Mapping the SF2/ASF Binding Sites in the Bovine Growth Hormone Exonic Splicing Enhancer

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SF2/ASF binding sites of bGH pre-mRNA
Summary

Splicing of the last intron (intron D) of the bovine growth hormone pre-mRNA requires the presence of a downstream exonic splicing enhancer (ESE). This enhancer is contained within a 115-nucleotide \( Fsp I-Pvu \) II (FP) fragment located in the middle of the last exon (exon 5). Previous work showed that the splicing factor SF2/ASF binds to this FP region and stimulates splicing of intron D \textit{in vitro}. However, the precise sequences recognized by SF2/ASF within the FP region had not been determined. Here we used multiple strategies to map the SF2/ASF binding sites and determine their importance for ESE function. Taking advantage of the fact that SF2/ASF ultraviolet (UV) cross-links specifically to RNA containing the FP sequence, we first mapped a major SF2/ASF binding site by UV cross-linking and reverse transcription. This strategy identified a 29-nucleotide SF2/ASF binding region in the middle of the FP sequence, comprising the 7-nucleotide purine-rich motif described previously. Interestingly, this binding region is neither sufficient, nor absolutely required for SF2/ASF-mediated splicing, suggesting the presence of additional SF2/ASF binding sites. The location of these additional sites was determined by electrophoretic mobility shift analysis (EMSA) of various subfragments of the FP sequence. Antisense 2’-O-methyl oligoribonucleotides
complementary to selected SF2/ASF binding sites block bGH intron D splicing. Thus, multiple SF2/ASF binding sites within the exonic splicing enhancer contribute to maximal enhancer activity.
Introduction

The involvement of a family of splicing factors called SR proteins in the splicing of numerous introns is well established (reviewed in refs. 1-3). Members of this family are characterized by one or two RNA-recognition motifs (RRMs) and a C-terminal domain with extensive arginine/serine repeats (RS domain). They include the proteins SF2/ASF, SC35, SRp20, SRp30c, 9G8, SRp40, SRp55, SRp75 and p54. These proteins are thought to be essential for splicing, given that splicing of synthetic pre-mRNA substrates does not occur in cytosolic S100 extracts, which contain only trace amounts of SR proteins, until at least one SR protein is added (4-6). SR proteins can promote binding of U1 small nuclear ribonucleoprotein particle (U1 snRNP) to 5' splice sites (7-10). In at least some pre-mRNAs, SR proteins may actually recognize and bind to the 5' splice site prior to the binding of U1 snRNP (11). At high concentrations, SR proteins can compensate for the removal or inactivation of U1 snRNP (12,13). However, it is clear that the role of SR proteins is more complex than just stimulating U1 snRNP binding to the 5' splice site.

Exon sequences that are generally purine-rich were discovered as global splicing enhancer signals, and thus, became known as Exonic Splicing Enhancers (ESE) (14-17). Evidence from yeast two-hybrid experiments has implicated SR proteins in forming bridging complexes between the ESE and
the 3' splice site, as well as between the 5' and 3' splice sites (18). In the
former example, SR proteins have been shown to be associated with ESE
sequences in the downstream exon (19-28). SR proteins have been shown
to bind directly to certain enhancer sequences, without requiring
intermediary proteins (19,21,22). However, in some cases it appears that
binding of SR proteins to the splicing enhancer sequences is indirect,
resulting from recruitment by other splicing factors to the enhancer
complex (20,23). SR proteins appear to be key players in enhancing
splicing, perhaps using their RS domains to recruit factors such as U2AF to
the 3' splice site (18,29).

The picture emerging suggests that at least some SR proteins bind
preferentially to purine-rich sequences (11,19,22,30,31), although non-
purine sequences can also function as splicing enhancers (24,32-36). Each
member of the SR protein family appears to have a different consensus
high affinity binding site(s), as determined by in vitro RNA-binding
selection analyses (SELEX) of SF2/ASF, SC35, 9G8, SRp40, B52 (Drosophila
SRp55) and RBP1 (Drosophila SRp20) (30,31,37-39). Distinct consensus
motifs were also determined for SF2/ASF, SRp55, SRp40, and SC35 using a
functional selection procedure (35,36). There is a significant resemblance
in nucleotide composition or a statistical match to a consensus motif
between sequences discovered as natural splicing enhancers and those
determined by binding or functional SELEX experiments (30,31,33-36,39).
However, little is known about the SR protein binding sites within these enhancer sequences, although it appears that SR protein-dependent splicing specificity is mainly due to the RRM domains (40,41).

Splicing of the last intron (intron D) of the bovine growth hormone (bGH) pre-mRNA requires the presence of a downstream splicing enhancer in the last exon (17,42). This ESE is contained within a 115-nucleotide \( Fsp \) I-\( Pvu \) II (FP) fragment in the middle of exon 5 (42). The SR protein SF2/ASF binds specifically to the FP region and, presumably as a result of this binding, it enhances splicing of bGH intron D \textit{in vitro} (19). Although there is evidence that a purine-rich sequence in the middle of the FP sequence is part of the bGH ESE sequence (17), it was not clear whether SF2/ASF binds to this purine-rich sequence and/or to adjacent regions. In this study, we take advantage of the fact that SF2/ASF binds to the FP sequence in the absence of other proteins, and utilize this simplified recognition event to study one of the earliest steps in splicing, ESE recognition. Mapping of SF2/ASF cross-links to the FP sequence and EMSA showed that there are multiple SF2/ASF binding sites within the FP sequence, including a site that is centered around the previously described purine motif (17). The functional significance of these SF2/ASF binding sites was established by specific inhibition of bGH intron D splicing by antisense 2'-\( O \)-methyl oligoribonucleotides complementary to selected
binding sites. Thus, multiple SF2/ASF binding sites within the FP element are required for maximal enhancer activity.
Experimental Procedures

