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SR proteins ASF/SF2 and 9G8 interact to activate enhancer-dependent intron D splicing of bovine growth hormone pre-mRNA in vitro

XINLAN LI,[1,3] MARY EILEEN SHAMBAUGH,[1] FRITZ M. ROTTMAN,[1] and JOSEPH A. BOKAR[1,2]

[1]Department of Molecular Biology and Microbiology, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4960, USA
[2]Department of Medicine, Case Western Reserve University, School of Medicine, Cleveland, Ohio 44106-4960, USA

ABSTRACT

The alternative splicing of the last intron (intron D) of bovine growth hormone (bGH) pre-mRNA requires a downstream exonic splicing enhancer (FP/ESE). The presence of at least one SR protein has been shown to be essential for FP/ESE function and splicing of intron D in in vitro splicing assays. However, in vitro reconstitution of splicing using individual purified SR proteins may not accurately reflect the true complexity of alternative splicing in an intact nucleus, where multiple SR proteins in varying amounts are likely to be available simultaneously. Here, a panel of recombinant baculovirus-expressed SR proteins was produced and tested for the ability to activate FP/ESE-dependent splicing. Individual recombinant SR proteins differed significantly in their activity in promoting intron D splicing. Among the recombinant SR proteins tested, SRp55 was the most active, SC35 showed very little activity, and ASF/SF2 and 9G8 individually had intermediate activity. At least one SR protein (ASF/SF2) bound to the FP/ESE with characteristics of a cooperative interaction. Most interestingly, low concentrations of ASF/SF2 and 9G8 acted synergistically to activate intron D splicing. This was due in part to synergistic binding to the FP/ESE. Splicing of bGH intron D is inherently complex, and is likely controlled by an interaction of the FP/ESE with several *trans*-acting protein factors acting both independently and cooperatively. This level of complexity may be required for precise control of alternative splicing by an exon sequence, which simultaneously is constrained to maintain translational integrity of the mature mRNA.

Keywords: 9G8, ASF/SF2, alternative splicing, bovine growth hormone, splicing enhancer, SR protein

INTRODUCTION

The removal of intervening sequences (introns) from pre-mRNAs takes place in large, dedicated ribonucleoprotein complexes called spliceosomes (reviewed in Reed & Palandjian, 1997; Nilsen, 1998; Staley & Guthrie, 1998). The productive assembly of spliceosomes on pre-mRNA requires the accurate identification of intron/exon borders by multiple *trans*-acting factors. Alternative splicing involves the varied inclusion of pre-mRNA sequences within the mature mRNA via intron retention, exon skipping, or the use of alternative 5′ and 3′ splice sites. A number of studies have described enhancer sequences, usually located in adjoining exons (exonic splicing enhancers, ESEs), that influence 5′ and 3′ splice site selection and/or splicing efficiency (reviewed in Fu, 1995; Manley & Tacke, 1996). Sequence elements within introns, as well as exon and intron length, also play roles in splice-site selection and splicing efficiency (Dominski & Kole, 1991; Sterner & Berget, 1993; Lou et al., 1995; Ryan & Cooper, 1996; Min et al., 1997). Thus, efficient and accurate splicing results from a complicated interaction of *cis*-acting elements with numerous *trans*-acting proteins and snRNAs in a coordinated fashion.

A general model for the function of splicing enhancers has emerged. A splicing enhancer serves as a binding site for one or more arginine/serine (SR)-rich proteins that can then activate a weak 5′ or 3 splice site of an adjacent intron. Splicing enhancers range from single SR protein-binding sites to clusters of binding sites for multiple identical or nonidentical SR proteins (Hertel & Maniatis, 1998). Initial examples of splicing enhancers focused on simple purine-rich elements that were fre-

Reprint requests to: Joseph A. Bokar, Department of Molecular Biology and Microbiology, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4960, USA; e-mail: jab5@po.cwru.edu.
[3]Current address: Novartis Agribusiness Biotechnology Research Inc., 3054 Cornwallis Road, Research Triangle Park, North Carolina 27709, USA.

quently found as short repeats (Watakabe et al., 1993; Xu et al., 1993; Dirksen et al., 1994; Tanaka et al., 1994). However, splicing enhancers are not restricted to purine sequences (Tian & Kole, 1995; Coulter et al., 1997; Tacke et al., 1997; Liu et al., 1998; Mayeda et al., 1999; Schaal & Maniatis, 1999). They are associated with both constitutive as well as alternatively utilized (regulated) splice sites. The strength of a splicing enhancer appears to be determined by several factors including the number of SR protein-binding sites, the relative activities of their cognate SR proteins, and the distance between the enhancer sequence and the intron (Graveley & Maniatis, 1998; Hertel & Maniatis, 1998).

SR proteins are considered essential splicing factors, as splicing does not occur in vitro in cytoplasmic S100 extract until at least one SR protein is added (Krainer et al., 1990; Ge et al., 1991; Zahler et al., 1992; Screaton et al., 1995). In addition to their roles as essential splicing factors, SR proteins are regulators of splice-site selection and alternative splicing (reviewed in Fu, 1995; Chabot, 1996; Manley & Tacke, 1996). SR proteins act early in spliceosome assembly, perhaps by facilitating the recognition of a functional 5′ splice site and/or complex formation at the 3′ splice site, and by facilitating bridging interactions across an intron or exon (Wu & Maniatis, 1993; Berget, 1995; Fu, 1995; Kennedy et al., 1998; Hertel & Maniatis, 1999). In the case of enhancer-dependent splicing, SR proteins bound to the enhancer are thought to facilitate the recruitment of the splicing machinery to adjacent splice sites, possibly by interacting with additional spliceosome components such as U1 snRNP, U2AF, and other SR proteins (Hertel & Maniatis, 1998).

The alternative splicing of bovine growth hormone (bGH) pre-mRNA has been used as a model system to determine the role of SR protein-splicing enhancer interactions in splice-site activation (Hampson et al., 1989; Sun et al., 1993a, 1993b; Dirksen et al., 1994, 1995). The fourth intron (intron D) of bGH pre-mRNA is variably retained or excised in mature bGH mRNA in both in vivo and in vitro splicing models. Intron D is bordered by weak splice sites, and its removal depends on a splicing enhancer present in an *Fsp*I-*Pvu*II restriction fragment located downstream from the intron D 3′ splice site. This splicing enhancer has been termed the FP/ESE. The SR protein ASF/SF2 was shown to bind to the FP/ESE and promote intron D splicing, presumably as a result of this interaction. A purine-rich sequence (GGAAG) that also binds SR protein could functionally replace the FP/ESE in a transfection assay.

Although a defined sequence with specificity for a single SR protein is sufficient to function as a splicing enhancer (Tacke & Manley, 1995; Tacke et al., 1997), splicing enhancers often are recognized by combinations of different SR proteins (Lavigueur et al., 1993; Staknis & Reed, 1994; Ramchatesingh et al., 1995; Yeakley et al., 1996). Likewise, the FP/ESE contains several subregions that individually act as splicing enhancers (Dirksen et al., 1995). It is not clear whether FP/ESE acts solely through the independent interaction of several SR proteins with enhancer sequences, or also involves some form of cooperative interaction of multiple SR proteins. In this study, we test the interaction of a variety of recombinant SR (rSR) proteins with the FP/ESE to better understand how a splicing enhancer functions. We show that cooperative binding of multiple molecules of an individual SR protein, as well as synergistic binding between different SR proteins, can contribute to the overall activity of the FP/ESE.

