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A Subset of SR Proteins Activates Splicing of the Cardiac Troponin T Alternative Exon by Direct Interactions with an Exonic Enhancer

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The cardiac troponin T pre-mRNA contains an exonic splicing enhancer that is required for inclusion of the alternative exon 5. Here we show that enhancer activity is exquisitely sensitive to changes in the sequence of a 9-nucleotide motif (GAGGAAGAA) even when its purine content is preserved. A series of mutations that increased or decreased the level of exon inclusion in vivo were used to correlate enhancer strength with RNA-protein interactions in vitro. Analyses involving UV cross-linking and immunoprecipitation indicated that only four (SRp30a, SRp40, SRp55, and SRp75) of six essential splicing factors known as SR proteins bind to the active enhancer RNA. Moreover, purified SRp40 and SRp55 activate splicing of exon 5 when added to a splicing-deficient S100 extract. Purified SRp30b did not stimulate splicing in S100 extracts, which is consistent with its failure to bind the enhancer RNA. In vitro competition of SR protein splicing activity and UV cross-linking demonstrated that the sequence determinants for SR protein binding were precisely coincident with the sequence determinants of enhancer strength. Thus, a subset of SR proteins interacts directly with the exonic enhancer to promote inclusion of a poorly defined alternative exon. Independent regulation of the levels of SR proteins may, therefore, contribute to the developmental regulation of exon inclusion.

Pre-mRNA splicing involves two transesterification reactions mediated by the spliceosome (11, 21). The mechanisms by which exon-intron borders are first defined within complex pre-mRNAs remain largely unknown. Although splice donor and acceptor sequences are fairly well conserved among pre-mRNAs, these elements are not sufficient to distinguish bona fide splice sites from unused cryptic splice sites present in many metazoan pre-mRNAs. Because splicing is so precise, additional controls for splice site selection must be present elsewhere in the pre-mRNA.

Results with vertebrate and invertebrate experimental systems have demonstrated that sequences within exons, exclusive of splice junctions, can play a role in splice site selection (13). One positively acting splicing element is a 13-nucleotide repeat in the female-specific exon of the *Drosophila doublesex* (*dsx*) gene. Two proteins, *transformer-2* and *transformer*, promote utilization of an upstream 3′ splice site via direct interactions with the repeats (12, 15, 22). Purine-rich splicing elements, called splicing enhancers, have recently been identified in several vertebrate exons (1, 6, 16, 19, 28, 31, 33, 35, 36) and in the *dsx* female-specific exon (20). Splicing enhancers are required for efficient splicing of the resident exon, and several have been shown to enhance splicing of heterologous exons (16, 19, 33, 35, 36). With one exception (16), splicing enhancers appear to activate splicing of the upstream intron (35). The enhancer in the fibronectin ED1 exon has been shown to promote binding of the U2 small nuclear ribonucleoprotein particle (snRNP) to the upstream branch site (19), which is consistent with this observation. The vertebrate splicing enhancers are commonly 6 to 13 nucleotides in length, are often present in multiple copies within an exon, and generally contain contiguous repeats of the motif GAR, where R is a G or an A (35). In vitro analysis with synthetic polypurine sequences demonstrated that purine richness alone is not sufficient for enhancer activity (29); however, the specific sequence requirements for enhancer activity have not been determined.

SR proteins are a highly conserved family of essential splicing factors (SRp20, SRp30a [SF2/ASF], SRp30b [SC35/PR264], SRp40, SRp55, SRp75, and 9G8). Individual SR proteins can complement splicing-deficient extracts (2, 9, 10, 18, 37), and association of the SR proteins with the pre-mRNA is required for the earliest steps of spliceosome assembly (8, 18). Interactions between SR proteins and pre-mRNA at the 3′ splice site (8) and 5′ splice site (41) have been demonstrated; SR proteins have been demonstrated to promote binding of U2 and U1 snRNPs to the 3′ and 5′ splice sites, respectively (7, 9, 17, 39). SR proteins have also been shown to bind to enhancers contained in exons from the fibronectin, bovine growth hormone, and avian sarcoma virus *env* genes (19, 27, 28) as well as to purine-rich and non-purine-rich enhancers in *dsx* exon 4 (20, 30). The results from those studies suggested that different exonic enhancers bind to and are activated by different members of the SR protein family. However, the nucleotide requirements for the vertebrate enhancers and therefore the specific targets for SR protein binding remain to be defined. In addition, a direct correlation between SR protein binding and activation of a vertebrate enhancer has been established for only one SR protein, SRp30a (28).

