for SR-BII. As a consequence, SR-BII differs from SR-BI in subcellular localization and cellular trafficking and, therefore, mediates lipid uptake and influences cholesterol trafficking in a functionally distinct manner from SR-BI [6,7].

Currently, it is not clear how SR-B alternative splicing is controlled at the molecular level. Our group has recently shown that estrogen can cause SR-BI reduction and SR-BII elevation in rat liver and that estrogen directly regulates SR-B alternative splicing [8]. We also identified ASF/SF2, as well as Tra2β, as important splicing factors involved in the SR-B splicing machinery [8]. However, the molecular mechanisms of this process are still not well understood. Mechanistic studies of splicing regulation by the pre-mRNA sequences involved in the alternative splicing event are critical to understanding the process.

Regulation of pre-mRNA splicing involves both *cis*- and *trans*-factors, which are, respectively, consensus sequence elements in the pre-mRNA, known as exon and intron splicing enhancers (ESEs and ISEs) and silencers (ESSs and ISSs), and cellular protein or ribonucleoprotein factors [9,10]. In the present study, using serial deletion and mutation analyses on a SR-B minigene, we have characterized the critical regulatory regions and sequences in both the upstream intron which is located upstream of the skipped exon and the skipped exon itself, and identified splicing regulatory elements. Further exploration of the interactions between these elements and the SR-B related splicing factor ASF/SF2 are discussed.

Materials and methods

Plasmids and oligonucleotides. The SR-B minigene, as illustrated in Supplemental Fig. 1, was constructed as previously described [8]. All the oligonucleotide primers for PCR and site-directed mutagenesis were purchased from Integrated DNA Technologies, Inc. (San Jose, CA).

Construction of SR-B minigene deletion/mutation expression plasmids. Serial deletions in intron 11 of SR-B minigene were generated using a unidirectional random deletion method. First, Kpn I and Mlu I restriction enzyme sites were generated at the 5′ end of intron 11 using the QuickChange site-directed mutagenesis kit (Stratagene), then unidirectional deletions were created using exonuclease III (Promega, Madison, WI). Aliquots removed at different

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time intervals from the exonuclease III reaction produced a series of deletions of different sizes. Then, the DNA was treated with Mung Bean Nuclease and the Klenow fragment of DNA Polymerase (Promega) to generate blunt ends. Finally, the DNA was ligated using the DNA Ligation Kit (Takara Bio Inc., New York, NY). Deletions within two defined sites were accomplished by restriction enzyme digestion, in which the restriction enzyme sites were generated using the QuickChange site-directed mutagenesis kit. Short deletions of 15 bp or less and nucleotide substitution with multiple thymine in exon 12 ESEs analyses were generated directly with the QuickChange site-directed mutagenesis kit. The deletion constructs were analyzed by restriction analyses and confirmed by sequencing.

Cell culture and plasmid transfection. The human hepatoma (HepG2) cell line, obtained from ATCC (Manassas, VA), were routinely maintained as previously described [8]. Plasmids were transfected into cells using Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's protocol. Cells were harvested 48 h post transfection and total cellular RNA was prepared.

Reverse transcription PCR of SR-B minigene. Total RNA was prepared using TRI reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol, treated with RNase-free DNase (TURBO DNA-free^{TM}, Ambion Inc., Austin, TX) and purified with RNeasy columns (QIAGEN Inc, Valencia, CA). Reverse transcription PCR (RT-PCR) reactions using 0.25 μg of total RNA and the Titan One Tube RT-PCR System kit (Roche Applied Science, Indianapolis, IN) were performed. Amplification of exogenous SR-B minigene transcripts were carried out as previously described [8].