RESULTS

SR proteins are necessary but not sufficient to activate bGH FP/ESE-dependent intron D splicing in S100 extract

Schematic representations of the splicing substrate and RNA gel mobility shift substrate used in this study are shown in Figure 1A. Previous studies have shown that the bGH FP/ESE sequence is required for splicing of intron D, both in transfected cells and in in vitro assays (Hampson et al., 1989; Sun et al., 1993a). ASF/SF2 specifically binds to the 115-nt FP/ESE and promotes intron D splicing when supplemented in nuclear extract (Sun et al., 1993b). Although the removal of intron D in limiting nuclear extracts is at least partially dependent on SR proteins, this system cannot be used to assess the unique activity of an individual SR protein because nuclear extract contains multiple SR proteins in significant amounts.

HeLa cell cytoplasmic S100 extract is a splicing-deficient fraction that contains only trace amounts of SR proteins. Most constitutive and some alternative splicing events can occur in S100 extract upon the addition of exogenous SR proteins. An in vitro splicing assay was performed using bGH-4D5 and S100 fraction combined with SR proteins purified from bovine pituitary (Fig. 1B) or with SR proteins purified from HeLa cells (data not shown). S100 plus SR proteins did not splice bGH-4D5. However, the addition of NF20/40 (a 20–40% ammonium sulfate fraction of nuclear extract) to S100 and SR proteins activated splicing of Intron D. This indicates that a splicing factor(s), in addition to SR protein, is limiting in S100 extract and is necessary for bGH FP/ESE-dependent intron D splicing. Western blot analysis using a monoclonal antibody (mAb104) that specifically recognizes SR proteins (Roth et al., 1990) confirmed that NF20/40 contains only trace amounts of SR proteins (data not shown). These results are similar to those observed by Tacke and Manley (1995) using the synthetic splicing substrate, GN. Thus, the S100 extract supplemented with NF20/40 represents an SR protein-dependent system to study the specificity of SR proteins in activating bGH intron D splicing.

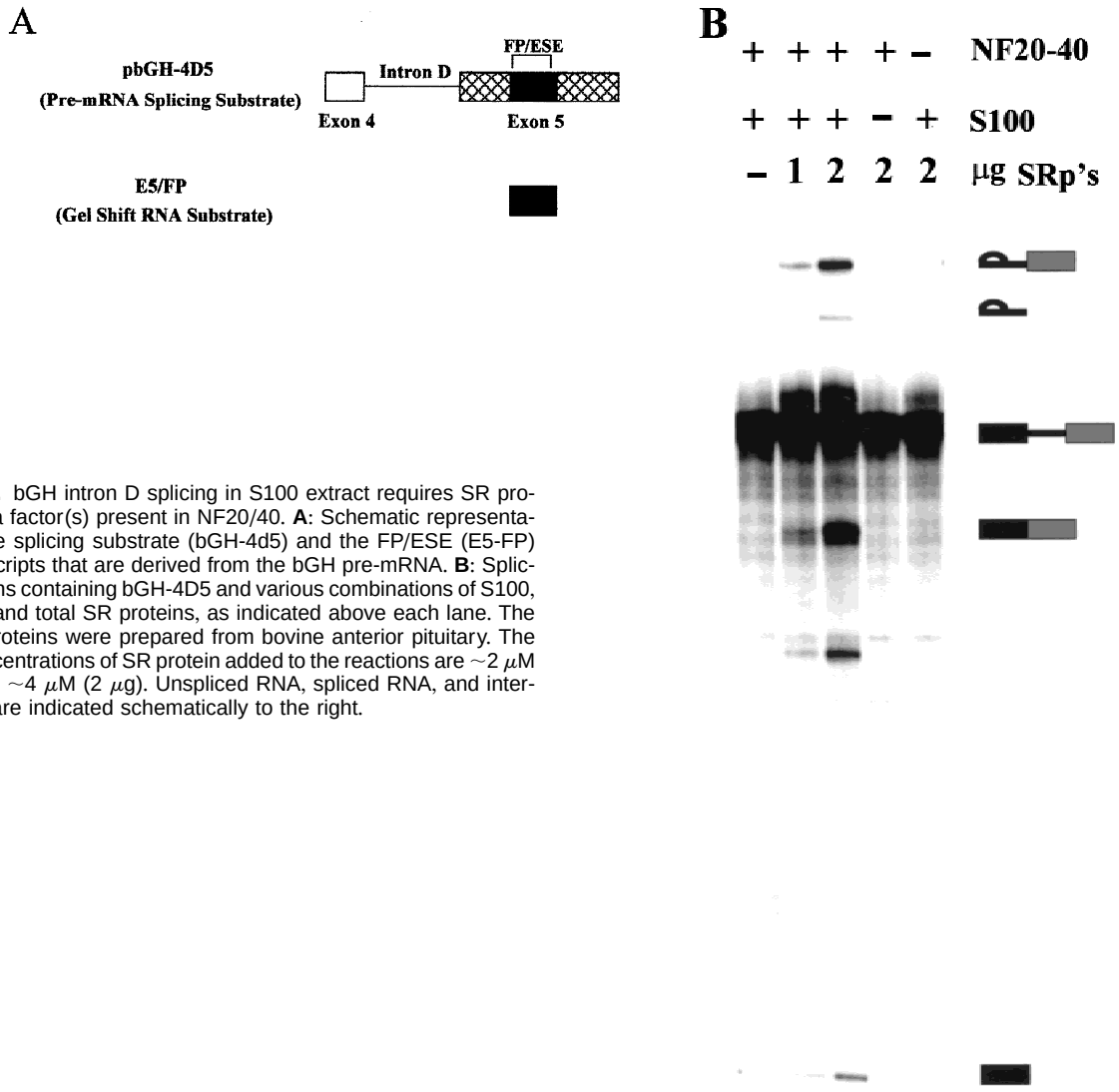

FIGURE 1. bGH intron D splicing in S100 extract requires SR proteins and a factor(s) present in NF20/40. **A**: Schematic representations of the splicing substrate (bGH-4d5) and the FP/ESE (E5-FP) RNA transcripts that are derived from the bGH pre-mRNA. **B**: Splicing reactions containing bGH-4D5 and various combinations of S100, NF20/40, and total SR proteins, as indicated above each lane. The total SR proteins were prepared from bovine anterior pituitary. The actual concentrations of SR protein added to the reactions are ~2 μM (1 μg) and ~4 μM (2 μg). Unspliced RNA, spliced RNA, and intermediates are indicated schematically to the right.

The activities of individual SR proteins differ in promoting bGH FP/ESE-dependent intron D splicing

Recombinant ASF/SF2, SC35, 9G8, and SRp55 were used to investigate the specificity of FP/ESE recognition. Six-histidine-tagged recombinant SR proteins were expressed using the BacPAK Baculovirus Expression system (Clontech), and purified using a Ni-agarose affinity resin. This system was chosen because the recombinant proteins could be purified free from endogenous SR proteins using an affinity technique that does not depend on characteristics of the SR protein itself, and because typical eukaryotic posttranslational modifications would be added. The individual rSR protein preparations were essentially homogeneous as assessed by SDS-PAGE stained with Coomassie blue (Fig. 2A). The activity of each rSR protein was assayed for the ability to promote splicing of a human β-globin pre-mRNA in S100 extract (Fig. 2B). The rSR proteins have roughly comparable activity in splicing human β-globin pre-mRNA. This is consistent with reports in the literature that many SR proteins are functionally redundant in stimulating constitutive splicing (Fu, 1995; Kramer, 1996; Manley & Tacke, 1996).