Materials. The plasmids pbGH-4D5 (43), pE5/FP (19) and pSP64-HβΔ6 (44) have been described. pSP64-HβΔ6 was subcloned into the pBS M13+ vector (Stratagene) to drive expression of β-globin from the T3 promoter. Plasmid pΔUV-XL was constructed by creating a polymerase chain reaction (PCR)-derived fragment of bGH using oligonucleotide BS-44 (5’-GCCTGTAGGGGAGGTGGAA-3’) and oligonucleotide ΔUV-XL (5’-ACGTCTCGTGCAGACCGTAG-3’) as upstream and downstream primers, respectively. This fragment was then subcloned into the Apa I and Rsa I sites of pbGH/4D5. Plasmid pUV-XL was constructed by creating a PCR-derived fragment of bGH using oligonucleotide T3 (5’-ATTAACCCTCACTAAAG-3’) and oligonucleotide UV-XL (5’-CTTATGCAGGTCCTTCCGGAAGCAGGAGAGCATGTTTGTG-3’) as upstream and downstream primers, respectively. This fragment was then subcloned into the Pst I and Pvu II sites of pbGH/4D5. Plasmids pE5/ΔFP/UV-XL and pE5/FP/ΔUV-XL were constructed by removing the Hind III-Sma I fragments from plasmids pUV-XL and pΔUV-XL, respectively. pE5/ΔFP plasmids containing the various FP subfragments inserted in place of the FP sequence were constructed by creating full-length PCR-derived plasmids containing the individual inserts using the Expand Long Template PCR System (Boehringer). In each case, the same upstream primer (5’-CTGTGCCTTCTAGTTGCC-3’) was used, whereas the
downstream primer varied according to the FP subfragment inserted: FP subfragment no. 1 (5'-CGCGTGCCTGACTGCGCATGTTTGTG-3'); FP subfragment no. 2 (5'-TAGTTCTTGAGCAGGCATGTTTGTG-3'); FP subfragment no. 3 (5'-CAGGTCCTTC CGGAAGGCATGTTTGTG-3'); FP subfragment no. 4 (5'-CAGGTCCTTC GCATGTTTGTG-3'); FP subfragment no. 5 (5'-TACGTCTCCGTCTTATG GCATGTTTGTG-3'); FP subfragment no. 6 (5'-CATGACCCTCAGGTGCATGTTTGTG-3'); FP subfragment no. 7 (5'-AGCGGCGGCACTTCCGTGATGTTTGTG-3'); FP subfragment no. 8 (5'-CTGGCCTCCCCGAAGCATGTTTGTG-3'); FP subfragment no. 9 (5'-CCCTCAGGTACGTCTCCGCATGTTTGTG-3').

Oligonucleotides T3 primer (5'-ATTAACCCTCACTAAAG-3') and FP-17mer (5'-CTGGCCTCCCCGAAGCG-3') were purchased from National Biosciences (NBI). 2'-O-methyl oligoribonucleotides B1 (5'-GmCmGmCmGm UmCmGmUmCmAmCmUmGm-3'), B2 (5'-GmUmUmCmUmUmGmAmGmCm AmGmCmGm-3'), B3 (5'-CmCmGmGmAmAmGmCmAmGmGmAmGmAm-3'), B4 (5'-CmUmUmAmUmGmCmAmGmGmUmCmCm-3'), B5 (5'-AmCmUm UmCmAmGmAmGmCmCmCmUmCm-3'), B6 (5'-CmAmGmGmUmGmGmCm UmCmCmCmCmCm-3'), B7 (5'-CmAmAmAmAmAmCmAmGmCmGmGmCm Am-3'), B8 (5'-CmGmGmGmGmGmAmGmGmGmGmGmGmGmGmCmAm-3'), were purchased from Oligos, Etc.

Human SF2/ASF (0.113 mg/ml) was purified from HeLa cell nuclear extract as described (45). 6-Histidine tagged recombinant human SF2/ASF (0.25 mg/ml) was expressed in Sf21 cells using the BacPAK Baculovirus.
Expression system (Clontech) and purified using a Ni-agarose affinity resin (Life Technologies) according to instructions of the manufacturer. Human recombinant hnRNP A1 (0.2 mg/ml) was expressed in *E. coli* and purified as described (47). Protein concentrations were determined by the dye-binding assay (Bio-Rad) with bovine serum albumin (BSA) as a standard. The anti-SF2/ASF monoclonal antibody (mAb96) is specific and does not cross-react with other SR proteins (48). The anti-hnRNP A1 mAb 4B10 was a generous gift from Drs. G. Dreyfuss and S. Piñol-Roma.