RNA–protein binding assay. The RNA–protein binding assay was carried out as previously described [11]. Briefly, SR-B minigene pre-mRNA was labeled with biotin-16-UTP (Ambion) using MAXIscript *in vitro* transcription T7 kit (Ambion). The labeled RNA was purified with ProbeQuant G-50 micro columns (GE Healthcare, Piscataway, NJ). The binding reactions were performed in 100 μl binding buffer consisting of 10 mM Hepes, 1 mM $MgCl_2$, 50 mM KCl, 5% glycerol, 1 mM DTT, 2.5 mM creatine phosphate, 0.01 mM ATP, 2.5 mg/mlL BSA, 0.1 μg/μl tRNA and 200 μg HeLa Nuclear Extract (Promega). After 15 min of incubation at room temperature, the reaction mixture was added to pre-washed 400 μl MagPrep Streptavidin beads (Novagen, San Diego, CA). The reaction was incubated for 1 h at room temperature to allow binding of the protein–RNA complex to the beads. The bead–RNA complex was then washed four times with binding buffer. After the final wash, 30 μl of 2× protein sample buffer was added to the beads and incubated for 10 min at 100 °C to allow dissociation of the protein from the complex. Protein samples were analyzed by SDS–PAGE electrophoresis and Western blotting.

Results

Critical regulatory regions in intron 11 involved in SR-B alternative splicing

To study the mechanism of SR-B pre-mRNA splicing, the SR-B minigene containing the minimum necessary genomic regions adjacent to exon 12 was used in *in vivo* expression analyses. The minigene expressed both the SR-BI (+exon 12) and SR-BII (−exon 12) isoforms in HepG2 cells, which resembled those of endogenous SR-B expression [4,8]. To examine the role of intron 11, initial serial deletions within intron 11 were generated. While preserving the immediate adjacent sequences to the intron/exon junction sites (5′-CAG/GUAAGUAGGC-3′ for exon 11/intron 11), mutant SR-B minigene constructs containing deletions ranging from 56 to 792 bp starting from +10 nt of intron 11, as illustrated in Fig. 1A,

