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Functions of SR proteins in the U12-dependent AT-AC pre-mRNA splicing pathway

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ABSTRACT

SR proteins play critical roles in the major pre-mRNA splicing pathway. A second pathway processes U12-dependent AT-AC introns. We demonstrate, by biochemical complementation, the requirement for SR proteins in splicing of AT-AC introns. Whereas SR proteins were sufficient to activate splicing of a P120 AT-AC intron, splicing of a sodium channel AT-AC intron required an additional nuclear fraction. Individual recombinant SR proteins promoted splicing of both substrates, but displayed marked preferences. SR proteins supported basal AT-AC splicing, and also splicing stimulation via a downstream enhancer or conventional 5′ splice site. Analysis of chimeric transcripts revealed that information dispersed throughout exons and introns dictates SR protein specificity and the requirement for the additional nuclear fraction. Thus, SR proteins function in both major and minor splicing pathways, and in coordinating the activities of both spliceosomes via exon definition. These results suggest that despite the substantial differences in intron consensus sequences and in four of the five snRNPs in each spliceosome, at least some of the interactions involving SR proteins are conserved between the two pathways.

Keywords: AT-AC introns; exon definition; exonic splicing enhancer; pre-mRNA splicing; SR proteins

INTRODUCTION

The removal of pre-mRNA introns is a complex process involving small nuclear ribonucleoprotein particles (snRNPs) and additional auxiliary protein factors, which form a sequential series of spliceosomal complexes on pre-mRNA. Numerous RNA–RNA, RNA–protein and protein–protein interactions are required to precisely excise each intron. These interactions involve conserved sequences common to most introns, including a 5′ splice site (5′ss), a branch point sequence (BPS), a polypyrimidine tract (Py tract), and a 3′ splice site (3′ss). A small subset of introns, called AT-AC or U12-dependent introns, have distinct conserved sequences at the 5′ss, BPS, and 3′ss, and are removed by a different spliceosome (reviewed in Wu & Krainer, 1999).

Splicing of AT-AC introns is similar in many ways to that of the conventional GT-AG or U2-dependent introns. Four of the five snRNAs (U1, U2, U4, and U6) that make up the conventional spliceosome have a structural and apparently functional homolog (U11, U12, U4atac, U6atac, respectively) in the AT-AC spliceosome (Hall & Padgett, 1996; Tarn & Steitz, 1996a, 1996b). U5 snRNA is common to both spliceosomes and Prp8, an integral U5 snRNP protein, appears to function in splicing of both types of introns (Tarn & Steitz, 1996b; Luo et al., 1999). The composition of purified U11 and U12 snRNP particles indicates that many of the protein components of the two snRNPs are the same as those in the U1 and U2 snRNPs (Will et al., 1999). In addition, U2- and U12-dependent introns both undergo a similar two-step splicing reaction (Tarn & Steitz, 1996a, 1996b).

Despite these similarities, there are several differences between the two spliceosomes. The 5′ss and BPS of AT-AC introns are highly conserved, in contrast to the degenerate consensus sequences of the GT-AG introns, and the characteristic Py tract between the 3′ss and BPS of conventional introns is absent in AT-AC introns (Wu & Krainer, 1996; Burge et al., 1998). The minor snRNPs are much less abundant than the major snRNPs (Montzka & Steitz, 1988) and several proteins isolated from the U11/U12 di-snRNP appear to be unique to the AT-AC spliceosome (Will et al., 1999). In addition, unlike the U1 and U2 snRNPs, which exist as individual particles and interact with the RNA separately, U11 and U12 form a di-snRNP and bind simultaneously to the 5′ss and BPS (Frilander & Steitz, 1999). These differences may reflect differences in the mech-

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anism by which the two types of splice sites are recognized, or they may reflect flexibility in the requirements for pre-mRNA splicing. In either case, a comparison of the two types of introns may lead to insights into general mechanisms of splicing and the evolution of the two splicing pathways.

Though progress has been made in determining the role of the minor snRNPs in AT-AC splicing, little is known about other factors required for AT-AC intron excision. In conventional splicing, a family of essential splicing factors, SR proteins, facilitates most snRNP–pre-mRNA interactions in the spliceosome (reviewed in Graveley, 2000). SR proteins are characterized by one or two RNA-recognition motifs (RRM) and a C-terminal domain rich in arginine/serine (RS) dipeptide repeats (Birney et al., 1993; Graveley, 2000). Two distinct, but not mutually exclusive, models have been proposed for SR protein function in conventional splicing. In the first model, SR proteins mediate protein–protein interactions spanning the intron. For this intron definition, SR proteins and other factors are proposed to act as a bridge between the 5′ss and 3′ss to bring the sites together and promote interactions required for splicing catalysis (Wu & Maniatis, 1993; Abovich & Rosbash, 1997; Hertel & Maniatis, 1999). In the second model, SR proteins mediate interactions across exons to facilitate intron removal by exon definition (Robberson et al., 1990). Many pre-mRNAs contain exonic splicing enhancer sequences (ESEs) that are required for efficient intron splicing and are recognized in a sequence-specific manner by individual SR proteins (reviewed in Blencowe, 2000; Graveley, 2000). Exon definition interactions can also occur via the 5′ss of the downstream intron (Robberson et al., 1990). This stimulatory activity requires U1 snRNP and SR proteins. The activity of SR proteins in intron definition and exon definition is not easily uncoupled, and thus, precise roles for SR proteins in splicing have been difficult to define. U12-dependent pre-mRNA splicing of AT-AC introns provides a means to test the activity of essential splicing factors, such as SR proteins, in a pathway that may have distinct roles for such proteins in splicing.

RESULTS

SCN4A AT-AC intron 2 is not spliced in S100 extract complemented with SR proteins

HeLa S100 extract contains all the components necessary for splicing of most U2-dependent GT-AG introns, except for SR proteins. Thus, the standard functional assay for SR protein activity is the ability to complement S100 extract (Krainer et al., 1990b). We used this assay to test whether SR proteins are required for splicing of AT-AC introns. An AT-AC pre-mRNA substrate derived from the SCN4A gene and consisting of exon 2, intron 2, and exon 3 (Wu & Krainer, 1996) was used for the S100 complementation assays (Fig. 1, SCN4AS). The SCN4AS AT-AC intron was not spliced in S100 extract alone, consistent with a requirement for SR proteins (Fig. 1, lane 2). However, splicing of SCN4AS was not rescued when SR proteins were added to the S100 extract (Fig. 1, lane 5). Neither the S100 extract nor the SR protein preparation inhibited AT-AC splicing in nuclear extract (not shown), suggesting that the lack of splicing was not due to the presence of an inhibitor. The inability of SR proteins to activate splicing in S100 extract indicates that splicing of the SCN4A AT-AC intron has additional or unique requirements for splicing that are not met by SR proteins.

SR proteins are required for SCN4A AT-AC intron splicing

To identify the splicing components that are missing from S100 extract and to establish a system for testing SR protein requirements in AT-AC splicing, we fractionated nuclear extract using selective precipitation by ammonium sulfate. Three nuclear extract fractions were generated by precipitation at 20% ammonium sulfate saturation, followed by precipitation at 60% ammonium sulfate (20−60%AS) and precipitation at 90% saturation (60−90%AS). Splicing reactions containing both the 20−60%AS and 60−90%AS fractions reconstituted β-globin and SCN4A splicing (not shown). SCN4A splicing was not observed with either fraction alone (Fig. 1, lane 3 and not shown) or when either fraction was added to S100 extract (Fig. 1, lane 4 and not shown). Because the bulk of SR proteins is found in the 60−90%AS fraction (Zahler, 1999 and not shown), the lack of splicing in S100 extract with the 20−60%AS fraction may be due to the absence of SR proteins in this fraction. Indeed, the SCN4A AT-AC intron was spliced when SR proteins were included with S100 extract and 20−60%AS (Fig. 1, lanes 6 and 7). This splicing reaction was as efficient as SCN4AS splicing in nuclear extract (Fig. 1, lane 1) and clearly demonstrates the requirement for SR proteins in SCN4A AT-AC intron splicing.