Mapping of specific SF2/ASF and hnRNP A1 ultraviolet (UV)-cross-links to the FP sequence by reverse transcription. [α-32P]GTP-labeled E5/FP RNA (16-18 ng/0.4 pmol) was incubated under standard splicing conditions (43) with either 266 ng of SF2/ASF purified to apparent homogeneity from HeLa cells or 400 ng of purified recombinant hnRNP A1 in 25-μl reactions at 30°C for 25 min. The reaction mixtures were then transferred to a microtiter plate and exposed to UV light (254 nm) at a distance of 4 cm for 15 min on ice using a UV lamp (15 Watts; G15T8, General Electric). Anti-SF2/ASF monoclonal antibody mAb96 (100 μl culture supernatant) and anti-hnRNP A1 mAb 4B10 (2.5 μl ascites fluid) were each prebound to 50 μl of protein A-agarose (50%) in 500 μl of immunoprecipitation (IP) buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% NP-40] for 1 hr and washed three times with 1 ml of IP buffer for
15 min at 4°C. The monoclonal antibody/protein A-agarose complexes were resuspended in 500 μl of IP buffer and the bound, cross-linked SF2/ASF-E5/FP RNA and hnRNP A1-E5/FP RNA reactions were added. The suspensions were mixed at 4°C for 2 hrs. After three washes with 1 ml of IP buffer for 15 min at 4°C, the cross-linked E5/FP RNAs were recovered by incubating the beads in 400 μl of 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM EDTA, 0.5% SDS, and 0.5 μg/μl Proteinase K for 30 min at 37°C with intermittent mixing, followed by phenol/chloroform extraction and ethanol precipitation.

The purified RNAs were then reverse transcribed with Superscript II (Life Technologies) under the following conditions. Equal counts of untreated, cross-linked and/or immunoprecipitated E5/FP RNAs were mixed with 10 pmol of either oligonucleotide FP-17mer or T3 primer in a volume of 6 μl of double distilled H2O and were denatured for 3 min at 90°C. After brief cooling on ice, each reaction was brought up to a final volume of 20 μl with reagents including buffers provided by the manufacturer, i.e., 20-40 U of RNase inhibitor (RNasin, Promega), 200 U of Superscript II, 5 μCi of [α-32P] dCTP, 0.5 μl of 100 μM dCTP, and 4.5 μl of a 2 mM mix of the remaining three dNTPs. Reverse transcription was done at 45°C for 1 hr. RNAs were degraded by addition of 20 μl of 0.4 M NaOH and incubation at 65°C for 30 min, and cDNAs were separated on 8% polyacrylamide gels along with appropriate controls.
**RNA Synthesis and In Vitro Splicing Reactions.** Capped RNAs were synthesized using T3 RNA polymerase for the bGH and β-globin pre-mRNAs and for the UV-XL and ΔUV-XL RNAs (produced from plasmids pE5/ΔFP/UV-XL and pE5/FP/ΔUV-XL linearized with Eco RI and Pvu II, respectively) and T7 RNA polymerase for the E5/FP RNA (produced from pE5/FP (19) linearized with either Bam HI or Pvu II) with conditions recommended by the manufacturer (Stratagene) using [α-32P] GTP to label the RNA. *In vitro* splicing reactions (12.5 µl) were performed with HeLa cell nuclear extract at 30°C for 75 min, as described (43). In the 2′-O-methyl oligoribonucleotide inhibition experiments, the oligoribonucleotides were pre-incubated with the pre-mRNAs at 30°C for 15-30 min before addition of the nuclear extract. In the order-of-addition experiments, either the oligoribonucleotide or purified SF2/ASF was pre-incubated with the pre-mRNA at 30°C for 30 min before addition of the remaining reagents.

**Electrophoretic Mobility Shift Assays (EMSA).** Uncapped, [α-32P] GTP-labeled E5/FP, E5/ΔFP (± FP subfragment inserts), E5/FP/ΔUV-XL and E5/ΔFP/UV-XL RNAs were synthesized for these assays. Each reaction (25 µl) contained 10 mM Tris-HCl (pH 7.9), 100 mM KCl, 1 mM DTT, 0.5 mM EDTA, 0.025% NP-40, 0.5 U/µl RNasin, 0.08 µg/µl BSA, 1 µg yeast tRNA,
10% glycerol, 125 ng of recombinant baculovirus SF2/ASF and approximately 3 fmol of the indicated RNA. The reactions were incubated at 30°C for 30 min and resolved on a non-denaturing 6% polyacrylamide gel containing 50 mM Tris, 50 mM glycine. Electrophoresis was performed at a constant voltage of 14 V/cm for 2 hr.
Results

**Mapping of SF2/ASF and hnRNP A1 cross-links in the bGH ESE.**

Our earlier work showed that SF2/ASF binds specifically to the 115-nucleotide FP/ESE sequence in exon 5, but not to the remaining regions of this exon (19). However, it was not clear whether this binding occurred at a purine-rich sequence centered around a 10-base-pair palindrome in the middle of the ESE, which can serve as a moderate splicing enhancer (17,42). Furthermore, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) was shown to counteract the SF2/ASF-mediated enhancement of intron D splicing, although it binds to the FP element with lower specificity than SF2/ASF (19). To determine more precisely where SF2/ASF and hnRNP A1 bind within the FP element, we took advantage of the fact that both SF2/ASF and hnRNP A1 can be cross-linked to the E5/FP RNA sequence in the absence of additional protein factors.