Individual rSR proteins were then tested for the ability to activate bGH intron D splicing in S100 extract supplemented with NF20/40 (Fig. 3, left panel). The addition of SRp55 strongly stimulated bGH intron D splicing. In contrast, ASF/SF2 and 9G8 were less active, and SC35 showed almost no activity. Thus, the individual rSR proteins differed significantly in their ability to promote bGH FP/ESE-dependent intron D splicing.

Synergistic activity between ASF/SF2 and 9G8, but not among other SR protein combinations, was observed for bGH FP/ESE-dependent intron D splicing

Our previous results indicated that purified human ASF/SF2 could promote bGH FP/ESE-dependent intron D

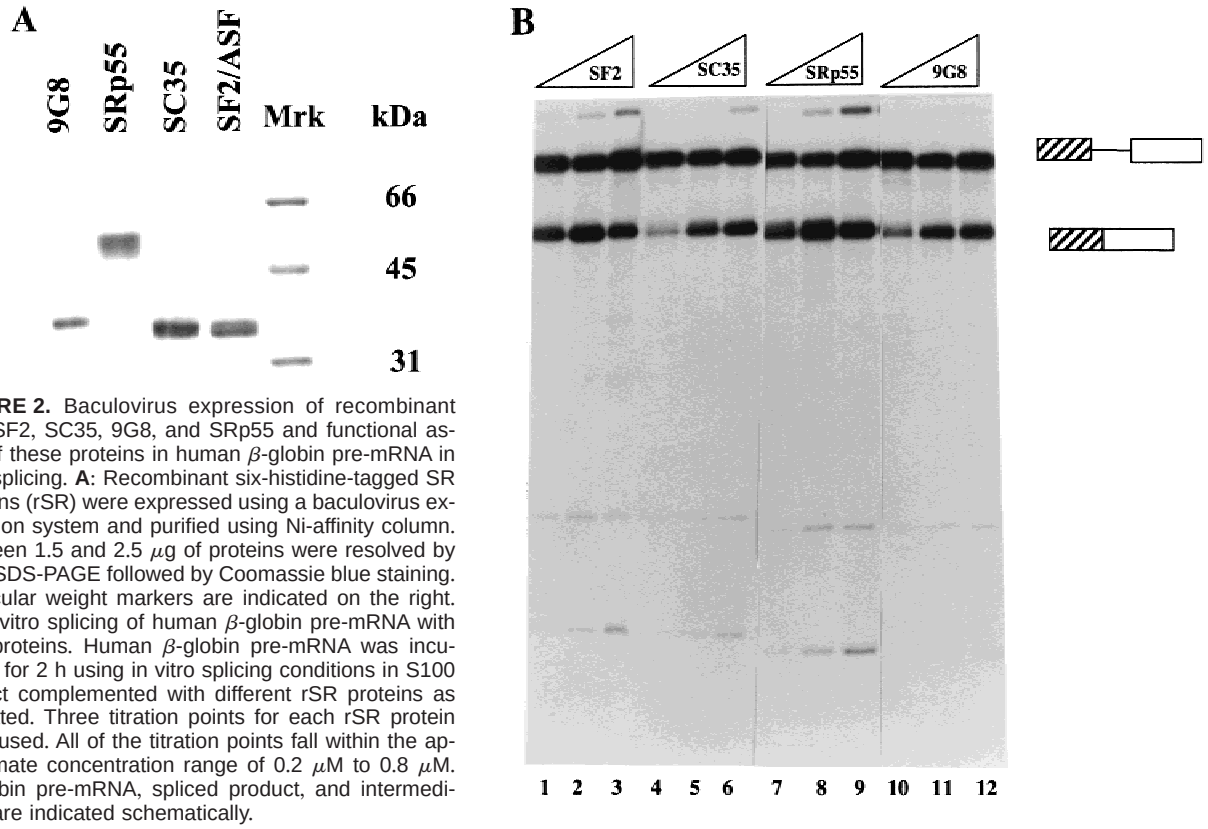

FIGURE 2. Baculovirus expression of recombinant ASF/SF2, SC35, 9G8, and SRp55 and functional assay of these proteins in human β-globin pre-mRNA in vitro splicing. **A**: Recombinant six-histidine-tagged SR proteins (rSR) were expressed using a baculovirus expression system and purified using Ni-affinity column. Between 1.5 and 2.5 μg of proteins were resolved by 12% SDS-PAGE followed by Coomassie blue staining. Molecular weight markers are indicated on the right. **B**: In vitro splicing of human β-globin pre-mRNA with rSR proteins. Human β-globin pre-mRNA was incubated for 2 h using in vitro splicing conditions in S100 extract complemented with different rSR proteins as indicated. Three titration points for each rSR protein were used. All of the titration points fall within the approximate concentration range of 0.2 μM to 0.8 μM. β-Globin pre-mRNA, spliced product, and intermediates are indicated schematically.

splicing efficiently when assayed by supplementation in HeLa cell nuclear extract (Sun et al., 1993b). We also tested the effect of the recombinant ASF/SF2 on intron D splicing in HeLa nuclear extract, and found that it efficiently promoted intron D excision (data not shown). In contrast, fairly limited activity was observed for recombinant ASF/SF2 in splicing bGH pre-mRNA when assayed in S100 extract supplemented with NF20/40 (Fig. 3, lanes 2–5). An important difference between these two systems is that nuclear extract contains all essential factors including SR proteins, whereas NF20/40-supplemented S100 extract contains only trace amounts of SR proteins. We hypothesized that optimal activity of ASF/SF2 in bGH intron D splicing requires an additional SR protein(s).

To formally test this hypothesis, various combinations of ASF/SF2, SC35, 9G8, and SRp55 were assayed for their effect on bGH intron D splicing in S100 extract supplemented with NF20/40 (Fig. 3, right panel). The combination of ASF/SF2 and 9G8 led to a significant stimulation of splicing at concentrations where each rSR protein individually had minimal activity (i.e., the lowest concentrations tested for each rSR protein in the left panel of Fig. 3). This level of activity appears, for two reasons, to be the result of a synergistic interaction between ASF/SF2 and 9G8. First, the splicing activity observed in the presence of both proteins is greater than the combined activities of each protein added separately at the same concentrations (Fig. 3, compare lane 19 to lanes 2 and 6). Second, the activity obtained in the presence of both proteins at a given concentration is higher than the activities observed with either protein at twice their concentration (Fig. 3, compare lane 19 to lanes 3 and 7). These results suggest some form of synergistic enhancement of splicing over and above an effect of total rSR protein concentration. We did not observe synergistic activity between ASF/SF2 and SC35, ASF/SF2 and SRp55, 9G8 and SRp55, SC35 and SRp55, or 9G8 and SC35. Thus, among the combinations of the four rSR proteins tested, only ASF/SF2 and 9G8 showed synergistic activity in bGH FP/ESE-dependent intron D splicing.