SR proteins mediate splicing in basal and enhancer-dependent AT-AC splicing

SCN4A AT-AC intron splicing is stimulated by a downstream 5′ss or an exonic splicing enhancer (Wu & Krainer, 1996, 1998). To determine if SR proteins mediate splicing of the basal SCN4A substrate and support stimulation of splicing by enhancer elements, splicing of three different SCN4A pre-mRNA substrates was compared. SCN4AS is described above, SCN4A+5′ss is identical to SCN4AS but has a conventional downstream 5′ss following exon 3 (Wu & Krainer, 1997) and SCN4A+Enh contains a synthetic purine-rich exonic splicing enhancer at the end of exon 3, but no downstream 5′ss (Wu & Krainer, 1998).

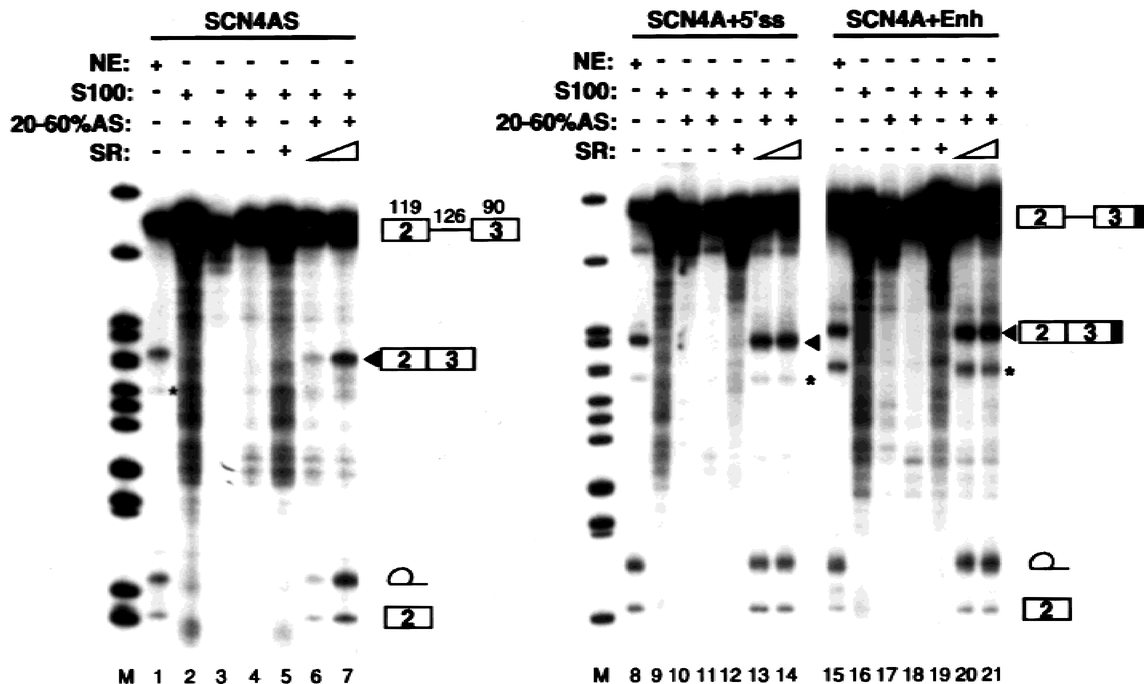

FIGURE 1. SR proteins activate splicing of the SCN4A AT-AC intron. Three different SCN4A pre-mRNA substrates were assayed for splicing in S100 complementation assays. The SCN4A basal substrate (SCN4AS, lanes 1–7) comprises intron 2 and flanking exon sequences; the SCN4A+5′ss substrate (+5′ss, lanes 8–14) includes a conventional 5′ss (9 nt) following exon 3; and the SCN4A+Enh substrate (+Enh, lanes 15–21) contains a model exonic splicing enhancer (19 nt) following exon 3. Splicing was performed in nuclear extract (lanes 1, 8, and 15), S100 extract alone (lanes 2, 9, and 16), S100 extract with total HeLa SR proteins (lanes 5, 12, and 19), S100 extract in combination with the 20–60%AS fraction (lanes 4, 11, and 18), and S100 extract with the 20–60%AS fraction and 0.15 μg (lanes 6, 13, and 20), or 0.3 μg (lanes 7, 14, and 21) of SR proteins. Pre-mRNA and spliced RNA products are shown schematically next to the panels. Sizes of exons (open box) and introns (lines) and stimulatory sequences (filled box) are indicated. The asterisk indicates a U2-dependent cryptic spliced product. Size markers are shown (M).

All three of the AT-AC pre-mRNA substrates were spliced in nuclear extract (Fig. 1, lanes 1, 8, and 15), though less accumulation of mRNA products was observed with SCN4AS relative to SCN4A+5′ss and SCN4A+Enh. This result is consistent with the previous study (Wu & Krainer, 1998) and confirms the activity of the downstream 5′ss and enhancer sequence in AT-AC intron splicing. The level of splicing stimulation by these elements was approximately twofold relative to the basal substrate. This stimulation is lower than that reported previously (Wu & Krainer, 1996, 1998) because the experiments presented here were performed under splicing conditions optimized in favor of the basal splicing reaction, which has a narrower optimal concentration of magnesium than the other substrates.

To determine the requirement for SR proteins in splicing of these RNA substrates, the reconstituted system described above was used. In addition to the basal SCN4A substrate, SCN4A splicing also was observed in the presence of a downstream 5′ss or enhancer sequence upon addition of SR proteins and the 20–60%AS fraction to S100 extract (Fig. 1, lanes 6–7, 13–14, and 20–21). The splicing efficiencies of SCN4A+5′ss and SCN4A+Enh in the reconstituted system nearly equaled those in nuclear extract. Splicing of SCN4A+5′ss and SCN4A+Enh pre-mRNAs was more efficiently complemented by SR proteins than splicing of the basal SCN4A substrate, as indicated by the greater accumulation of mRNA products (cf. Fig. 1, lanes 6, 13, and 20). These results suggest that SR proteins not only function in basal AT-AC splicing, but also support the stimulation of splicing by a downstream enhancer or 5′ss.

Recombinant SR proteins function in AT-AC intron splicing

Total SR protein preparations may contain low abundance proteins specific for AT-AC splicing, in addition to the known SR proteins. To determine whether AT-AC intron splicing requires a distinct subset of SR proteins, we tested the ability of individual recombinant SR proteins to activate AT-AC intron splicing in the reconstituted system (Fig. 2A). The SCN4AS substrate (Fig. 2A, lanes 1–4) was spliced upon the addition of recombinant 9G8 (first row), and recombinant SRp55 (second row). However, splicing was undetectable in the presence of recombinant SF2/ASF (third row) or SC35 (fourth row). In contrast, 9G8, SRp55, and SF2/ASF activated splicing of an SCN4A substrate containing a