In separate reactions, purified SF2/ASF and recombinant hnRNP A1 were UV cross-linked to the E5/FP RNA under splicing conditions, as previously reported (19). The UV cross-linked complexes were immunoprecipitated with the corresponding antibodies and the purified RNAs were then analyzed by primer extension after digestion of the protein with proteinase K. The data obtained with SF2/ASF and hnRNP A1 are shown in Figures 1A and 1B, respectively. The precise location of the
Cross-links was determined by comparison to a dideoxy sequencing ladder of untreated E5/FP RNA (Figures 1A and 1B, lanes 1-4), taking into account that reverse transcriptase terminates DNA synthesis at the nucleotide that immediately precedes a cross-link site (49). To identify bands due to secondary structure-mediated strong stops, untreated RNA was analyzed (lane 5). To assign bands due to RNA-RNA crosslinks or other photodamage, E5/FP RNA was UV-cross-linked in the absence of protein and reverse transcribed (lane 6). To rule out bands resulting from nicking of the RNA during the experiment, RNA was incubated in the presence of protein and immunoprecipitated but not UV-irradiated (lane 7). Finally, to identify specific protein-RNA cross-links, RNA was incubated in the presence of protein and then either reverse transcribed directly (lane 8) or immunoprecipitated and then reverse transcribed (lane 9). It was expected that specific cross-links would appear in lane 8 and be enriched in lane 9. Cross-links judged to be specific for SF2/ASF or hnRNP A1 were compiled and are shown in Figure 2. The relative size of the vertical bar over or under a specific nucleotide in Figure 2 reflects the degree to which a particular cross-link can be distinguished from background bands in the control lanes. The SF2/ASF cross-links are centered around the purine-rich sequence described previously (underlined in Figure 2; see ref. 17), whereas the hnRNP A1 cross-links are spread throughout the 3’ half of the FP sequence. The partial overlap of the SF2/ASF and hnRNP A1 cross-links
provided a potential explanation for how hnRNP A1 counteracts the SF2/ASF-mediated stimulation of intron D splicing. However, attempts to determine if this overlap is important for the antagonistic effects of SF2/ASF and hnRNP A1 were unsuccessful. Nevertheless, the fact that different bands are observed with SF2/ASF and hnRNP A1 further supports the conclusion that these bands are a result of specific cross-links to these proteins, and not an artifact of the procedure.

A 29-nucleotide sequence containing the SF2/ASF cross-links binds to SF2/ASF, but is not sufficient to maximally enhance intron D splicing. To determine if the cross-links identified above are indeed from a functional SF2/ASF binding site, a 29-nucleotide sequence containing the nucleotides that cross-link to SF2/ASF was either deleted from the bGH RNA substrate (ΔUV-XL, Figure 2) or inserted in place of the entire 115-nucleotide FP element (UV-XL, Figure 2). If SF2/ASF functional binding is confined to this region, it might be expected that ΔUV-XL would behave like the FP deletion (ΔFP), i.e., it would not splice in vitro, even with the addition of SF2/ASF (19,43), whereas the insertion mutant UV-XL would behave like the wild-type pre-mRNA. Figure 3 shows the results of this experiment. The wild-type bGH substrate spliced with moderate efficiency using the standard amount of nuclear extract (lane 1), whereas the FP-deleted substrate (ΔFP) failed to splice under these conditions (lane
4), as previously reported. Surprisingly, ΔUV-XL still spliced, albeit less efficiently (~50% of wild-type; lane 3), and UV-XL spliced poorly under these conditions (~20% of wild-type, lane 2). To test specifically for the requirement for SF2/ASF in splicing, the reactions were performed in dilute nuclear extract supplemented with optimal amounts of recombinant baculovirus-produced SF2/ASF (23,50). Under dilute nuclear extract conditions, the four substrates spliced very poorly (lanes 5, 7, 9, and 11). Addition of 0.125 µg of recombinant SF2/ASF led to increased splicing of both the wild-type substrate (lane 6) and the ΔUV-XL substrate (lane 10). However, the level of splicing of the ΔUV-XL substrate was consistently lower than that of the wild-type substrate in parallel reactions supplemented with a range of SF2/ASF concentrations (data not shown). As expected, the ΔFP substrate did not splice after supplementation with recombinant SF2/ASF (lane 12). Interestingly, the UV-XL substrate spliced upon supplementation of the dilute nuclear extract with recombinant SF2/ASF (lane 8), but at lower levels than the wild-type and ΔUV-XL substrates. This difference was consistent over a range of SF2/ASF concentrations (data not shown).

The question remains as to why UV-XL spliced so poorly in comparison to wild-type and ΔUV-XL substrates, given that cross-links to SF2/ASF were only observed within the 29-nucleotide (UV-XL) sequence. Perhaps the 29-nucleotide sequence is indeed a strong SF2/ASF binding
site which is rendered inefficient in splicing of the UV-XL substrate due to removal of other sequences required for splicing stimulation. These other sequences could be additional, weaker SF2/ASF binding sites not detected by the cross-link mapping assay or they could be binding sites for additional splicing factors, such as other SR proteins, required to stimulate splicing. To address this question, an electrophoretic mobility shift assay (EMSA) was developed to monitor SF2/ASF binding to the various bGH exon 5 sequences used in Figure 3. Figure 4 shows that SF2/ASF bound efficiently to RNA containing only the FP element (E5/FP, lane 2), but not to RNA containing an FP-deleted bGH exon 5 (E5/ΔFP, lane 8). This result is consistent with previous experiments showing that E5/FP, but not E5/ΔFP, serves as an efficient trans-competitor of intron D splicing and cross-links specifically to SF2/ASF (19,43). Interestingly, deletion of the 29-nucleotide sequence from E5/FP (E5/FP/ΔUV-XL) still allowed very efficient binding of SF2/ASF (Figure 4, lane 4). In contrast, exon 5 RNA containing the 29-nucleotide sequence replacing the entire FP sequence (E5/ΔFP/UV-XL) bound to SF2/ASF (lane 6) less efficiently than both wild-type E5/FP and E5/FP/ΔUV-XL RNAs (compare lane 6 with lanes 2 and 4). These results are consistent with the results in Figure 3 and suggest that, while the UV-cross-link/reverse transcription experiment (Fig. 1A) identified an important SF2/ASF binding site, there are additional SF2/ASF binding sites
in the FP sequence not detected by UV-cross-linking that are required for maximal enhancer activity.