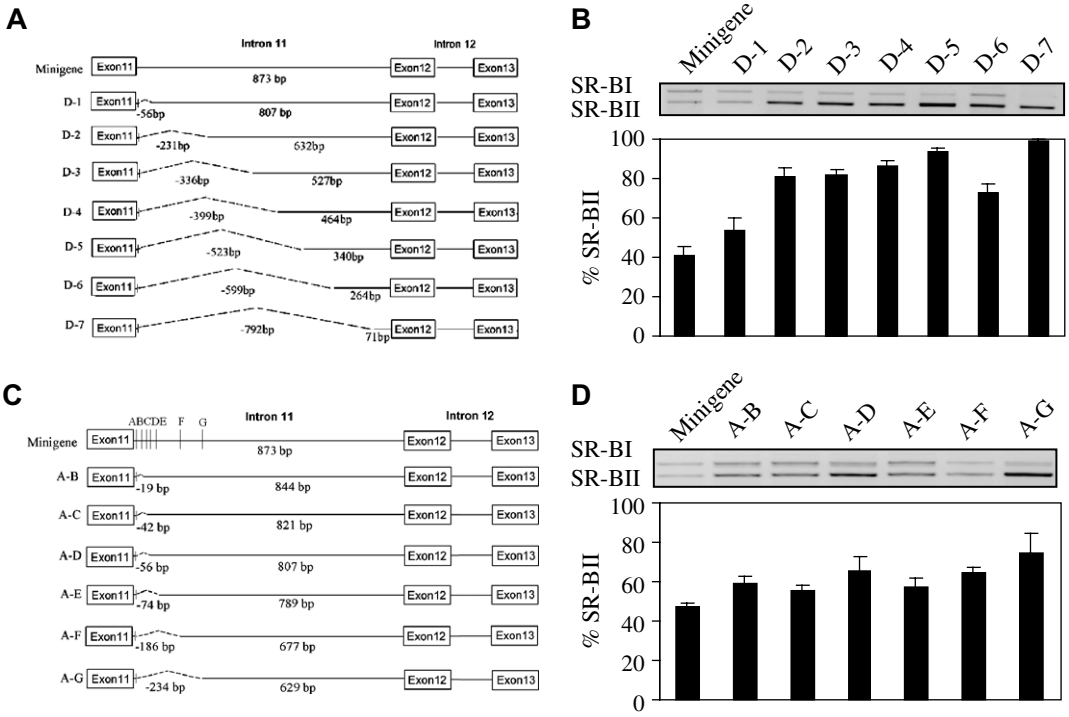

Fig. 1. Analyses of intron 11 in the regulation of SR-B minigene splicing. (A) Structure of mutant SR-B minigene constructs with serial deletions in intron 11. The deletions are indicated by dashed lines and the lengths of deletions and the remaining sequences are indicated under the lines. (B) *In vivo* splicing of the mutant constructs. The mutant constructs (D-1 to D-7) were used in the transfection. RNA was isolated and subjected to RT-PCR. (C) Structure of mutant constructs with serial deletions within +10 to +244 nt region in intron 11. (D) *In vivo* splicing of the mutant constructs. RNA was isolated and subjected to analyses with RT-PCR. All percentages of SR-BII isoform were determined by densitometry and are graphically illustrated. Mean + SEM from three independent experiments is presented.

were generated. These SR-B minigene subclones, D-1 to D-7, were used to transfect HepG2 cells to investigate which sequences in intron 11 were involved in the regulation of the alternative splicing. Generally, all deletion constructs had higher rates of SR-BII expression compared to the wild type SR-B minigene, indicating that important SR-BI expression enhancing regulatory elements reside within the deleted regions (Fig. 1B). In light of the splicing isoforms shifts from SR-BI to SR-BII observed, the regions of +10 to +241 nt (comparing D-2 with original minigene), +419 to +533 nt (comparing D-5 with D-4), and +609 to +802 nt (comparing D-7 with D-6) in intron 11 were more likely to contain enhancers for SR-B exon 12 inclusion, whereas the region of +533 to +609 nt (comparing D-6 with D-5) was likely to contain silencers for SR-B exon 12 exclusion.

Intronic splicing enhancers for SR-B alternative splicing in intron 11

Next we focused on two regions in intron 11: regions at +10 to +241 nt and +419 to +533 nt. We generated an additional series of constructs with deletions within the +10 to +244 nt region as illustrated in Fig. 1C. The deletions ranged from 19 to 234 bp. *In vivo* expression of these mutants in HepG2 cells showed deletions of +10 to +29 nt (comparing A-B to original minigene), +52 to +66 nt (comparing A-D to A-C) and +196 to +244 nt (comparing A-G to A-F) greatly affected the splicing pattern (Fig. 1D).

To analyze the region of +419 to +533 nt, we generated a third set of constructs with deletions within the +244 to +615 nt region, retaining the 244 nt sequence at the 5′ end of intron 11. The constructs were designated as G-J, G-K, G-H, and G-I as illustrated in Fig. 2A. *In vivo* expression of these constructs showed that the G-H and G-I, especially G-H, deletions yielded dramatically higher rates of SR-BII (Fig. 2B). Thus, the region of +399 to +536 nt (region K-H), the common deletion region in G-H and G-I constructs, is indicated to contain important regulatory elements for SR-B alternative splicing. To further confirm this, we generated constructs with smaller deletions between +399 and +615 nt, including the exact deletion of K-H and other constructs designated as K-I and H-I (Fig. 2A). *In vivo* expression showed that K-H and K-I constructs did yield high expression rates of SR-BII compared to the G-H construct, confirming that the K-H region (+399 to +536 nt) contains crucial regulatory elements.

Next, we used RESCUE-ISE as a prediction tool [12] to analyze the putative intronic splicing enhancers (ISEs) in intron 11. RESCUE-ISE predicts ISEs hexamers based on computational methods and statistical analysis of genome sequences with experimental validation in several different species [12]. One hundred and twenty-nine hexamers were identified in the intron 11 regions of interest (data not shown). Combined with the results from the deletion analyses, we examined small hexamers or hexamer cluster regions which fall within the regions of interest, i.e., regions +10 to +29 nt, +52 to +66 nt, +196 to +244 nt, and +399 to +536 nt. Six constructs containing deletions of these potential ISEs were generated and designated as Δ1–Δ7 (Fig. 2C). *In vivo* expression showed that the Δ1 and Δ3 constructs yielded higher expression