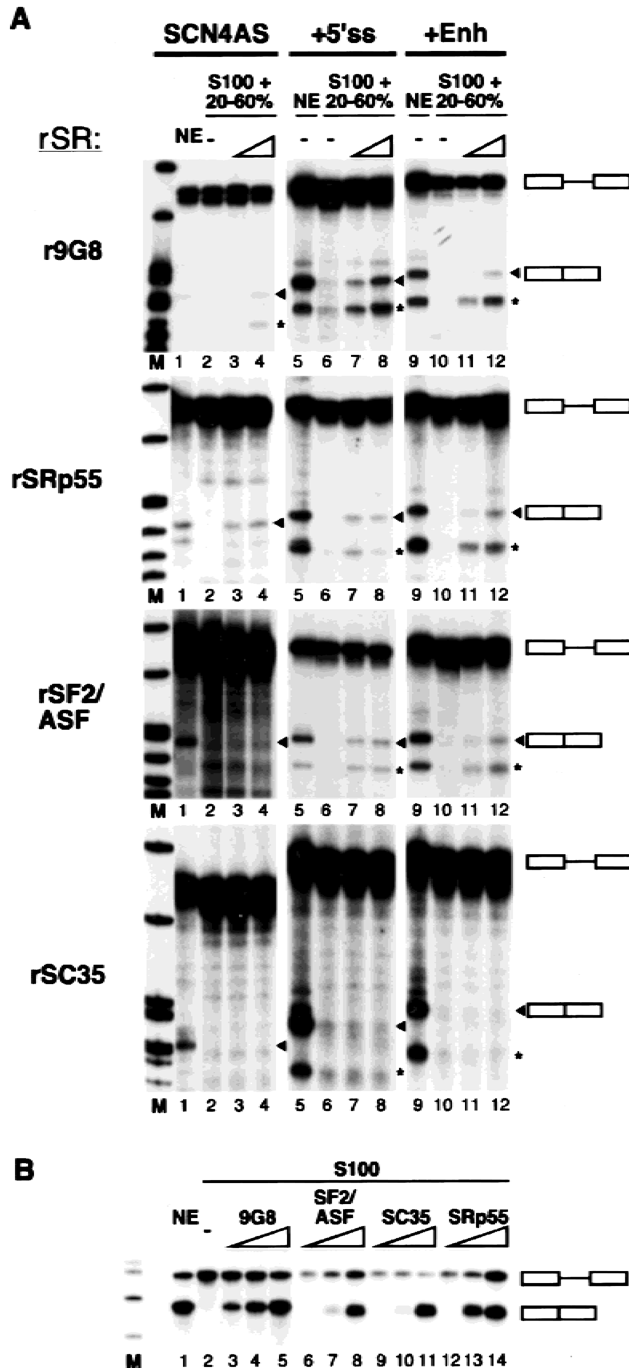

FIGURE 2. Individual SR proteins function in AT-AC splicing in vitro. **A**: SCN4AS (lanes 1–4), SCN4A+5′ss (lanes 5–8), and SCN4A+Enh (lanes 9–12) were tested for splicing in the presence of purified recombinant SR proteins. AT-AC intron splicing was assayed in S100 extract with the 20–60%AS fraction and recombinant 9G8 (r9G8, first row; lane 7: 1 pmol; lanes 3, 8, 11: 2 pmol; lanes 4, 12: 4 pmol), rSRp55 (second row; lanes 7, 11: 1.25 pmol; lanes 3, 8, 12: 2.5 pmol; lane 4: 5 pmol), rSF2/ASF (third row; lanes 3, 7, 11: 4 pmol; lanes 4, 8, 12: 8 pmol) or rSC35 (fourth row; lanes 3, 7, 11: 2.5 pmol; lanes 4, 8, 12: 5 pmol). Individual panels are different exposures used to optimally show the splicing of each substrate. **B**: Splicing of β-globin pre-mRNA in S100 extract complemented with recombinant SR proteins: 9G8 (2, 4, 8 pmol), SF2/ASF (1, 2, 4 pmol), SC35 (2, 4, 8 pmol), SRp55 (1, 2, 4 pmol).

downstream 5′ss (+5′ss, Fig. 2, lanes 5–8) or exonic splicing enhancer (+Enh, Fig. 2, lanes 9–12). An obvious stimulation of splicing by a downstream 5′ss or enhancer was observed upon addition of SF2/ASF, relative to the basal SCN4AS substrate (third row). Recombinant 9G8 (first row; lanes 3, 8, and 11) and SRp55 (second row; lanes 3, 8, and 12) more efficiently activated splicing of SCN4A+5′ss and SCN4A+Enh than SCN4AS. SC35 did not function in splicing of the SCN4A AT-AC intron in the reconstituted S100 extract splicing system with any of the substrates (fourth row). All of the recombinant proteins were active in splicing of a conventional intron from the human β-globin gene, with no dramatic difference in relative activity among the individual proteins (Fig. 2B). These results demonstrate that conventional SR proteins can activate SCN4A AT-AC intron splicing, although there is SR protein specificity for basal splicing, as well as for exon definition interactions.

SR proteins activate P120 AT-AC intron splicing in S100 extract

The requirement for SR proteins and the 20–60%AS fraction observed in SCN4A splicing may be general features of AT-AC intron splicing or they may represent substrate-specific features. To distinguish between these possibilities, we tested splicing of the P120 AT-AC intron F. The P120 substrate consists of exon 6, intron F and exon 7 (Fig. 3). The in vitro removal of this intron by the U12-dependent splicing pathway was previously shown (Tarn & Steitz, 1996b). We also tested the effect of a downstream conventional 5′ss using a P120 substrate with a consensus GT-AG 5′ss following exon 7 (P120S+5′ss). The U12-dependence of splicing of P120S+5′ss was demonstrated by the loss of splicing in nuclear extract upon oligonucleotide-directed RNase H digestion of U12 snRNA (Fig. 3, lanes 12–14) but not upon U2 snRNA digestion by the same procedure (Fig. 3, lanes 9–11). U12-dependent splicing of SCN4A+5′ss was previously shown (Wu & Krainer, 1996) and was used here as a control for U12 and U2 inactivation by RNase H digestion, as both U12-dependent AT-AC splicing (Fig. 3, lanes 1–7) and cryptic splicing via the U2-dependent splicing pathway (Fig. 3, lanes 1–7, asterisk) were observed from this substrate.

Next, we tested the requirement for SR proteins and the 20–60%AS fraction in P120 AT-AC intron splicing. Splicing of a basal P120 RNA transcript lacking a downstream 5′ss (P120S) was nearly undetectable in nuclear extract (Fig. 4A, lanes 1 and 2). However, low levels of splicing were seen when SR proteins were added to the S100 extract (Fig. 4, lane 6). P120S splicing was not improved in S100 extract supplemented with the 20–60%AS fraction with or without SR proteins (not shown). P120 splicing was strongly activated in nuclear extract when a conventional 5′ss (P120S+

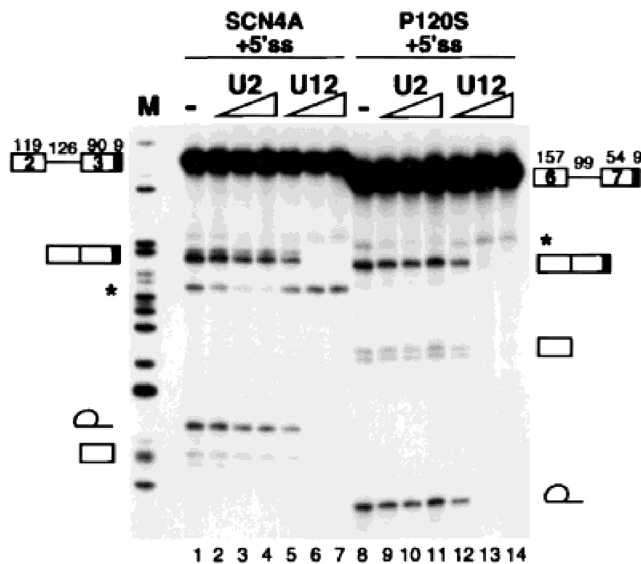

FIGURE 3. U12-dependent splicing of P120 AT-AC intron. U12-dependent splicing of SCN4A+5′ss and P120S+5′ss substrates is demonstrated by oligonucleotide-directed RNase H digestion of U2 and U12 snRNA in HeLa nuclear extract. U2-specific oligonucleotides, at a final concentration of 50, 100, 200 nM (lanes 2–4, 9–11) and U12 at 5, 50, 250 nM (lanes 5–7, 12–14) were preincubated in nuclear extract for 15 min. Control reactions were preincubated in the absence of oligonucleotides (lanes 1, 8). The mobilities of the precursors, intermediates, and products are indicated. The structure of the P120+5′ss substrate is shown with sizes of introns and exons. The asterisks indicate spliced products generated by use of U2-dependent cryptic splice sites.