**SF2/ASF binding sites are located throughout the FP sequence.**

To more precisely map the additional SF2/ASF binding sites present in the FP sequence, this region was divided into 14-17 nucleotide subfragments, which were inserted into the FP-deleted exon 5 (E5/ΔFP) in place of the FP sequence. These RNAs were used in the EMSA described above. The results of this experiment are shown in Figure 5. 125 ng of recombinant baculovirus SF2/ASF shifted 30% of the E5/ΔFP RNA (lane 1), while 92% of the E5/FP RNA is shifted by this amount of SF2/ASF (Figure 4, lane 2). This result is consistent with the basal levels of cross-linking to E5/ΔFP RNA observed previously (19,43). Interestingly, RNA containing subfragment no. 6 is shifted by only 19% (lane 7). This extent of binding represents a drop compared to E5/ΔFP RNA (30%), suggesting that insertion of subfragment no. 6 disrupted a E5/ΔFP SF2/ASF binding site that spans the region of the insertion, but that this FP 76-89 fragment does not bind SF2/ASF to any detectable levels. Therefore, we considered RNA containing subfragment no. 6 to provide a good baseline with which to compare the other FP subfragments. Using this assumption, the FP subfragments were divided into three groups. The first group consists of the FP subfragments (nos. 6 and 8) that show little or no binding to
SF2/ASF. These two subfragments are located in the 3’ end of the FP sequence. The second group consists of the FP subfragments (nos. 1, 3 and 7) that show intermediate levels of binding to SF2/ASF. Note that subfragment no. 3 contains one of the cross-links mapped in Figure 1A. The third group consists of the FP subfragments (nos. 2, 4, 5 and 9) that show the highest levels of binding to SF2/ASF. This group includes subfragment no. 4 which contains most of the cross-links mapped in Figure 1A, reinforcing the fact that the cross-links do represent an important SF2/ASF binding site. Most importantly, this experiment confirmed that there are multiple SF2/ASF binding sites in the FP sequence and, as a first approximation, defined where these binding sites are located.

In an attempt to correlate the binding of SF2/ASF to the FP sequence with ESE function, we designed 2’-O-methyl oligoribonucleotides complementary to different regions of bGH exon 5 and tested them for their ability to inhibit splicing in vitro. These modified oligoribonucleotides bind stably to complementary RNAs, the hybrids are resistant to RNase H cleavage that occurs in crude extract when antisense DNA is used, and splicing can be inhibited when important pre-mRNA elements are targeted (51-53). Eight 2’-O-methyl oligoribonucleotides complementary to the region of the mapped SF2/ASF cross-links (B3 and B4, Figure 6), other regions in the FP sequence (B1, B2, B5 and B6) or downstream of the FP sequence (B7 and B8) were incubated in in vitro splicing reactions of bGH
pre-mRNA substrates at a concentration of 0.5 µM each (Figure 6). At concentrations higher than 0.5 µM, we began to observe non-specific effects for some of the oligoribonucleotides, i.e. inhibitory effects that are not dependent on annealing of the oligoribonucleotide to the RNA substrate (data not shown). Because of the need to keep the oligoribonucleotide concentrations as low as possible in order to avoid non-specific effects, we found that, in contrast to some studies (51,52), inhibition was improved by pre-incubating the oligoribonucleotides with the splicing substrate for 15-30 minutes at 0°C prior to addition of the nuclear extract.

As can be seen in Figure 6, oligoribonucleotides B7 and B8 do not significantly inhibit bGH pre-mRNA splicing (lanes B7 and B8) which is consistent with the fact that E5/ΔFP RNA does not contain strong SF2/ASF binding sites (19). Oligoribonucleotide B6 also does not significantly affect splicing, consistent with the EMSA data in Figure 5 showing little or no SF2/ASF binding to subfragment no. 8, which is complementary to oligoribonucleotide B6. In addition, oligoribonucleotide B5, which spans a weak and a moderate SF2/ASF binding sites (FP subfragments 6 and 7), has only a minor effect on intron D splicing (lane 6). On the other hand, oligoribonucleotides B3 and B4 significantly inhibit bGH pre-mRNA splicing (lanes 4 and 5). These two oligoribonucleotides are complementary to the 29-nucleotide UV-XL sequence, and thus, it would be expected that they inhibit bGH pre-mRNA splicing by blocking specific SF2/ASF binding sites.
Indeed, a combination of these two oligoribonucleotides, which covers the entire region of SF2/ASF cross-links, almost completely inhibits bGH intron D splicing (not shown). Interestingly, oligoribonucleotide B2, which is complementary to a strong SF2/ASF binding site (FP subfragment no. 2), inhibits bGH pre-mRNA splicing only moderately. On the other hand, oligoribonucleotide B1, which is complementary to a moderate SF2/ASF binding site (FP subfragment no. 1), gives the strongest effect observed on bGH pre-mRNA splicing, along with oligoribonucleotide B3. As a control, oligoribonucleotides B2-B5 have no effect on β-globin, adenovirus major late (AdML), fushi-tarazu (ftz), or placental growth factor (PlGF) pre-mRNA splicing reactions (data not shown). The data in figures 5 and 6 support the conclusion that SF2/ASF binds to several functional sites within the FP sequence and that several functional sites are required for maximal ESE activity.
Discussion