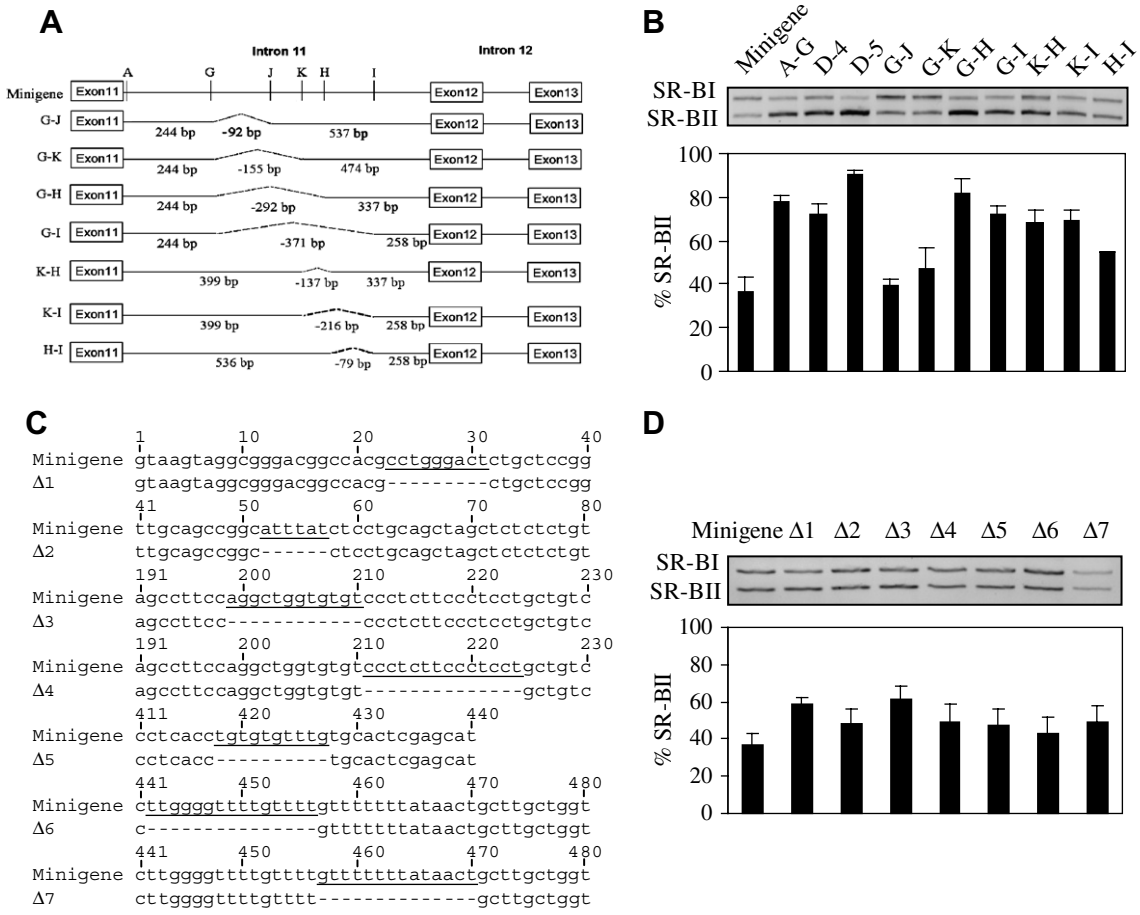

Fig. 2. Additional analyses of intron 11 in the regulation of SR-B minigene splicing. (A) Structure of the mutant constructs with deletions within +419 to +513 nt region in intron 11. Different deletions between G (+244 nt) and I (+605 nt) were generated. (B) *In vivo* splicing of the mutant constructs. A-G, D-4, and D-5 as shown is Fig. 1 are used as comparisons. (C) Structure of the mutant constructs with deletion of the putative ISEs in SR-B intron 11. The constructs were designated as Δ1–Δ7. (D) *In vivo* splicing of the mutant constructs.

levels of SR-BII compared to original SR-B minigene, increasing the expression of SR-BII by 58% and 120%, respectively, whereas Δ2, Δ4–Δ7 did not seem to alter SR-B alternative splicing (Fig. 2D). These relatively modest changes are comparable to the corresponding deletion constructs of A-B and A-G. This result indicates that the two hexamer clusters, sequences of +23 to +31 nt (cctgggact) and +199 to +210 nt (aggctggtgtgt), are ISEs involved in SR-B alternative splicing. Although K-H region (+399 to +536 nt) gave us more impressive changes, current analyses based on computational prediction did not recognize ISEs in that region.