A more extensive study of the synergistic activity of ASF/SF2 and 9G8 in bGH intron D splicing was performed using various amounts of ASF/SF2 and a low fixed amount of 9G8 (Fig. 4, left panel). No splicing of intron D is detectable with two low concentrations of 9G8 (0.23 and 0.46 μM) in S100 extract supplemented with NF20/40. Likewise, the extent of splicing seen with the two lowest concentrations of ASF/SF2 (0.12 and 0.23 μM) is negligible. However, when the lowest concentration of 9G8 (0.23 μM) is combined with various concentrations of ASF/SF2 (0.12–0.46 μM), dramatic stimulation of intron D splicing is observed. Interestingly, this synergistic activity is more obvious at the lower ASF/SF2 concentrations. Similar results are apparent in Figure 4 (right panel), with significant splicing occurring with the combination of 0.23 μM ASF/SF2

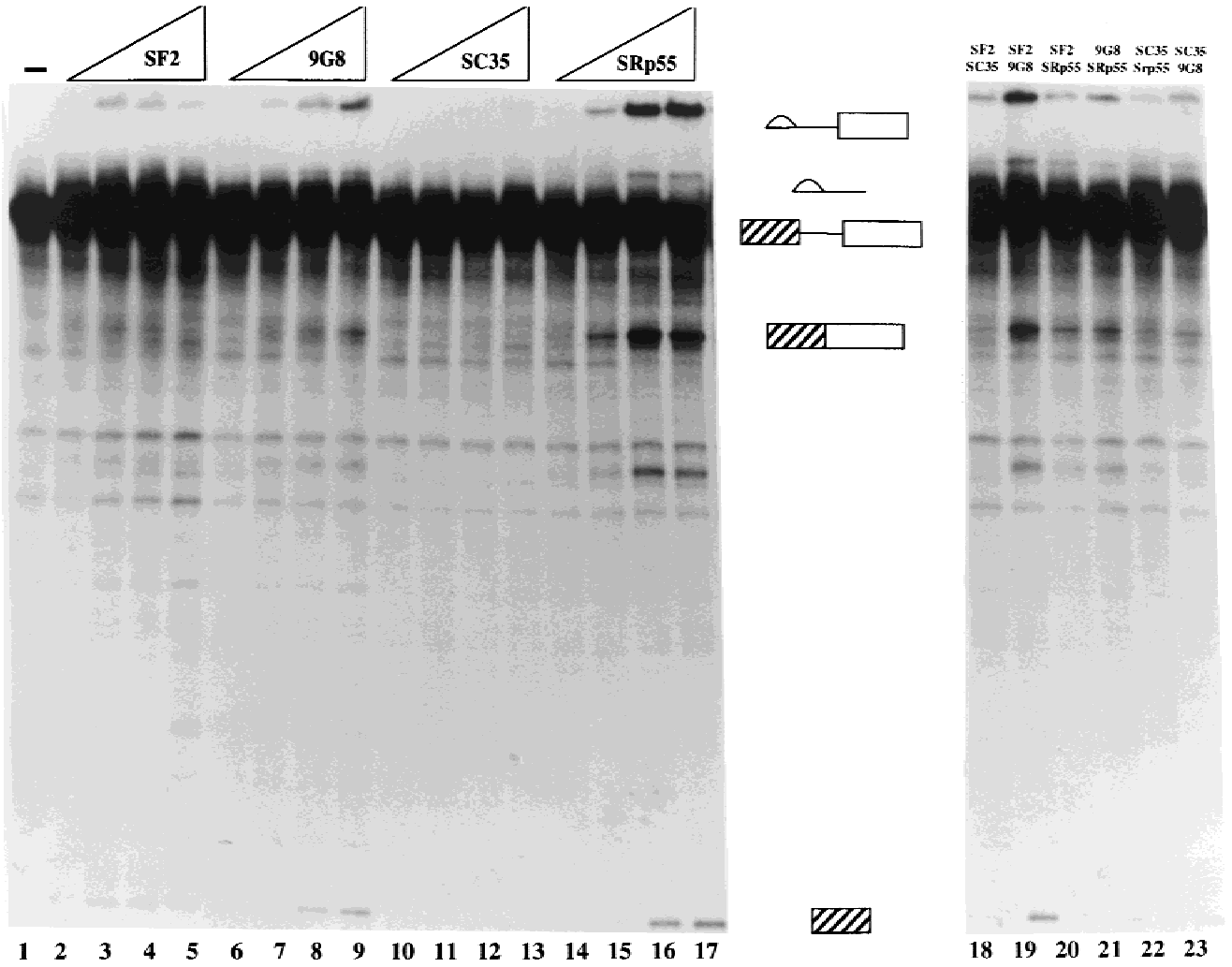

FIGURE 3. The activities of individual SR proteins differ in their ability to promote splicing of bGH intron D. bGH pre-mRNA was incubated using in vitro splicing conditions with NF20/40 supplemented S100 extract alone (lane 1) or complemented with different individual rSR proteins (lanes 2–17) or various combinations of two rSR proteins (lanes 18–23) as indicated. In assays using individual rSR proteins (lanes 2–17), four titration points were used. The amounts of rSR proteins were determined based upon their comparable activity with human β-globin pre-mRNA in a standard S100 complementation assay. In every case, the amount of SR protein added falls within the approximate concentration range of 0.1 μM to 0.8 μM. In studies using various combinations of two rSR proteins (lanes 18–23), the amount of each rSR protein used was equivalent to the lowest amounts (i.e., the initial titration points) in the left panel of the figure, and are as follows: SF2: 0.23 μM; 9G8: 0.23 μM; SC35: 0.23 μM; and SRp55: 0.1 μM. For example, lane 19 compares with lanes 2 +6. Pre-mRNA, spliced product, and intermediates are indicated schematically.

and 0.23 μM 9G8 (Fig. 4, lane 9). The inclusion of additional 9G8 (0.46 μM, Fig. 4, lane 10) only slightly increased the level of splicing activity beyond the level seen with 0.23 μM. However, in the absence of ASF/SF2, a 9G8 concentration of 0.92 μM is needed for significant splicing activity (Fig. 4, lanes 11 and 12), again illustrating the increased sensitivity (functional synergism) of the combination of these SR proteins as compared to ASF/SF2 or 9G8 individually.

ASF/SF2 and 9G8 bind synergistically to bGH FP/ESE

Native gel mobility shift assays were employed to test whether the synergistic enhancement of splicing could be attributed to synergistic binding of recombinant ASF/SF2 and 9G8 to bGH FP/ESE. A constant amount of RNA (5 fmol) was incubated with ASF/SF2 in increasing concentrations over a range of 0–96 nM. A dominant band and at least one minor band, corresponding to protein/RNA complexes, were detected (Fig. 5A, left panel). The fraction of RNA bound in the protein/RNA complexes was determined by PhosphorImager analysis. The mean results of three separate experiments are plotted (Fig. 5A, right panel). The curve has a striking sigmoid shape, which is indicative of multiple ASF/SF2 molecules binding to the RNA in a cooperative fashion. The apparent K_d for the interaction is approximately 60 nM, which is in excellent agreement with the affinity reported for other SR