Splicing of bGH intron D is dependent on the presence of a downstream ESE sequence and on the splicing factor SF2/ASF (19,42). SF2/ASF stimulates intron D splicing, presumably by binding to the ESE. A number of different ESEs have now been described in several genes, in which it is clear that SR proteins are involved in ESE-mediated splicing enhancement (19-28). However, little is known about the specific nucleotide contacts made by the SR proteins. To our knowledge, there are only three examples in which mapping of interactions between an SR protein and specific nucleotides within the ESE has been attempted (32,54,55). In one of these studies, chemical modification interference was used to map contacts between an SR protein and specific nucleotides (55), while in the other two studies, cross-links to an SR protein were mapped to one or more RNase-derived fragments in the immediate vicinity of a single labeled nucleotide introduced into the pre-mRNA (32,54).

In the case of bGH intron D, it was not clear exactly where SF2/ASF is binding and whether there are multiple binding sites. Though there was evidence to suggest that purine sequences are required (17,56), sequences surrounding the purine-rich motif also appear to be involved. In order to better ascertain where SF2/ASF binds in the FP sequence and to determine which ribonucleotides may be primarily involved in this interaction, we
took advantage of the fact that SF2/ASF cross-links specifically to the FP sequence even in the absence of other proteins (19). We reasoned that points of contact between SF2/ASF and the FP sequence could be determined by mapping cross-linking sites between them, thereby identifying the region(s) of SF2/ASF binding. This mapping was accomplished by reverse transcription of E5/FP RNA that had been cross-linked to SF2/ASF and immunoprecipitated by anti-SF2/ASF antibody mAb96. This strategy has been used to map RNA-RNA cross-links in other systems (49,57), however, to our knowledge, it has never been used to map protein-RNA cross-links. With this technique we were able to detect cross-links between SF2/ASF (or hnRNP A1) and specific ribonucleotides of the FP sequence (Figure 2) since reverse transcriptase terminates DNA synthesis at the nucleotide that immediately precedes a cross-link site (49). Two observations concerning this technique must be made here. The bands arising due to specific cross-links are not very pronounced relative to background. Therefore, it is possible that some specific cross-links were missed. Presumably, the reason we are able to detect this SF2/ASF binding site(s) in the FP sequence is because of its strength and/or specificity relative to non-specific background binding. While hnRNP A1 led to cross-links at both purine and pyrimidine residues, we only observed cross-links at pyrimidine residues with SF2/ASF. This difference probably arises because the formation of a cross-link depends on both the photoreactivity
of individual amino acid side chains and of nucleotide bases (58,59). Additionally, there is a preference for pyrimidines over purines in cross-linking (58). Thus, it is possible that SF2/ASF does in fact bind to the purine motif identified earlier (17,30), but that this interaction does not lead to formation of a cross-link.

Deletion of a 29-nucleotide sequence (UV-XL) containing this putative SF2/ASF binding site(s) led to only a 50% reduction in the SF2/ASF-mediated stimulation of intron D splicing as compared to wild-type bGH pre-mRNA (Figure 3). In addition, this sequence by itself functioned only as a moderate splicing enhancer, even though it clearly bound SF2/ASF (Figure 4). Although it is possible that these effects were caused by the deletion mutants affecting the secondary structure of bGH pre-mRNA, we do not believe this to be the case. Extensive analyses of bGH mRNA secondary structure, using Zuker’s computer algorithm (60-62) in combination with previous experimental data obtained with structure-sensitive probes (for example, RNAses T1 and V1) has not suggested any obvious alterations. Instead, we favor the hypothesis that additional SF2/ASF binding sites are required for maximal enhancer activity. The presence of additional SF2/ASF binding sites in the FP sequence was confirmed by the fact that ∆UV-XL RNA was significantly shifted by SF2/ASF in the EMSA (Figure 4). In fact, ∆UV-XL RNA shifted even better than wild-type FP RNA. This observation raises the possibility that deletion
of the UV-XL sequence activated a new/cryptic SF2/ASF binding site which, together with the normal SF2/ASF binding sites in the FP sequence, compensated for the loss of the UV-XL binding site. Apparently this compensation results in even better SF2/ASF binding. However, if this assumption is true, this new/cryptic SF2/ASF binding site does not appear to be functional, since the ΔUV-XL pre-mRNA never splices better than the wild-type bGH pre-mRNA (Figure 3). Thus, the precise location of SF2/ASF binding sites within the enhancer and the interrelationship between the various SF2/ASF binding sites appear to be important factors in determining enhancer function.

In an attempt to locate the additional SF2/ASF binding sites, the FP sequence was divided into similar sized subfragments of 14-17 nucleotides. These subfragments were inserted into bGH exon 5 in place of the entire FP sequence. EMSA analysis of these FP subfragments showed that they could be divided into three groups: those that showed little or no binding to SF2/ASF (subfragment nos. 6 and 8), those that showed moderate binding to SF2/ASF (subfragment nos. 1, 3 and 7), and those that showed strong binding to SF2/ASF (subfragment nos. 2, 4, 5 and 9). The SF2/ASF cross-links that were mapped in Figure 1A reside in subfragments that showed moderate binding (subfragment no. 3 contains 1 cross-link; Figure 5) and strong binding (subfragment no. 4 contains 3 cross-links) to SF2/ASF. The fact that SF2/ASF binds strongly to
subfragment no.4, which contains most of the mapped SF2/ASF cross-links, shows that there is good agreement between these two completely different methods (cross-link mapping and EMSA) for determining SF2/ASF binding sites.