Splicing regulatory elements in exon 12

Generally, both the skipped exon and the upstream intron contain critical regulatory elements [13]. To investigate whether exon 12 might be involved in splice site selection, we analyzed the exon 12 sequence with three different web-based computational prediction programs: RESCUE-ESE [12], ESE-Finder [14] and PESX [15]. The putative ESEs are shown in Fig. 3A. To determine whether they are functional or not, small regions (6–12 nt) containing these putative elements were replaced with multiple thymine nucleotides within exon 12 (TE1–TE14 in Fig. 3A and Supplemental Fig. 2). These substitution constructs were analyzed since a deletion in an exon may cause alteration of the structure of ESEs, thus affecting splicing activity. Substituting thymine/uridine residues for ESEs has been widely used to analyze ESE function [16]. *In vivo* expression results showed that TE1, TE3, TE10–TE14 have a strongly altered splicing pattern, with shifts from SR-BI to SR-BII (Fig. 3B). In contrast, TE4–TE8 showed decreased SR-BII levels or insignificant changes. These results confirm that TE1, TE3, TE10–TE14 are normally functioning as ESEs which enhance exon 12 involvement in SR-B splicing. Interestingly, with TE4–TE10, a longer splicing form (upper band, Fig. 3B upper panel) appeared to be spliced at different splice sites compared to SR-BI, and the sizes of the splicing products were observed to be between SR-BI and SR-BII. Although the significance of these splice site changes has yet to be determined, the effects caused by loss of function of the regulatory elements as a consequence of base pair substitution are obvious and these indicate that the regulatory elements residing in exon 12 have important roles in maintaining accurate SR-B alternative splicing. We also did deletion analysis of these predicted ESEs and the deletions, except for TE3∼TE6, caused similar splicing pattern changes (data not shown).

Interaction of the splicing factor ASF/SF2 and the regulatory elements

Our previous studies showed that the splicing factor ASF/SF2 was involved in the regulation of SR-B gene alternative splicing [8]. Overexpression of ASF/SF2 caused a shift from SR-BII to SR-BI. Splicing factors function in the splicing machinery by interacting with intronic or exonic elements, thus it is interesting to know whether ASF/SF2 is interacting with intron 11 and/or exon 12 regulatory elements. Using a RNA–protein binding assay, we determined that ASF/SF2 proteins were binding to the SR-B minigene exon 12 and also to the intron 11 pre-mRNA (Fig. 4A). Therefore, we co-transfected the mutant SR-B constructs with ASF/SF2. Results showed that with the intron 11 mutant constructs Δ1–Δ7, overexpression of ASF/SF2 consistently induced a shift from SR-BII to SR-BI in a manner similar to the wild type SR-B minigene (Fig. 4B). With the exon 12 mutant constructs, overexpression of ASF/SF2 did not induce a decrease in SR-BII levels in TE1, TE3–TE14 constructs, as it did with the original minigene and the TE2