5′ss) or a synthetic purine-rich splicing enhancer (P120S+Enh) was present downstream of the 3′ exon (Fig. 4A, lanes 1, 7, and 13). In addition, sequences representing high-score recognition motifs for SF2/ASF (Liu et al., 1998) were also able to function as enhancers of P120S splicing (not shown). Though the P120S+5′ss and P120S+Enh transcripts were not spliced in S100 extract alone (Fig. 4A, lanes 8 and 14), splicing was strongly stimulated upon addition of SR proteins, with an efficiency equal to or greater than that in nuclear extract (Fig. 4A, lanes 9–12 and 15–18).

Individual recombinant SR proteins also stimulated splicing of the P120S+5′ss pre-mRNA (Fig. 4B). Recombinant 9G8 (Fig. 4B, lanes 4–5), SC35 (Fig. 4B, lanes 6–7), SRp55 (Fig. 4B, lanes 8–9), and SF2/ASF (Fig. 4B, lanes 12–13) were titrated into S100 extract to achieve maximal splicing. At the optimized concentrations, little difference in activity was observed among recombinant SR proteins.

SR proteins are required for spliceosomal complex A formation

To determine the step at which SR proteins function in AT-AC intron splicing, spliceosome complex assembly was assayed. Stable spliceosomal complexes were not detected with SCN4A substrates incubated in nuclear extract or in S100 extract with the 20–60%AS fraction and SR proteins, precluding analysis of complex assembly on this substrate. However, spliceosomal complexes A and B were formed on P120S+5′ss pre-mRNA in in vitro splicing reactions containing nuclear extract (Fig. 4C, lanes 1–6). Spliceosomal complex C could be detected after a longer exposure (not shown). Two distinct A complexes were observed as described previously (Tarn & Steitz, 1996b; Frilander & Steitz, 1999). No spliceosomal complexes were formed in S100 extract alone (Fig. 4C, lanes 7–12). However, when S100 extract was complemented with recombinant SC35, formation of complexes A and B was stimulated (Fig. 4C, lanes 13–18). These data show that SR proteins are required for spliceosomal complex A assembly on the P120 AT-AC intron.

P120 exon sequences enhance splicing in vitro

Previous reports showed that splicing of a P120 basal substrate in nuclear extract requires inactivation of U1 or U2 snRNP (Tarn & Steitz, 1996a, 1996b). Our P120S basal substrate was not spliced in nuclear extract (Fig. 4A) with or without inactivation of the major splicing pathway by RNase H digestion with U2-specific oligonucleotides (not shown). One difference between this and the previous studies is the length of the downstream exon 7. Our P120 substrate has a truncated exon 7 of 54 nt, whereas previous studies used a substrate with a longer exon 7. To investigate the effect of exon length on splicing, we tested the splicing of a P120 transcript with a 95-nt exon 7 (Fig. 5, top, P120L). Even without inactivation of the major splicing pathway, the addition of these 41 nt of exon 7 strongly enhanced splicing relative to P120S (Fig. 5, lane 1, top panel and Fig. 4A, lane 1). This splicing enhancement was also seen in S100 extract complemented with SR proteins (Fig. 5, top panel, lanes 3–5) or recombinant SF2/ASF (Fig. 5, lanes 6–8), SC35 (Fig. 5, lanes 9–11) and SRp55 (Fig. 5, lanes 12–14).

Interestingly, unlike results with the P120S substrate, for which splicing was strongly stimulated when a 5′ss was present following exon 7 (Fig. 4), little stimulation of splicing was seen upon addition of a downstream 5′ss to P120L (P120L+5′ss; Fig. 5, middle panel). The presence of the enhancer sequence at the 3′ end of exon 7 caused an unexpected reduction in splicing of P120L+Enh relative to P120L (Fig. 5, bottom panel, lanes 1–14). These results suggest the presence of a natural exonic splicing enhancer within P120 exon 7.

In vitro splicing of SCN4A/P120 chimeric transcripts

To identify regions of the SCN4A and P120 transcripts that confer or alleviate the requirement for the 20–