In an attempt to correlate the binding of SF2/ASF to the FP sequence with ESE function, antisense 2'-O-methyl oligoribonucleotides were designed that were complementary to several of the FP subfragments (Figure 5) or to sequences downstream of the FP region. These 2'-O-methyl oligoribonucleotides had varying effects on bGH pre-mRNA splicing that were generally consistent with the observed binding of SF2/ASF to the FP subfragments. For example, oligoribonucleotides B6-B8 did not significantly affect splicing, oligoribonucleotide B5 inhibited splicing only slightly and oligoribonucleotides B3 and B4 strongly inhibited splicing, consistent with the binding of SF2/ASF as determined by EMSA (cf. Figures 5 and 6). The effect of oligoribonucleotides B3 and B4 on splicing is consistent with the fact that they block the region of mapped SF2/ASF cross-links (Figure 2). This region also contains the purine motif previously shown to be part of the bGH ESE (17) as well as the best match to one of the SF2/ASF binding SELEX winner sequences (RGAAGAAC; see ref. 30). Interestingly, oligoribonucleotide B1 inhibits splicing more efficiently than oligoribonucleotide B2, even though FP subfragment no.1 contains a moderate SF2/ASF binding site and FP subfragment no.2 contains a strong
binding site (Figure 5). This result is consistent with the observation that
the best binding sites are not necessarily the best functional sites (35).
Indeed, while FP subfragments nos.1 and 2 both contain good matches to
the optimal SF2/ASF binding site determined by functional SELEX
(SRSASGA, where S represents G or C, and R represents purine; see ref. 35),
subfragment no. 1 contains a better match (GUGACGA) to this SELEX
sequence than subfragment no. 2 (CUCAAGA; the comparison is based on
the high-score motif analysis used in ref. 35). This may explain why
oligoribonucleotide B1 has a stronger inhibitory effect than
oligoribonucleotide B2. Thus, the functional activity of SF2/ASF in bGH
intron D splicing as determined by the antisense 2'-O-methyl
oligoribonucleotide inhibition experiment (Figure 6) correlates with
binding of SF2/ASF to the various FP subfragments (Figure 5), though the
best binding sites are not necessarily the best functional sites.

In summary, we have defined multiple sites of functional SF2/ASF
binding in the bGH ESE. It appears that these multiple SF2/ASF binding
sites are all involved in enhancer complex formation and stimulation of
intron D splicing. Thus, the bGH ESE has similarities to the ESE found in the
doublesex female-specific exon, in which binding of SR proteins to multiple
enhancer elements is required for maximal enhancer activity and in which
multiple elements have additive enhancement effects (20,32,63). We were
able to detect contacts between SF2/ASF and specific ribonucleotides
within one of these binding sites using a UV-cross-link mapping strategy. The remaining functional binding sites were identified using two different strategies: EMSA and antisense 2'-O-methyl oligoribonucleotide inhibition experiments. Thus, multiple strategies were required to gain a clearer understanding of SF2/ASF binding to the bGH ESE, one of the earliest steps in splicing of bGH intron D. It appears that this ESE is a composite of multiple SF2/ASF functional binding sites that have additive effects. This arrangement of elements results in a robust ESE that is highly resistant to mutational inactivation.
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Footnotes

Abbreviations:
bGH, bovine growth hormone;
ESE, exonic splicing enhancer;
FP, Fsp I-Pvu II;
ΔFP, Fsp I-Pvu II deletion;
EMSA, electrophoretic mobility shift assay;
UV, ultraviolet.

Acknowledgements

We thank Joseph Bokar, Melody Stallings-Mann and Mary Eileen Shambaugh for critically reading the manuscript. We thank Hong-Xiang Liu and Luca Cartegni for providing the high-score functional motif analysis of bGH SF2/ASF binding sites. This work was supported by Public Health Service grant DK32770 from the National Institute of Health to F.M.R. A.M. and A.R.K. were supported in part by National Institute of Health grant GM42699.

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Figure Legends

FIG. 1. Mapping of specific SF2/ASF and hnRNP A1 UV cross-links to the bGH FP sequence by reverse transcription. (A) RNA derived from the FP splicing enhancer sequence in exon 5 of bGH pre-mRNA (E5/FP RNA) was incubated with purified SF2/ASF under splicing conditions. Following UV-irradiation and immunoprecipitation, RNAs were reverse transcribed using an oligonucleotide that hybridizes to the 3' end of the FP sequence (FP-17mer). Bullets to the right of the gel represent specific SF2/ASF cross-links. The bar to the left of the gel indicates the location of the purine motif previously shown to be part of the bGH ESE (17). The first five lanes represent reverse transcription of E5/FP RNA that was untreated, in the presence of: lane 1, ddGTP; lane 2, ddATP; lane 3, ddTTP; lane 4, ddCTP; lane 5, buffer. Lanes 6-9 represent reverse transcription of E5/FP RNA that was treated in various ways. Presence or absence of SF2/ASF protein, UV-cross-linking and immunoprecipitation is indicated with + or - marks above each lane, respectively. (B) E5/FP RNA was incubated with recombinant hnRNP A1 under splicing conditions. Lanes are the same as in (A), with the exception that hnRNP A1 protein and specific antibody was used instead of SF2/ASF. Bullets and bracket to the right of the gel represent specific hnRNP A1 cross-links.
FIG. 2. Location of specific SF2/ASF and hnRNP A1 cross-links within the FP region. Specific cross-links identified in Figure 1 are shown superimposed on the FP sequence. SF2/ASF cross-links are shown above, and hnRNP A1 cross-links below, the FP sequence. The size of the vertical bar over a specific ribonucleotide reflects the degree to which a particular cross-link can be distinguished from background bands in the control lanes. UV-cross-links near the 3′ end of the FP sequence were identified by performing the experiment with E5/FP RNA synthesized from plasmid E5/FP linearized at a Pvu II site in the vector and reverse transcribed with an oligonucleotide complementary to the T3 promoter (T3 primer). The underlined nucleotides represent the purine motif previously shown to be part of the bGH ESE (17). The lines above the sequence indicate the nucleotides which are included in constructs UV-XL and ΔUV-XL.