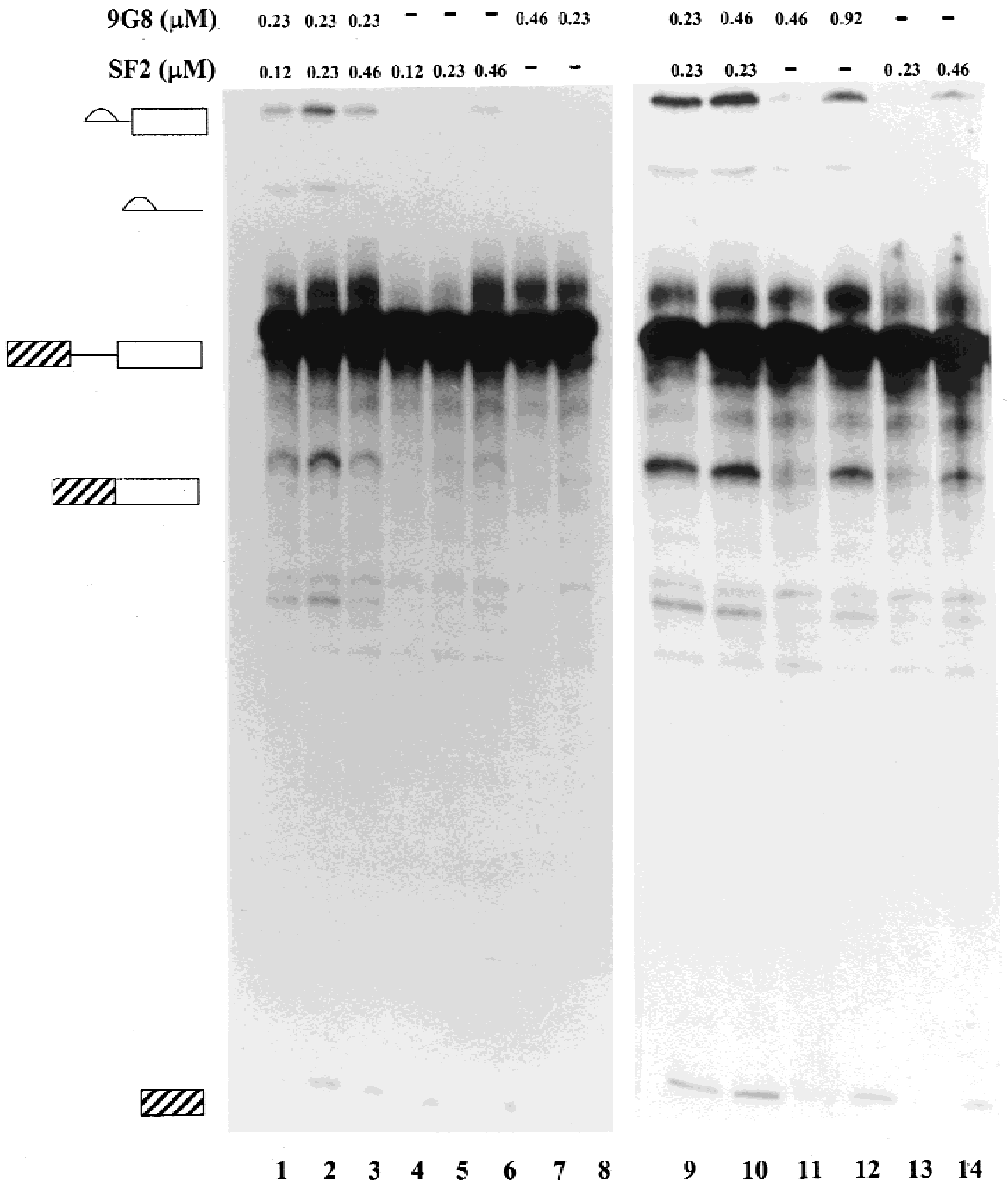

FIGURE 4. ASF/SF2 acts synergistically with 9G8 in stimulating splicing of bGH intron D. Splicing assays with bGH pre-mRNA were performed as those in Figure 3, except that only ASF/SF2 and 9G8 were studied. The left panel and the right panel were from independent experiments. The concentrations of the rSR proteins included in each assay are indicated above each lane. Pre-mRNA, spliced product, and intermediates are indicated schematically.

protein/RNA interactions (Nagel et al. 1998). The stoichiometry of the complexes is not known, but a Hill plot of the same data has a slope of approximately five, which is consistent with multiple binding sites (not shown). A similar experiment was performed with 9G8 over a concentration range from 0 to 160 nM (Fig. 5B, left panel). In contrast to the ASF/SF2 curve, the 9G8 binding curve is not sigmoidal and instead is approximately linear (Fig. 5B, right panel). 9G8 binds with somewhat lower affinity, with a K_d = 100 nM. The progressively decreasing mobility of the 9G8-RNA complexes with increasing 9G8 concentration also suggests that multiple 9G8 molecules may interact with the FP/ESE RNA. However, a Hill plot of the 9G8 binding data has a slope of only 1.6 (not shown), much lower than that observed for ASF/SF2 binding.