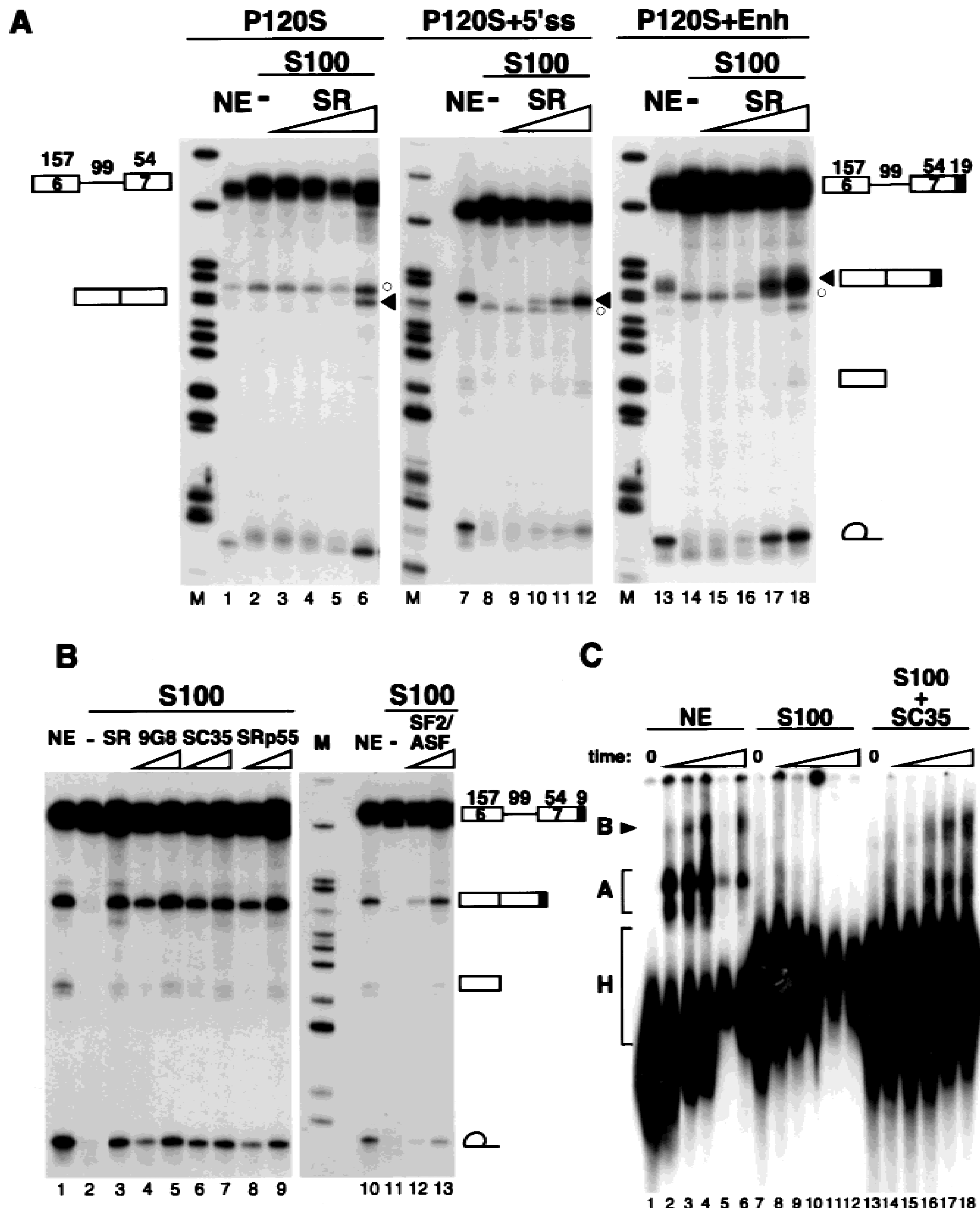

FIGURE 4. SR proteins activate P120 AT-AC intron splicing. **A**: Three P120 pre-mRNA substrates were assayed for splicing in S100 extract with SR proteins. The P120S substrate (lanes 1–6) comprises only the intron and flanking exon sequences; P120S+5′ss (lanes 7–12) includes a conventional 5′ss at the 3′ end of the downstream exon; and the P120S+Enh substrate (lanes 13–18) has a synthetic exonic splicing enhancer at the 3′ end of the downstream exon. The S100 extract was complemented with total HeLa SR proteins (0.025, 0.05, 0.1, 0.2 μg, lanes 3–6, 9–12, and 15–18). Arrowheads indicate spliced products. Open circles indicate a degraded substrate fragment. **B**: Recombinant SR proteins activate P120 AT-AC intron splicing in S100 extract. Total SR proteins (0.1 μg, lane 3), r9G8 (2, 4 pmol, lanes 4–5), rSC35 (4, 8 pmol, lanes 6–7), rSRp55 (1, 2 pmol, lanes 8–9), and rSF2/ASF (2, 4 pmol, lanes 12–13) were incubated with P120+5′ss RNA in S100 extract. **C**: Native-gel analysis of spliceosomal complexes formed on P120S+5′ss pre-mRNA. In vitro splicing reactions containing either nuclear extract (lanes 1–6), S100 extract (lanes 7–12), or S100 extract and 10 pmol of rSC35 (13–18) were carried out under standard conditions for 0, 30, 60, 120, 180, or 240 min and analyzed on a nondenaturing polyacrylamide gel.

60%AS fraction in the S100 complementation assays, chimeric transcripts were constructed in which the upstream exon, intron or downstream exon of SCN4AS was replaced with the corresponding sequence from the P120L transcript (Fig. 6, top). When either exon 2 of SCN4A was substituted with exon 6 of P120L (PSS substrate) or intron 2 of SCN4A was replaced with intron F of P120L (SPS), the 20–60%AS fraction was

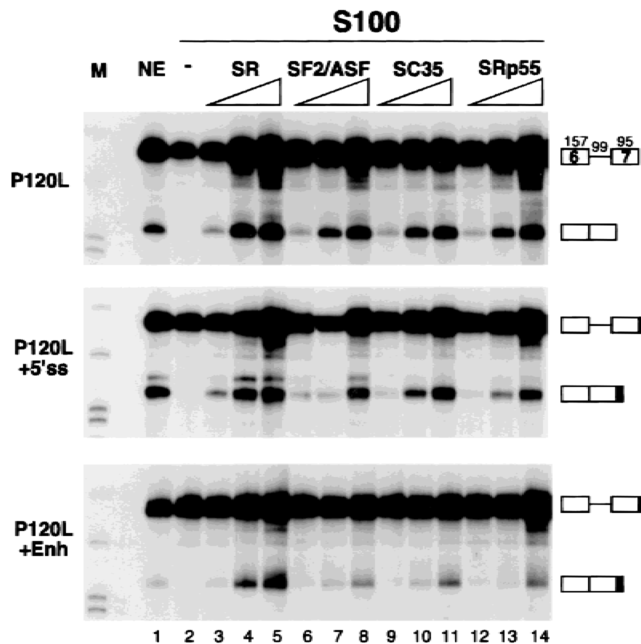

FIGURE 5. P120 exon 7 sequences enhance splicing. Basal splicing of a P120 transcript with an extended downstream exon. P120L (top panel), P120L+5′ss (middle panel), and P120L+Enh (bottom panel) transcripts were used in in vitro splicing assays containing nuclear extract or S100 extract complemented with SR proteins. Total Hela SR proteins (0.05, 0.1, and 0.2 μg, lanes 3–5), rSF2/ASF (0.05, 1, 2 pmol, lanes 6–8), rSC35 (2, 4, 8 pmol, lanes 9–11) or rSRp55 (0.05, 1, 2 pmol, lanes 12–14) were incubated with the corresponding substrate RNAs in S100 extract. The structure of each P120L RNA is shown next to each panel, with sizes of exons and introns indicated.

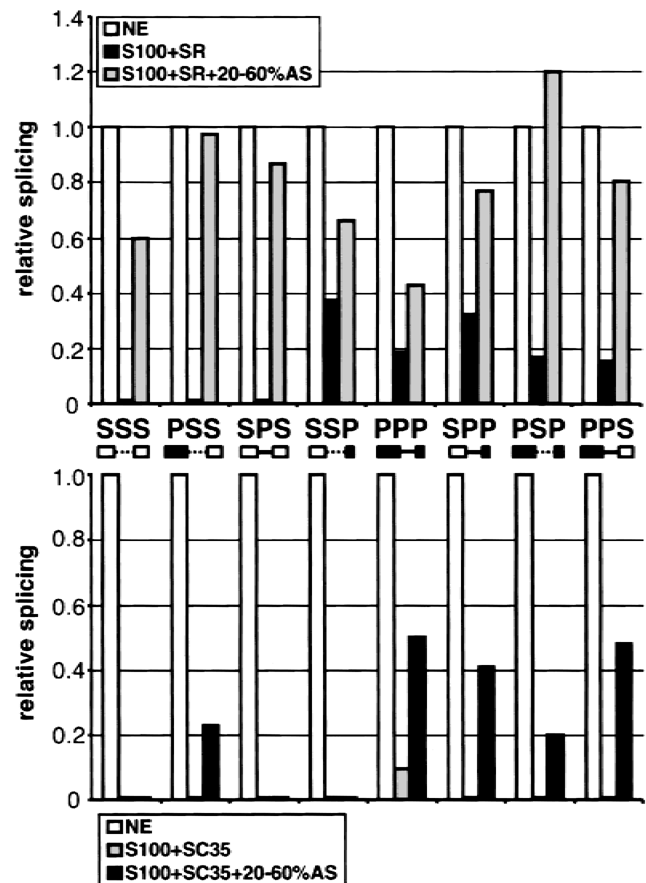

FIGURE 6. Splicing of SCN4A/P120 chimeric RNA. Quantitation of in vitro splicing assays of chimeric splicing substrates in nuclear extract (NE), S100 extract with total SR proteins (top, 0.15 μg) or recombinant SC35 (bottom, 8 pmol) or S100 extract with the 20–60%AS fraction and either SR proteins (top) or recombinant SC35 (bottom). Splicing efficiency for each substrate in nuclear extract was arbitrarily set at 1 and splicing in the complemented S100 extracts was normalized relative to this value. Splicing was quantitated using the ratio of lariat RNA/(lariat + pre-mRNA). For consistency, lariat RNA was used to quantitate splicing because only two possible intron lariats were produced from chimeric substrates, whereas each chimera produced a spliced mRNA product of different size and stability. Diagrams of substrates are shown beneath the corresponding name, with SCN4A exons and introns as white boxes and hatched lines, respectively, and P120 exons and introns as solid boxes and lines, respectively. SSS is SCN4AS and PPP is P120L.

required, in addition to SR proteins, for complementation of splicing in S100 extract. However, when SCN4A exon 3 was replaced with the P120 exon 7 fragment from the P120L transcript (SSP), SR proteins alone were sufficient for splicing in S100 extract (Fig. 6, top). Thus, either P120L exon 7 sequences activate splicing to bypass the need for the 20–60%AS fraction, or SCN4A exon 3 sequences block splicing in the absence of the 20–60%AS fraction.