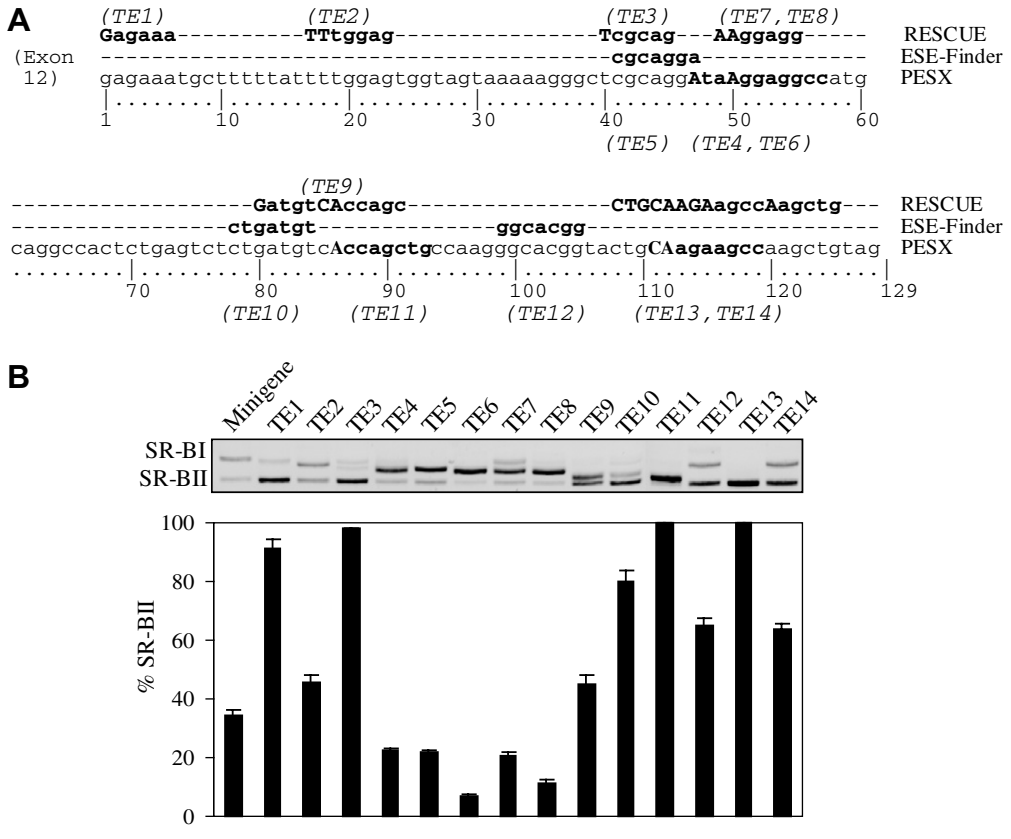

Fig. 3. Analyses of SR-B Exon 12 splicing regulatory elements. (A) Exon 12 ESEs predicted by RESCUE-ESE, ESE-Finder, and PESX. Sequences of putative ESEs are depicted in bold font. The capitalized letters represent the beginning of the 6-nt (for RESCUE-ESE) or 8-nt (for PESX) elements. Mutant constructs (TE1–TE14) with multiple thymine substitution of these elements were constructed and described in detail in Supplemental Fig. 2. (B) *In vivo* splicing of the mutant constructs.