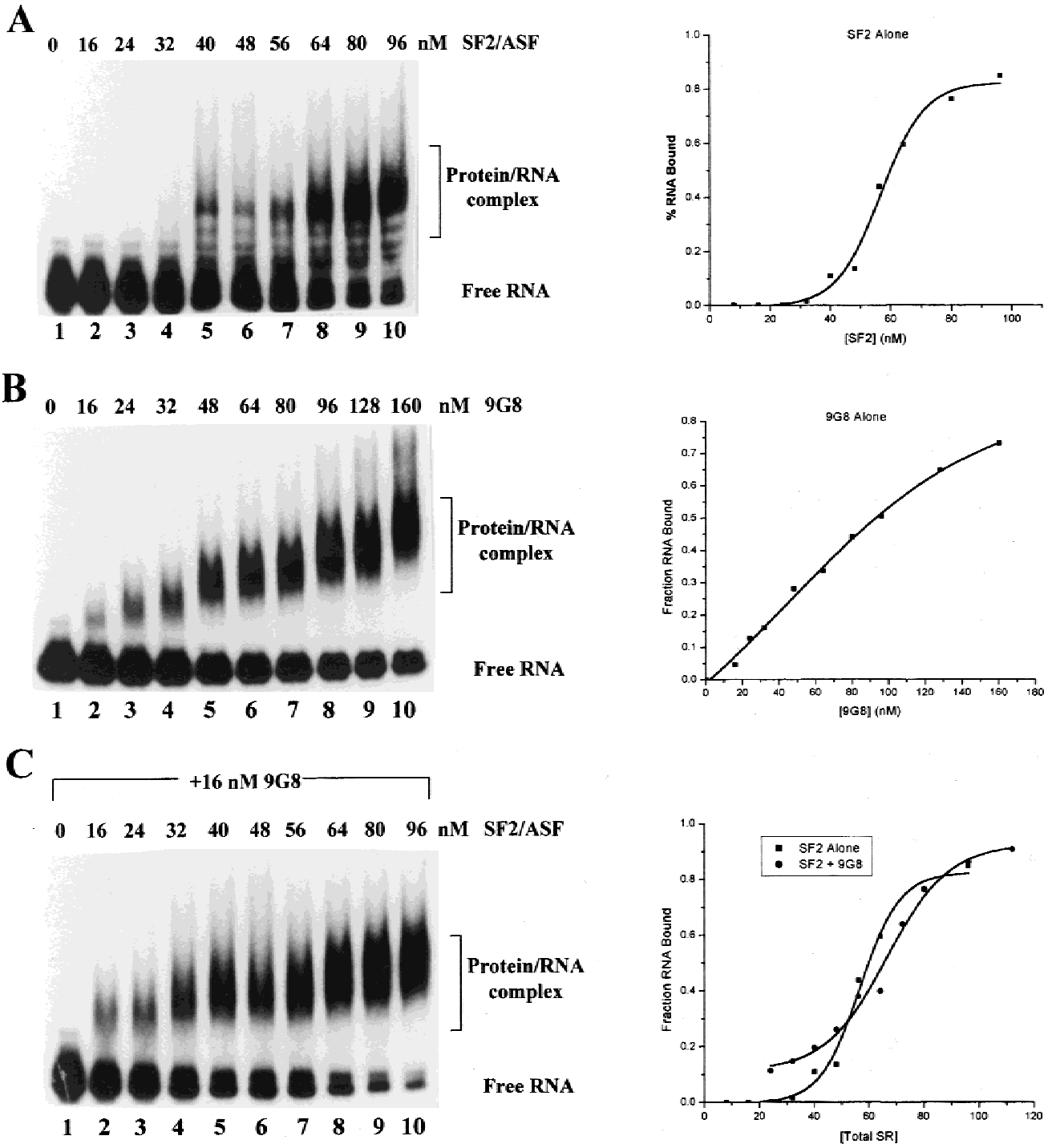

FIGURE 5. ASF/SF2 and 9G8 binding to bGH FP/ESE. **A**–**C**: Five femtomoles of ^{32}P-labeled FP/ESE RNA substrate was incubated with increasing amounts of ASF/SF2 (**A**), 9G8 (**B**), or a combination of a low fixed amount of 9G8 with increasing amounts of ASF/SF2 (**C**) and resolved in 6% nondenaturing polyacrylamide gel and visualized by autoradiography. In gel **A**, ten titration points of ASF/SF2 spanning 0–96 nM were used. In gel **B**, the amounts of 9G8 range from 0–160 nM. In gel **C**, the experiment was performed as in **A**, except 16 nM 9G8 was added to each binding reaction. Each of these experiments was performed independently, at least three times. The fractions of bound and unbound RNA were quantified using a PhosphorImager, and the mean values plotted against protein concentration as shown in the respective right-hand panels (not every point on the curves was present in the representative experiment photographed for the figure). Best-fit curves were generated using Origin 6.0 (Microcal Software Inc.).

This difference is consistent with the apparent lack of cooperative binding of 9G8 to the RNA substrate.

To investigate further the interactions that lead to the synergistic splicing stimulation observed with the combination of 9G8 and ASF/SF2, gel mobility shift assays were performed using a mixture of these rSR proteins. At lower concentrations (16, 24, and 32 nM, Fig. 5A, lanes 2–4), ASF/SF2 shows almost no binding to the FP/ESE. However, the addition of a low level of 9G8 (16 nM), which alone binds only 4.6% of the RNA (Fig. 5B, lane 2), to these low concentrations of ASF/SF2 results in significant binding to FP/ESE RNA (ranging from 12–20% bound, Fig. 5C). The binding activity in the presence of these two proteins (at the lower concentrations) is significantly higher than the combined binding of the individual proteins (compare Fig. 5C, lanes 2–4 to 5A and B, lanes 2–4, respectively). The fraction of bound and unbound RNA was determined by PhosphorImager analysis, and the mean values from the three independent determinations were plotted versus total rSR protein concentration (Fig. 5C, right panel). The binding curve for ASF/SF2 alone (from Fig. 5A) is superimposed for comparison. Two points are apparent: First, there is no "left shift" of the binding curve, indicating that the K_d for rSR protein binding is not significantly different with the combination. Second, the fraction of RNA bound is significantly higher than would be expected, but only at low total rSR protein concentrations. This point is more readily apparent when the data is summarized as in Figure 6. For example, notice that approximately 20% of the RNA is complexed with protein when incubated with 24 nM ASF/SF2 and 16 nM 9G8. This fraction exceeds a simple additive effect by fourfold, and exceeds the fraction of RNA bound in 40 nM ASF/SF2 by approximately twofold. At higher ASF/SF2 concentrations, the combination of ASF/SF2 with 16 nM 9G8 showed very similar binding activity to ASF/SF2 alone at the same concentrations. These data illustrate that ASF/SF2 and 9G8 cooperate in FP/ESE RNA binding, and are consistent with our observation that ASF/SF2 and 9G8 stimulate bGH intron D splicing synergistically at low concentrations.

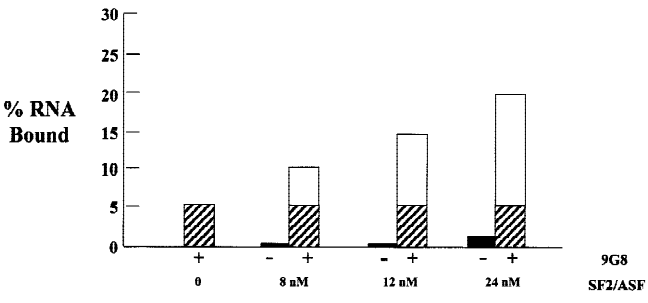

FIGURE 6. Synergistic binding of 9G8 and ASF/SF2 to FP/ESE. A subset of the data from Figures 6B and 6C is replotted in a format that more readily illustrates the synergistic binding of 9G8 (16 nM) and ASF/SF2 (8–24 nM). The crosshatched bars represent the fraction of bound RNA that is accounted for by the 9G8 alone. The negligible binding due to the ASF/SF2 alone is shown to the left in each pair of data. The open bars represent the excess binding that is attributed to the interaction of ASF/SF2 and 9G8.

DISCUSSION

Previous studies have demonstrated that splicing of bGH intron D requires the presence of the FP/ESE sequence in the downstream exon (Hampson et al., 1989; Sun et al., 1993a). One SR protein, ASF/SF2, was shown to stimulate intron D splicing in nuclear extract, presumably through a specific interaction with the FP/ESE (Sun et al., 1993b). In the present study, we have further examined the FP/ESE region and have found that it has a broad specificity, being recognized and bound by all four of the rSR proteins tested. These rSR proteins bind directly and efficiently to the FP/ESE in the absence of other proteins (Fig. 5A,B; the binding studies of SC35 and SRp55 are not shown). This is in contrast with the *Drosophila* female-specific splicing of *doublesex* (*dsx*) pre-mRNA, where the specific binding of an SR protein (9G8 in HeLa extracts, and RBP1 in *Drosophila* cell extracts) to the *dsx*RE requires the *Drosophila* splicing regulators, Tra and Tra2 (Tian & Maniatis, 1993; Lynch & Maniatis, 1996).

Although ASF/SF2 and 9G8 are both capable of binding to the FP/ESE directly, a difference was noted between these two interactions. The interaction of ASF/SF2 with the FP/ESE is portrayed by a sigmoid binding curve characteristic of a cooperative interaction, whereas 9G8 binds in an almost linear fashion. The apparent K_d for these proteins are 60 nM and 100 nM, respectively, in good agreement with other estimates of specific protein–RNA interactions (Berglund et al., 1998; Nagel et al., 1998). In addition, synergistic binding to FP/ESE was observed between ASF/SF2 and 9G8 at low concentrations of the two rSR proteins. At higher ASF/SF2 concentrations (above 40 nM), where ASF/SF2 shows efficient binding by itself, no obvious synergy between ASF/SF2 and 9G8 in binding to the FP/ESE was observed. Presumably, very efficient binding of ASF/SF2 alone (possibly due to the aforementioned cooperativity) masks the synergy between two different molecules.