To distinguish between these possibilities, similar chimeric substrates were constructed in the context of the P120L substrate (Fig. 6, top). Replacement of P120L exon 6, intron F, or exon 7 with the SCN4A exon 2, intron 2, or exon 3, respectively (SPP, PSP, or PPS substrates) had little effect on splicing in S100 extract in the presence of SR proteins, relative to the P120 parent substrate (PPP). Although the 20–60%AS fraction stimulated splicing in S100 extract in the presence of SR proteins for all of the substrates, this fraction was not required for splicing. This result suggests that SCN4A exon 3 alone does not specifically block splicing in S100 extract in the absence of the 20–60%AS fraction. Additionally, the P120L exon 7 fragment does not appear to be specifically required for P120L splicing in S100 extract complemented with SR proteins (PPS substrate). Rather, it appears that the pre-mRNA sequences that determine the 20–60%AS fraction requirement are dispersed throughout the SCN4A substrate, because exon 3 is necessary (SSP) but not sufficient to confer this requirement to the P120 substrate (PPS substrate).

As shown above (Figs. 2 and 5), SC35 complemented splicing of P120 but not SCN4A substrates in S100 complementation assays. We used the chimeric substrates to identify the substrate regions that are responsible for SC35 specificity. Splicing of the chimeric substrates was assayed in the presence of SC35 in S100 extract, with or without addition of the 20–60%AS fraction. Interestingly, splicing of an SCN4A substrate in which exon 2 was replaced with P120 exon 6 (PSS

substrate) was complemented by SC35 in S100 extract with the 20–60%AS fraction. This result suggests that SC35 acts via sequences within exon 6 of P120 to activate splicing. However, a P120 substrate in which exon 6 was replaced with SCN4A exon 2 (SPP substrate) also was spliced in S100 extract in the presence of the 20–60%AS fraction and SC35, suggesting that P120 exon 6 is not the sole mediator of SC35 activity. In addition, none of the chimeric substrates were spliced in the absence of the 20–60%AS fraction, suggesting that total SR proteins, but not SC35, can bypass the requirement for the 20–60%AS fraction in splicing of SSP, SPP, PSP, and PPS substrates (Fig. 6). Taken together, these results indicate that SR protein specificity is determined by interactions between multiple exonic and intronic regions of the pre-mRNA substrate, and illustrate the complexity and likely combinatorial control in the splicing of any single intron.

DISCUSSION

The discovery that members of the SR protein family of splicing factors, which are involved in multiple steps of conventional splicing, are also involved in AT-AC intron splicing has implications for the mechanisms of splicing via two distinct pathways. The basic functions of SR proteins appear to be similar in both splicing reactions, and SR proteins appear to have a role in the interplay between the two pathways during exon definition.

Substrate-specific requirements for splicing of SCN4A and P120 AT-AC introns

We found that SR proteins function in splicing of the two AT-AC introns that were tested, although the precise requirements were different. Splicing of the SCN4A, but not the P120, AT-AC intron required an additional factor(s), besides SR proteins, that is not present in S100 extract. These results suggest that the contribution of the activity present in a 20–60%AS fraction of nuclear extract is not essential for AT-AC splicing. Rather, the 20–60%AS fraction likely contains at least one factor that is specifically required for splicing of some AT-AC introns. Alternatively, the 20–60%AS fraction may supplement the S100 extract with a factor that is already present in limiting amounts in this extract, and is required at a higher concentration for SCN4A splicing compared to P120 splicing. The 20–60%AS fraction does not appear to supplement the reaction with the AT-AC-specific snRNPs, because the P120 intron is spliced by the same minor snRNPs and does not require the 20–60%AS fraction. Furthermore, SCN4A in vitro splicing in nuclear extracts with inactivated U12 or U6atac snRNAs was rescued by S100 extract but not by the 20–60%AS fraction (not shown). These observations suggest that the S100 extract contains sufficient minor snRNPs to support splicing.

SCN4A/P120L chimeric transcripts were used to test the basis of the substrate-specific dependence on the 20–60%AS fraction. Substitution of just SCN4A exon 3 with P120 exon 7 eliminated the requirement for the 20–60%AS fraction (SSP substrate, Fig. 6). At the same time, none of the P120 substitutions with SCN4A exons or intron (SPP, PSP, PPS substrates) rendered the substrates dependent upon the 20–60%AS fraction for splicing in S100 complementation assays. These results indicate that the P120 exon 7 fragment bypasses the requirement for the 20–60%AS fraction in the SSP substrate, suggesting that the exon can activate splicing in the absence of that fraction. Alternatively, the 20–60%AS fraction may function to overcome a splicing block that is associated in part with the SCN4A exon 3. However, another portion of the SCN4A substrate, in addition to the downstream exon, must also be required for this putative block, because SCN4A exon 3 was not sufficient to confer 20–60%AS fraction-dependence on the P120 substrate (PPS substrate). The splicing efficiency of the chimeric substrates in nuclear extract did not correlate with the dependence on the 20–60%AS fraction (not shown), and thus the overall efficiency of splicing does not determine the requirement for the 20–60%AS fraction.

The requirement for the 20–60%AS fraction activity may not be unique to a subset of AT-AC introns. A similar nuclear extract fraction was shown previously to be required for complementation of splicing of a synthetic U2-dependent RNA substrate in S100 extract supplemented with rSF2/ASF (Tacke & Manley, 1995). Additionally, the bovine growth hormone intron D (F. Rottman, pers. comm.) as well as the β-tropomyosin intron 5 (D. Helfman, pers. comm.) require a similar nuclear fraction, in addition to SR proteins, to activate splicing in S100 extract. Though it is possible that one factor is responsible for the splicing activity in all these substrates, many nuclear factors are present in these crude fractions.

Interestingly, the pair of cryptic U2-dependent splice sites of SCN4A also requires the 20–60%AS fraction, in addition to SR proteins, for splicing in S100 extract (Fig. 1). This observation suggests that the two mutually exclusive splicing pathways are somehow linked in this pre-mRNA and the two different pairs of sites have similar requirements. On the other hand, splicing of the cryptic and the AT-AC sites can occur independently of each other, as demonstrated by inactivation of either pathway (Fig. 3). One interesting possibility is that the 20–60%AS fraction is required at a very early step, common to both spliceosome assembly pathways and before commitment to one of them, perhaps to allow recognition of the RNA in the region surrounding the AT-AC and cryptic GT-AG splice sites.

The 20–60%AS fraction may contain a splicing coactivator. Splicing coactivators interact with factors that bind to pre-mRNA, such as SR proteins and snRNPs,

to promote splicing. An example of a coactivator is the SRm160/300 complex (Blencowe et al., 1998). A HeLa nuclear extract fraction containing SRm160/300 enhances splicing of all tested substrates when added together with SR proteins to S100 extracts, but is only specifically required for efficient splicing of a subset of pre-mRNAs (Blencowe et al., 1998). Likewise, our results show that splicing of P120 as well as the chimeric substrates is stimulated by simultaneous addition of the 20–60%AS fraction and SR proteins to S100 extract, though only SCN4A and a subset of the chimeric substrates strictly require the 20–60%AS fraction for splicing (Fig. 6). One important distinction between the activities of the SRm160/300 fraction and the 20–60%AS fraction, however, is that SRm160/300 increases splicing efficiency of U2-dependent introns in the presence of SR proteins but is not absolutely required for splicing (Blencowe et al., 1998), whereas the SCN4A AT-AC intron is not spliced at all in the absence of the 20–60%AS fraction, even upon addition of excess SR proteins. Although the 20–60%AS fraction described here has properties of a splicing coactivator, the factor responsible for this activity is probably not SRm160/300, which is soluble at high concentrations of ammonium sulfate (Blencowe et al., 1995) and therefore should not be enriched in the 20–60%AS fraction.