FIG. 3. The region that cross-links to SF2/ASF has weak enhancer activity, whereas exon 5 lacking this region has moderate enhancer activity. Wild-type, UV-XL, ΔUV-XL, and ΔFP bGH pre-mRNAs were incubated in the presence of a standard amount of nuclear extract (48%, lanes 1-4) or in dilute nuclear extract (24%) supplemented with either 0 µg (lanes 5, 7, 9, and 11) or 0.125 µg (lanes 6, 8, 10, and 12) of baculovirus-produced recombinant SF2/ASF: wild-type pre-mRNA, lanes
5+6; UV-XL pre-mRNA, lanes 7+8; ∆UV-XL pre-mRNA, lanes 9+10; ∆FP pre-mRNA, lanes 11+12. S, pre-mRNA substrates; P, spliced products; L, lariats containing the intron and the exon; I, lariats containing the intron only; E, free 5' exon. ∆UV-XL and ∆FP are bGH pre-mRNAs in which the 29-nucleotide sequence or the entire 115-nucleotide FP sequence have been deleted, respectively. UV-XL is a bGH pre-mRNA in which the 29-nucleotide sequence replaces the entire 115-nucleotide FP sequence.

FIG. 4. SF2/ASF binds efficiently to both the UV-XL and ∆UV-XL sequences. 3 fmol of E5/FP, E5/∆FP, E5/FP/∆UV-XL or E5/∆FP/UV-XL RNA substrate was incubated with either 0 µg (lanes 1, 3, 5, and 7) or 0.125 µg (lanes 2, 4, 6, and 8) of baculovirus-produced recombinant SF2/ASF at 30°C for 30 min and resolved on non-denaturing 6% polyacrylamide gels containing 50 mM Tris, 50 mM glycine. E5/FP, lanes 1 and 2; E5/FP/∆UV-XL, lanes 3 and 4; E5/∆FP/UV-XL, lanes 5 and 6; E5/∆FP, lanes 7 and 8. S, shifted RNA; F, Free RNA.

FIG. 5. SF2/ASF binds to multiple sequences within the FP region. 3 fmol of E5/∆FP substrate, with or without insertion of FP subfragments nos. 1-9, was incubated with 0.125 µg of baculovirus-produced recombinant SF2/ASF at 30°C for 30 min and resolved on non-denaturing 6% polyacrylamide gels containing 50 mM Tris, 50 mM glycine (shown on
the left). E5/ΔFP without an insert, lane ΔFP; insertion of subfragments nos.1-9 are shown in lanes 1-9, respectively. Quantification of the shifted and unshifted RNAs was obtained using Multi-Analyst/MacIntosh software (Bio-Rad Laboratories) and is expressed as the percentage (%) of shifted RNA/total RNA (shown on the right). The values shown in the bar graph represent the mean±S.D. from 4 experiments, with the exception of E5/FP (n=3). The sequences of each FP subfragment inserted into E5/ΔFP are shown at the bottom of the figure superimposed on the FP sequence.

FIG. 6. **Antisense 2'-O-methyl oligoribonucleotides specifically inhibit splicing of bGH.** Oligoribonucleotides B1 through B8 were added at a final concentration of 0.5 µM each to *in vitro* splicing reactions with bGH pre-mRNA. The oligoribonucleotides were pre-incubated with the pre-mRNAs for 20 min at 0°C prior to addition of the nuclear extract. Spliced products were separated on 8% denaturing polyacrylamide gels (shown on the left). *Symbols* are as described in the Fig. 3 legend. Quantification of the bGH spliced products (shown on the right) was obtained using a PhosphorImager (Molecular Dynamics Inc.), and splicing efficiency was expressed as the percentage (%) of spliced products/total bGH mRNA. The values shown in the bar graph represent the mean±S.D. from at least 5 experiments (for no oligo control, n=7; for oligoribonucleotides B2-B5, n=6). The locations of oligoribonucleotides B1 through B6 are shown at the
bottom of the figure superimposed on the FP sequence. The locations of oligoribonucleotides B7 and B8 are not shown, but they are complementary to separate exon 5 sequences downstream of the FP region.
A
Sequencing ladder
CTAG - + + +
SF2/ASF
Immunoprecipitation
UV-cross-linking

B
Sequencing ladder
CTAG - + + +
hnRNP A1
Immunoprecipitation
UV-cross-linking
Mapping the SF2/ASF Binding Sites in the Bovine Growth Hormone Exonic Splicing Enhancer

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J. Biol. Chem. published online July 3, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001126200

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