Most studies that have investigated specific sequences that bind to SR proteins have identified binding sites of approximately 10 nt. The FP/ESE used in this study is 115 nt in length, and can potentially contain numerous overlapping and nonoverlapping SR protein binding sites. The Hill coefficients for ASF/SF2 binding and 9G8 binding are both suggestive of multiple binding sites for these proteins. The identification of specific regions of the FP/ESE that are essential for binding of these SR proteins, either individually or cooperatively, is beyond the scope of this study. However, some comments are in order. Purine-rich sequences from 4 to 14 nt can functionally replace the FP/ESE in

transfected cell model of Intron D splicing (Dirksen et al., 1994, 1995), and several purine-rich regions can be identified within the FP/ESE sequence. Dirksen et al. have divided the FP element into nine overlapping 14–17 nt subregions, and they have shown that most of these bind to recombinant ASF/SF2 in gel mobility shift assays, albeit with varying affinities (Dirksen et al., 2000). Tacke and Manley (1995) used the SELEX technique to identify three consensus sequences for ASF/SF2 binding. There is at least one good match for each of these consensus sequences within FP/ESE (Fig. 7). We have not yet tried to map specific 9G8 binding sites within the FP/ESE. However, Cavaloc et al. (1999) have identified 9G8 binding sequences by using the SELEX technique, and they proposed two 9G8 consensus binding sites. There are also good matches to each of these two consensus sequences in FP/ESE (Fig. 7). Whether these are the actual binding sites for 9G8 and how these sites interact with nearby ASF/SF2 binding sites remain to be determined.

The functional significance of the synergistic binding activity between ASF/SF2 and 9G8 in bGH intron D splicing was demonstrated by in vitro splicing assays. Very inefficient splicing was observed when low concentrations of ASF/SF2 and 9G8 were individually added to the NF20/40-supplemented S100 extract. However, when the two proteins were added in combination, a substantial increase in splicing activity was observed. The increased activity with both SR proteins is higher than the sum of their individual activities, and higher than the maximal activity that each individual protein can achieve. Thus, our data suggest that the optimal activities of ASF/SF2 and 9G8 in bGH intron D splicing require a synergistic interaction between the two proteins. The enhanced splicing activity appears to be mediated at least partly through synergistic binding of the rSR proteins to the FP/ESE.

Interestingly, the actual SR protein concentrations in the splicing experiments, including the range where the synergistic effect of SR protein concentration on splicing occurs, are much higher than the concentrations used in the binding experiments. The SR protein concentrations necessary to activate splicing in Figure 1 (i.e., SR proteins prepared from bovine anterior pituitary) are in the 2 to 4 μM range. The SR protein concentrations necessary to activate splicing in Figures 2 through 4 (i.e., individual recombinant SR proteins expressed in a baculovirus system) are in the 0.2 to 0.8 μM range, an order of magnitude difference. The concentrations at which synergistic binding is seen in the gel mobility shift experiments are, in turn, another order of magnitude lower than those observed in the splicing experiments. One explanation for these discrepancies is that binding of purified SR proteins to "naked" RNA is a highly efficient process, whereas there are numerous competing RNA binding proteins in nuclear extract, S100, and NF20-40. Therefore, it may not be reasonable to compare the different conditions directly. These two types of experiments are consistent in principle, although the absolute concentrations at which the synergism is observed are discrepant.

Cooperative binding alone may not completely account for the synergistic functional activity measured in the splicing assays, despite the evidence for synergistic binding of ASF/SF2 and 9G8 at low concentrations of the two proteins. In the gel shift assays, high concentrations of ASF/SF2 lead to much higher levels of rSR protein–RNA complex than is observed with the combination of 9G8 and ASF/SF2 at concentrations where cooperativity is observed. If splicing enhancement is simply a function of the extent of occupancy of the FP/ESE by SR proteins, one would expect the most efficient splicing to occur at the higher concentrations of ASF/SF2, conditions where as much as 80% of the RNA is complexed with SR proteins (as assayed by the gel retardation assay). In fact, more efficient splicing occurs with the lower concentrations of ASF/SF2 and 9G8. This suggests that there are differences in SR protein–RNA complexes that define functional versus nonfunctional assemblies. This implies that downstream

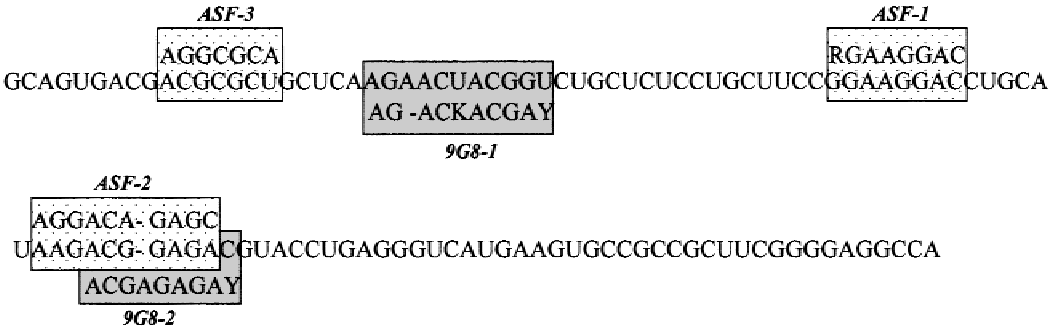

FIGURE 7. Comparison of FP/ESE to SELEX-derived SR protein-binding consensus sequences. The sequence of the bGH FP/ESE is shown in alignment with several putative consensus-binding sequences for ASF/SF2 and 9G8. ASF binding consensus sequences 1–3 are from Tacke and Manley (1995). 9G8 binding consensus sequences 1 and 2 are form Cavaloc et al. (1999). Y: pyrimidine; R: purine; K: G/U.

events such as protein–protein interactions between the bound SR proteins and other components of the spliceosome may occur more efficiently with the heterotypic SR protein complex. As the concentration of ASF/SF2 is increased, 9G8 may be displaced. Although the occupancy of the FP/ESE by SR protein (i.e., ASF/SF2) is higher, the efficiency with which the homotypic SR protein complex stimulates splicing is lower. Alternatively, it also remains possible that cooperative binding alone does completely account for the synergistic functional activity. The apparent discrepancies between the gel-shift experiments and the splicing assays may be due to inherent differences in the assay conditions. There are numerous competing RNA-binding proteins in the extract, and it may therefore not be valid to compare the two conditions directly.

The enhancing activities of these two SR proteins, in general, do not depend on each other. ASF/SF2 and 9G8 individually can activate constitutive splicing and certain ESE-dependent splicing (Krainer et al., 1991; Cavaloc et al., 1994; Tacke & Manley, 1995; Lynch & Maniatis, 1996; Liu et al., 1998). Therefore, the synergistic activity between ASF/SF2 and 9G8 seen in our experiments is most likely intrinsic to the bGH pre-mRNA. The synergy between ASF/SF2 and 9G8 contrasts with the apparent lack of synergy observed among other rSR protein combinations. SRp55 enhances intron D splicing very efficiently without the requirement for other SR proteins. An SRp55 consensus sequence identified in an in vitro SELEX experiment is highly enriched in the bGH FP/ESE, compared to the remainder of exon 5 (Liu et al., 1998). Another rSR protein, SC35, was highly active in a standard S100 complementation assay with β-globin pre-mRNA, but showed no enhancing activity in intron D splicing. Furthermore, no cooperative activity of SC35 with SF2/SASF, SRp55, or 9G8 was observed. These results suggest that the observed effects of SRp55 and the cooperative activity between ASF/SF2 and 9G8 are not a simple reflection of their general splicing stimulation, but rather are specific to the bGH FP/ESE.