A splicing activator, RNPS1, has been described, which is distinct from a splicing coactivator in that it probably contacts the pre-mRNA directly (Mayeda et al., 1999). RNPS1 stimulates splicing when added together with limiting SR proteins to S100 extracts but exhibits little substrate specificity. However, RNPS1 is not responsible for the 20–60%AS activity, as addition of recombinant RNPS1 and SR proteins to S100 extract did not activate SCN4A splicing (not shown).

Recombinant SR protein activity in AT-AC splicing

Our results show that individual SR proteins can activate splicing of both SCN4A and P120 AT-AC introns. Differences in recombinant protein activity between the two substrates were observed (Figs. 2A and 4B). An obvious difference between the SR protein requirements for splicing of P120 and SCN4A pre-mRNAs is that SC35 did not activate splicing of any of the SCN4A substrates but was functional for P120 splicing in S100 extract. The results from experiments using SCN4A/P120L chimeric splicing substrates suggest that P120 exon 6 is important for SC35 activity in splicing of P120, given that splicing of the PSS substrate was activated by recombinant SC35 in the S100 complementation assay (Fig. 6). A recent study showed that a region of the 5′ exon is required for establishing U12/U6atac and U6atac/5′ss interactions during AT-AC spliceosome assembly (Frilander & Steitz, 2001). Perhaps specific sequences within P120 exon 6 are recognized by SC35 and promote spliceosomal interactions at the 5′ss. However, other regions of the substrate must also be important in determining SC35 specificity, as splicing was still observed in S100 extract with SC35 as the sole SR protein when exon 6 in the P120 substrate was replaced with SCN4A exon 2 (SPP substrate).

All of the chimeric substrates required the 20–60%AS fraction for splicing in S100 extract when SC35 was the only SR protein. This result differs from splicing in S100 extract in the presence of total SR proteins for some of the chimeric substrates, which did not require the 20–60%AS fraction. Thus, total SR proteins can bypass the requirement for the 20–60%AS fraction in splicing of some, but not all, of the AT-AC splicing substrates. These observations are consistent with the idea that the 20–60%AS fraction is a splicing coactivator that is only required for splicing of particular substrates and perhaps functions with a subset of SR proteins in a substrate-specific manner.

Another difference in the activity of the recombinant SR proteins was the inability of SF2/ASF to activate SCN4AS splicing (Fig. 2A). In this case, unlike with SC35 complementation, a downstream 5′ss or enhancer sequence stimulated splicing when SF2/ASF was the sole SR protein. The lack of activity with the basal substrate suggests that SF2/ASF cannot mediate interactions spanning the SCN4A intron. The absence of SCN4AS splicing may also indicate the presence of an intrinsic splicing enhancer in SCN4A that is recognized by 9G8 and SRp55 but not by SF2/ASF. The presence of an SF2/ASF-specific splicing silencer in SCN4A cannot be ruled out.

The differential activities of the recombinant SR proteins in the splicing of the SCN4A+Enh substrate may be due to specificity of the heterologous enhancer for a specific SR protein. However, it seems an unusual coincidence that the SCN4A+5′ss substrate showed the same SR protein specificity. Also, if the heterologous enhancer favors interactions with a specific SR protein, then the P120+Enh substrate should show the same trend. However, all the recombinant SR proteins stimulated P120+Enh splicing equally. These results suggest that differences in splicing are likely due to substrate-specific differences among SR proteins, rather than to overall recombinant protein activity or heterologous enhancer or downstream 5′ss specificity.

P120 exon 7 sequences stimulate splicing of the upstream AT-AC intron

We have further investigated the role of SR proteins in AT-AC splicing by analyzing intrinsic *cis*-acting sequences that may be involved in splicing. P120S pre-mRNA, with an exon 7 truncated 54 nt downstream of the intron F 3′ss, was not efficiently spliced in nuclear extract (Fig. 4). However, extending the exon to include 95 nt of exon 7 strongly stimulated splicing (Fig. 5),

suggesting that the 41-nt element comprises part or all of a natural splicing enhancer. This enhancer activity was recapitulated in S100 extract complemented with SR proteins. The identification of a natural AT-AC exonic splicing enhancer confirms that ESE activity is important in AT-AC splicing. The 41-nt element of P120L contains a purine-rich region similar to those found in other ESEs (Wu & Krainer, 1999). Moreover, using available score matrices for SF2/ASF, SRp55, and SC35 to identify sequences with SR protein recognition motifs (Liu et al., 1998, 2000), high scores for all three SR proteins were found in the 41-nt region (not shown). A downstream 5′ss was not able to significantly stimulate splicing of the P120L substrate, suggesting that the enhancer activity and the downstream 5′ss activity are mutually exclusive. In addition, the presence of the heterologous ESE (P120+Enh) appeared to abrogate the activity of the natural splicing enhancer. Although multiple enhancers have been shown to act additively (Hertel & Maniatis, 1998), in the case of P120L splicing the natural and heterologous enhancers appear to counteract each other.

Spliceosome assembly on AT-AC introns

SR proteins are required early in the splicing reaction in conventional intron splicing (Krainer et al., 1990a). The earliest identified spliceosomal complex in AT-AC splicing is the A complex, which is an ATP-dependent complex containing U11 and U12 snRNAs (Tarn & Steitz, 1996b; Frilander & Steitz, 1999). Our results suggest that SR proteins stimulate these interactions to promote A complex assembly (Fig. 4C). In conventional splicing, SR proteins stimulate stable binding of U1 snRNP to the 5′ss (Kohtz et al., 1994), and U2 snRNP to the BPS (Tarn & Steitz, 1995) as well as association of U4/U6•U5 with the pre-mRNA (Roscigno & Garcia-Blanco, 1995) and formation of U2-U6 base pairing (Tarn & Steitz, 1995). Analogous functions of SR proteins in AT-AC intron splicing remain to be determined. It is remarkable, however, that SR proteins function in a similar manner in both splicing pathways, considering that SR protein activities, at least for splicing of U2-dependent introns, involve the stimulation of highly specific interactions between the major snRNPs and the GT-AG splice sites.

Models for SR protein activity in AT-AC intron splicing

Because many of the activities of the SR proteins appear to be conserved between the two splicing pathways, a comparison can be made using the known interactions and functions of SR proteins in U2-dependent splicing to predict possible roles of SR proteins in AT-AC splicing (Fig. 7). We have addressed the role of SR proteins in interactions spanning the intron in the absence of exonic splicing enhancer signals (basal splicing), as compared to exon definition interactions mediated by an ESE or downstream 5′ss. The basal SCN4A substrate was spliced in nuclear extract, and P120S basal splicing was nearly undetectable. Similar results were obtained in S100 extract complemented with SR proteins in the presence (SCN4AS) or absence (P120S) of the 20–60%AS fraction, except that at high concentrations of SR proteins, a low level of P120S splicing was detected (Fig. 4A). These results suggest that SR proteins can mediate intron definition in AT-AC splicing, though, we cannot rule out the possibility that SR proteins act via intrinsic ESEs present in the basal substrates.