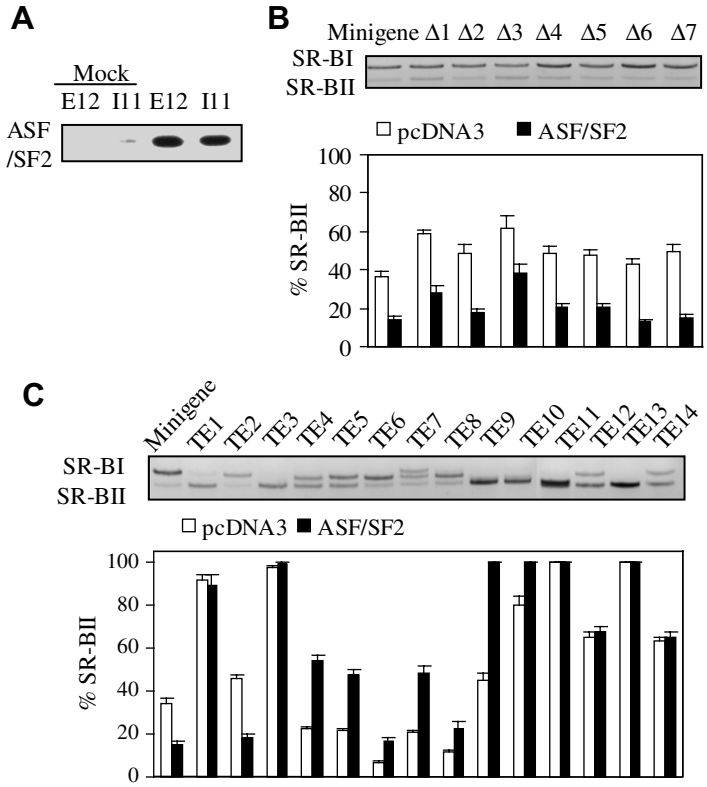

Fig. 4. Interactions between the splicing factor ASF/SF2 and intron 11 and exon 12. (A) *In vitro* RNA–protein binding assay was performed. In mock controls the unlabeled UTP was used instead of biotin-16-UTP. (B) Intron 11 mutant constructs of SR-B putative ISE deletions were co-transfected together with ASF/SF2. pcDNA3 was used as the vector control and the photograph is similar to the one shown in Fig. 2D. (C) Exon 12 multiple-thymine substitution constructs of SR-B minigenes were co-transfected together with ASF/SF2. pcDNA3 was used as the vector control and the photograph is similar to the one shown in Fig. 3B.

mutant. This suggests that the ASF/SF2 protein does interact with multiple exon 12 pre-mRNA sequences, most likely through binding of the protein factor and the regulatory elements.

Discussion

The control mechanisms for the regulation of alternative exon selection in SR-B gene expression are completely unknown. In this study, we have used deletion and mutation analyses of the SR-B minigene to identify the *cis*-regulators of alternative splicing. We have determined the critical regulatory regions in intron 11 and have identified two intronic splicing enhancer sequences. We have also analyzed exonic regulatory sequences in exon 12. Mutation or deletion of these sequences dramatically changes the splicing pattern of the SR-B transcript. Lastly, the splicing factor ASF/SF2 has been shown to interact with SR-B intron 11 and exon 12.

In mammalian cells regulation of splicing involves both the *cis*-regulatory elements and protein *trans*-factors [10]. Our data support that multiple regulatory regions reside in both the intron and exon regions. Intron 11 has at least four regions, +10 to +29 nt, +52 to +66 nt, +196 to +244 nt, and +399 to +536 nt, that are important for efficient exon 12 inclusion. Two intronic enhancers have been identified from the predicted ISEs within these regions. Replacement of the putative ESEs in exon 12 with multiple thymine residues greatly affected SR-B splicing, suggesting the importance of the sequences and structure of exon 12 in SR-B alternative splicing. Due to unknown mechanisms, replacement of some of the exonic sequences with multiple thymines induced different splice sites. Thus, the exon 12 regulatory sequences play roles not only in the determination of whether or not to include exon 12, but also in the correct selection of splice sites for SR-BI. Our identification of the critical positive regulatory sequences in exon 12 suggests a possible mechanism for the exon 12 inclusion-exclusion or splice site selection control, in which the splicing regulatory elements in exon 12 guarantee the accurate and correct inclusion of exon 12 itself.

The regulatory sequences affect the alternative splicing through the binding of regulatory protein factors [17]. ASF/SF2 modulates the function of ESEs through direct or indirect binding [18], then takes part in ESE activation and affects alternative splicing by gradually shifting splice-site selection from a distal site to proximal sites [19]. Our results have shown that the mutation of some exonic regulatory elements caused elimination of ASF/SF2 effects on SR-B splicing, suggesting that disruption of either the ESEs structure or the interactions between the protein factors and ESEs causes a loss of the ASF/SF2 function. Thus, ASF/SF2 interacts with exon 12, and might also interact with some unknown ISEs in intron 11, and can, subsequently, enhance the inclusion of exon 12 in SR-B splicing.

In summary, we have shown that *cis*-regulatory elements in intron 11 and exon 12 play critical roles and the splicing factor ASF/SF2 is associated with these sequences. Elucidation of the mechanisms controlling alternative splicing of SR-B pre-mRNA will enhance our understanding of how SR-B expression is regulated at the pre-mRNA splicing level, and will further our understanding of how SR-B regulation functionally affects the biological process of cholesterol metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.05.007.

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