To our knowledge, cooperative binding of mammalian SR proteins to an ESE has not been previously reported, although it has been extensively postulated. Hertel and Maniatis (1998) suggested that cooperative binding of SR proteins, and/or multiple interactions between the bound SR proteins and different components of the splicing machinery, might occur at multisite enhancers. Such cooperativity may be analogous to the cooperativity seen between the bound activators of the transcription machinery (Ptashne & Gann, 1997). The *Drosophila dsx* splicing enhancer provides the most appropriate model of cooperativity for comparison to the bGH FP/ESE. Lynch and Maniatis (1996) showed that the *Drosophila* splicing proteins Tra, Tra2, and the SR protein RBP1 bind cooperatively as a heterotrimeric complex to each of six repeat elements (*dsx*RE). They performed a similar experiment using mammalian SR proteins with *Drosophila* Tra, Tra2, and the *dsx*RE. In this case, 9G8 replaces RBP1 in the complex. Also, Tra, Tra2, and the *Drosophila* SR protein dSRp30 bind cooperatively to a separate purine-rich element (PRE) that lies between repeats five and six on the *dsx*RE. When this experiment is performed with mammalian SR proteins substituting for the *Drosophila* factors, ASF/SF2 is detected within the complex. Therefore, in these in vitro binding studies, both 9G8 and ASF/SF2 bind to the *dsx* enhancer cooperatively with Tra and Tra2, illustrating that they are capable of cooperative interactions with other (SR-like) RNA binding proteins. To our knowledge, our current study is the first to show that 9G8 and ASF/SF2 (or any other mammalian SR proteins) can bind cooperatively to an ESE as a homotypic (in the case of ASF/SF2) and heterotypic complex.

The presence of multiple regulatory elements in the FP/ESE, as in the *dsx*RE, might serve as a mechanism for a fine-tuned regulation in vivo, and suggest several hypotheses: First, the intracellular levels of SR proteins are likely to be lower than those used in vitro, and the presence of multiple ESE regulatory elements might be essential for splice site activation, presumably by increasing the probability of a productive interaction between the enhancer complex and the complexes assembled at the weak 5′ and/or 3′ splice sites (Wu & Maniatis, 1993; Staknis & Reed, 1994; Hertel & Maniatis, 1998). Second, regulation of splicing via an ESE containing multiple regulatory elements, which are recognized by different SR proteins, may be more sensitive to cellular changes in the levels of individual SR proteins. Finally, the presence of multiple elements might reflect the evolutionary constraints for ESEs to maintain translational integrity. By incorporating multiple elements, an ESE can compromise between the affinity of individual elements for specific factors and the capability to encode functional proteins. In this respect, it is worthwhile to note that a single base mutation within an enhancer element without changing the encoded amino acid was found to inactivate enhancer function (Schaal & Maniatis, 1999). In summary, using bGH pre-mRNA as a model splicing system, we have demonstrated that SR protein/splicing enhancer function involves both the independent interaction of certain SR proteins and the synergistic interaction of multiple SR proteins with the enhancer. These findings illustrate the flexibility of splicing control involving SR proteins and enhancer sequences.

MATERIALS AND METHODS

Plasmid construction for protein expression

The coding sequences of ASF/SF2, SC35, 9G8, and SRp55 were obtained by PCR amplification using upstream primers

containing their native start codons, and downstream primers containing 6-histidine-coding sequences. The template plasmids were provided by Akila Mayeda (Cold Spring Harbor Laboratory, USA) and Gavin Screaton (Cambridge University, United Kingdom). The amplified products were subcloned into pBacPAK8 baculovirus transfer vector (Clontech) at the *Sma*I site. All constructs were sequenced prior to transfection.

The oligonucleotides used for PCR were:

ASF/SF2: AGCTGGATCCATCATGTCGGGAGGTGGTG and TCAATGGTGATGGTGATGATGTGTACGAGAGCGAGATC;
SC35: ACGTGGATCCAGAGCTATGAGCTACGG and TCAG TGGTGATGATGATGATGAGAGGACACCGCTCC;
9G8: AGCTGGATCCATCATGTCGCGTTACGG and TCAGT GATGGTGGTGATGATGGTCCATTCTTTCAGG;
SRp55: AGCTGGATCCGACATGCCGCGCGTC and TCAAT GGTGATGGTGATGATGATCTCTGGAACTCGACC.

Baculovirus expression of recombinant SR proteins

Sf21 cells were grown at 27 °C and ambient pCO_2. Following the initial transfection, the viral titer was increased through three rounds of amplification. Protein production was performed using 2 mL of virus stock ($\sim 1 \times 10^8$ pfu/mL) to infect $2–3 \times 10^7$ Sf21 cells in a 150 cm^2 flask. Four flasks of Sf21 cells were used to produce each recombinant protein. Forty-eight hours after the infection, the cells were harvested, rinsed in PBS, and the cell pellet was resuspended in Ni-agarose binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 0.5% NP-40) and lysed by sonication. The recombinant protein was then purified using a Ni-agarose affinity resin (Qiagen) according to the instructions of the manufacturer. The eluted fractions from the affinity column were then pooled and dialyzed against BC100 buffer (20 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.5 mM EDTA, 20% glycerol) and stored at −80 °C.

Preparation of nuclear extract and nuclear fraction NF20/40

HeLa nuclear extracts and S100 extracts were prepared as described in Dignam et al. (1983), except that Tris was substituted for HEPES in Buffer D (therefore, referred to here as Buffer E). NF20/40 was prepared similar to the method described by Tacke and Manley (1995). Nuclear extract (1 mL, in Buffer E) was diluted with 1 mL of buffer E minus glycerol. Ammonium sulfate was added to 20% saturation, the mixture was incubated with rocking for 30–60 min at 4 °C for 30 min, and was centrifuged for 30 min at 30,000 × *g*. Ammonium sulfate was added to the supernatant to 40% saturation. The mixture again was rocked at 4 °C for 1 h and centrifuged as above. The pellet was rinsed with buffer E containing 40% ammonium sulfate and resuspended in 0.7 mL of buffer E. After overnight dialysis against buffer E, the dialysate was centrifuged in a microfuge for 5 min to remove precipitated material, and the supernatant was stored at −80 °C.

RNA synthesis and in vitro splicing reactions

Capped (for splicing) and uncapped (for gel shift) RNAs were synthesized from linearized plasmids pbGH-4D5 and pE5/FP as previously described (Fig. 1A; Sun et al., 1993a). In vitro splicing reactions were performed as described (Sun et al., 1993a), except that the nuclear extract was replaced by S100 and/or NF20/40 and the incubation time was 120 min instead of 75 min.

RNA gel mobility shift assays

Binding reactions contained 10 mM Tris-Cl, 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, varying concentrations of rSR proteins and 2.5–5 fmol of the indicated RNA. The reactions were incubated at 30 °C for 30 min and resolved on a nondenaturing 6% polyacrylamide gel (80:1 acrylamide:bisacrylamide) containing 50 mM Tris and 50 mM glycine. Electrophoresis was performed at a constant voltage of 14 V/cm for 2–3 h at 4 °C.

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