If intron definition in AT-AC splicing occurs in an SR protein-dependent manner, it is tempting to envision a mechanism analogous to that proposed for conventional splicing, in which SR proteins interact with U11 snRNP, instead of U1 snRNP, and with factors at the 3′ss to bridge the intron through a network of protein–protein interactions (Fig. 7). In conventional splicing, SR proteins interact with the RS domain of a U1 snRNP protein, U1-70K, at the 5′ss (Wu & Maniatis, 1993; Kohtz et al., 1994). Recently, a 35-kDa protein with homology to U1-70K was found in purified U11 snRNP

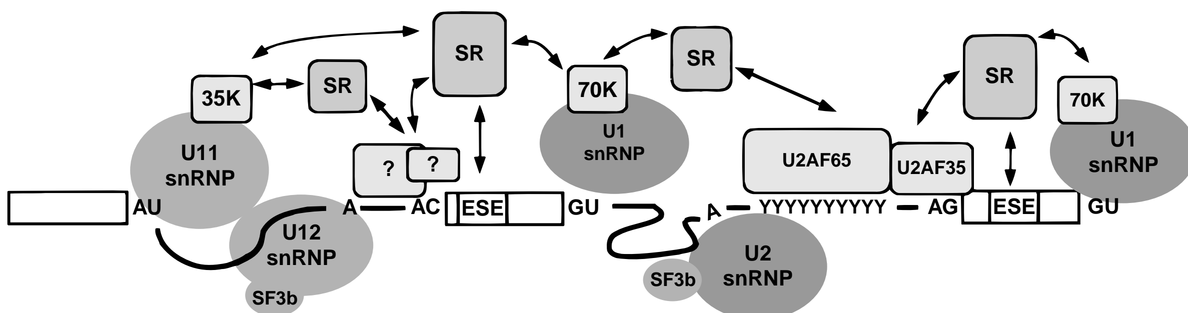

FIGURE 7. Model for SR protein functions in AT-AC intron splicing. For comparison, SR protein interactions in a downstream GT-AG intron are depicted. SR protein interactions spanning introns and exon definition via an exonic splicing enhancer or a downstream 5′ss are illustrated. Arrows indicate potential RNA–protein and protein–protein interactions.

(Will et al., 1999). This protein may be a target for SR protein interactions in the AT-AC splicing pathway.

Efficient splicing of the SCN4A and P120 substrates with a 5′ss or enhancer in the downstream exon is consistent with the ability of SR proteins to stimulate splicing via downstream stimulatory elements. It was shown previously that U1 snRNP is dispensable for ESE activity in AT-AC splicing, but required for the enhancement by a downstream 5′ss (Wu & Krainer, 1996, 1998). The combined results suggest that SR proteins can support both types of splicing enhancement, though they likely act by two distinct mechanisms, one involving interactions with U1 snRNP at the downstream 5′ss, and the other functioning independently of U1 snRNP. In the case of a downstream 5′ss, it is likely that, similar to conventional splicing, SR proteins interact with U1 snRNP bound at a downstream 5′ss, and act as a bridge by interacting with factors at the upstream AT-AC 3′ss (Fig. 7). It will be of interest to see if an AT-AC 5′ss can also stimulate splicing of an upstream intron, which would suggest that U11 snRNP can fulfill the same function as U1 snRNP in enhancing upstream intron splicing from a downstream 5′ss.

U1 snRNP is a likely target for SR protein interactions at the 5′ss in an exon definition model for splicing. However, a target at the AT-AC 3′ss in both intron and exon definition models for AT-AC splicing is difficult to predict. A downstream conventional 5′ss has been shown to increase $U2AF^{65}$ binding to the upstream conventional 3′ss (Hoffman & Grabowski, 1992). One model for ESE activity proposes that SR proteins interact with the enhancer and recruit $U2AF^{35}$ to the upstream 3′ss, thereby promoting intron splicing (Zuo & Maniatis, 1996; Guth et al., 1999; Blencowe, 2000). A role for U2AF in AT-AC splicing has not been identified. AT-AC introns lack the Py tract that $U2AF^{65}$ interacts with in conventional introns (Zamore & Green, 1989). Likewise, $U2AF^{35}$ specifically recognizes the conventional 3′ss sequence AG/G (Wu et al., 1999), which is absent in many AT-AC introns. U2AF may not be required for AT-AC splicing and SR proteins may act through a different mechanism to enhance AT-AC intron splicing. Alternatively, unknown factor(s) common to both splicing pathways may be the primary target for SR proteins in enhancer-mediated stimulation of splicing. Further comparative analysis of protein requirements in AT-AC and conventional splicing is expected to yield important insights into splice-site recognition mechanisms and the evolution of both splicing pathways.

MATERIALS AND METHODS

RNA substrates

SCN4A template plasmids pSP64-SCN4A (SCN4A+5′ss), pSP64-SCN4AS, and pSP64-SCN4AENH12 (SCN4A+Enh) were linearized with *Xba*I, *Bam*HI, and *Eco*RI, respectively, and transcribed with SP6 RNA polymerase (Wu & Krainer, 1996, 1998). The P120 plasmid pSP64-P120 was used in PCR to amplify a 462-bp fragment of human genomic P120 comprising 12 nt upstream of exon 6, intron F, exon 7, and 7 nt of intron G. The 5′ primer was flanked with a *Hin*dIII site and the 3′ primer with a *Sac*I site, which were used after digestion to insert the fragment into corresponding sites in plasmid pSP64. The plasmid was linearized with *Eco*RI, which digests 54 nt into exon 7, and transcribed with SP6 RNA polymerase to make P120S pre-mRNA. The same P120 and SCN4A plasmids were used as a PCR templates for all additional substrates. P120 RNA substrates transcribed from either pSP64-P120S or from a PCR product gave similar results in in vitro splicing assays. The SCN4A/P120L chimeric RNA transcripts were constructed by overlap-extension PCR and transcribed with T7 RNA polymerase. Substrates transcribed from PCR products contained either the entire SCN4A exon 2 (119 nt) or P120 exon 6 (157 nt); the entire SCN4A intron 2 (126 nt) or P120 intron F (99 nt); and the entire SCN4A exon 3 (90 nt) or 54 nt (P120S) or 95 nt (P120L, SSP, SPP) of P120 exon 7. β-globin pre-mRNA was made from pSP64-H$\beta\Delta$6 as described (Krainer et al., 1984).

HeLa cell extracts, fractions, and recombinant proteins

Nuclear and S100 extracts were prepared as described (Mayeda & Krainer, 1999b). Ammonium sulfate fractionation was carried out as described (Murray et al., 1999). Purified total HeLa SR proteins were prepared as described (Zahler, 1999). Recombinant SF2/ASF was expressed in *Escherichia coli* and purified (Screaton et al., 1995). Recombinant SC35 was expressed in baculovirus (virus stock kindly provided by T. Maniatis) and purified as described (Tian & Maniatis, 1993). Recombinant SRp55 and 9G8 expressed in baculovirus were a generous gift from F. Rottman.

In vitro splicing and spliceosome assembly assays

Conditions for in vitro transcription and splicing were described previously for β-globin (Mayeda & Krainer, 1999a) and the SCN4A substrates (Wu & Krainer, 1996, 1997). P120 substrate preparation was essentially identical. In vitro splicing of β-globin was carried out for 2–4 h as described (Mayeda & Krainer, 1999a) except that 32 mM HEPES (pH 7.3) was used and nuclear extract made up 40% of the reaction. SCN4A in vitro splicing was performed similarly, except that 2.5 mM ATP was used. SCN4A and chimeric substrates were spliced in the presence of 5–5.5 mM $MgCl_2$. P120 pre-mRNA splicing reactions were similar, but the concentration of $MgCl_2$ was 4–5 mM. Chimeric RNA substrates were spliced in the presence of 5 mM $MgCl_2$. Splicing reactions were incubated at 30 °C for 5–5.5 h. Oligonucleotide-directed RNase H digestion of snRNAs from nuclear extract was carried out as described (Wu & Krainer, 1996). Quantitation of splicing was carried out with a FujiX Bas 2000 phosphorimager. Percent splicing was determined by the molar ratio of mRNA/(pre-mRNA + mRNA). Native-gel splicing complex assays were done with 0.5 mg/mL heparin as described (Konarska, 1989).

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