Previous work characterized a splicing enhancer in the alternative exon (exon 5) of the cardiac troponin T (cTNT) gene (3, 5, 35). The cTNT gene is expressed only in cardiac and skeletal muscle, where exon 5 inclusion predominates in the embryo and exon skipping predominates in the adult (4). In

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this study, we localized the core splicing enhancer activity to a 9-nucleotide sequence and defined specific nucleotide requirements for enhancer activity in vivo. Enhancer mutants with a range of activities were then used to correlate enhancer strength in vivo with sequence-specific enhancer-protein interactions in vitro. Using a series of point mutations that increase and decrease the level of enhancer activity in vivo, we demonstrated a direct correlation between enhancer strength and the relative binding affinity of four SR proteins: SRp30a, SRp40, SRp55, and SRp75. Two SR proteins that bind to the enhancer, SRp40 and SRp55, activate splicing of exon 5. In contrast, SRp30b, which shows a low affinity for the enhancer, does not activate splicing of exon 5. These results demonstrate sequence-specific binding of a subset of at least four SR proteins to the cTNT exon 5 enhancer and directly demonstrate a correlation between SR protein binding in vitro and enhancer activity in vivo.

MATERIALS AND METHODS

Plasmid construction, transfection, and RNA analysis. The ΔPB.SA plasmid (Fig. 1B) was derived from ΔPB (35) by generating a *Sal*I site (one nucleotide substitution) and an *Asp*718 site (two nucleotide substitutions) 99 and 71 nucleotides upstream and downstream from the exon, respectively. These substitutions do not affect splicing in vivo (unpublished data). Site-directed mutagenesis was performed on the exon by PCR with the megaprimer approach (26). All mutations were confirmed by sequencing. QT35 and primary chicken breast muscle cultures were transiently transfected and RNA was analyzed by primer extension as described previously (35). Primer extension products were quantitated directly from the gel with a Betagen Betascope 603 analyzer.

In vitro splicing. Synthesis of splicing substrates, preparation of HeLa cell nuclear extracts, and in vitro splicing assays were performed as described previously (3) except that splicing reactions were performed in 10 μl by using 5 μl of nuclear extract (50 μg of total protein). The E45SA RNA substrate differs from the E45 RNA previously described (3) by a single nucleotide change to introduce a *Sal*I site 99 nucleotides upstream of exon 5 and by deletion of a 36-nucleotide *Bam*HI fragment from the polylinker at the 5′ end of the RNA. Plasmid templates were cleaved at a natural *Mae*III site so that the RNA contained 8 nucleotides of intron 5. Plasmid templates for all mutant exon competitor RNAs except the HET RNA were derived from the respective minigenes listed in Fig. 1A. HET RNA contained the 30-nucleotide exon 2 from skeletal troponin I. To construct the HET plasmid, a synthetic exon 2 was blunt end cloned into Bluescript (Stratagene), which was digested with *Sac*I and *Asp*718 and treated with T4 DNA polymerase. The template was cut with *Rsa*I and transcribed with T7 RNA polymerase.

Competitor RNAs were synthesized under standard conditions in 100-μl reaction mixtures containing 3 μg of template DNA, 4 mM GATC, and 5 to 10 μCi of [^{32}P]UTP (800 mCi/mmol) for quantitation. RNAs were visualized by UV shadowing and were gel isolated. Splicing and UV cross-linking competitions were performed by mixing substrate and competitor RNAs prior to addition to the splicing reaction mixture. Preincubating reaction mixtures with competitor RNA prior to adding substrate did not show any additional competition (data not shown).

Analysis of spliceosome assembly was performed by native gel electrophoresis as described by Zillmann et al. (40) under our standard splicing conditions.

UV cross-linking and immunoprecipitation. UV cross-linking was performed with 1.0×10^5 to 2.0×10^5 cpm of substrate (2×10^6 cpm/μg) labeled with [^{32}P]GTP and [^{32}P]UTP. Splicing reaction mixtures (10 μl) containing 4 μl of nuclear extract were incubated at 30°C for 10 min. The reaction mixtures were placed on an aluminum block prechilled in ice water and were UV irradiated 4 cm from a germicidal lamp (Philips G15T8) for 8 min. Samples were digested with RNase A (0.5 μg) and RNase T_1 (0.5 μg) at 37°C for 30 min. An equal volume of protein loading buffer was added, and the samples were denatured at 90°C and run on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel. Sizes were determined with prestained markers (low range; Bio-Rad). The specificity of the anti-SR protein monoclonal antibody is similar to that of monoclonal antibody MAb 104 (22a, 24, 37). Anti-SF2/ASF monoclonal antibody was a generous gift from A. Krainer (Cold Spring Harbor Laboratory). For immunoprecipitation reactions, 150 to 300 μl of supernatant was prebound to protein G-Sepharose (20 μl of Gammabind beads; Pharmacia) in IP buffer (10 mM $NaPO_4$ [pH 7.0], 150 mM NaCl, 10 mM EDTA) for 1 h at room temperature. UV cross-linking reaction mixtures were incubated with prebound antibody in 40-μl reaction mixtures containing IP buffer at room temperature for at least 2 h. After four washes with IP buffer, bound proteins were removed from the beads in 40 μl of SDS loading buffer at 75°C for 4 min and loaded onto a denaturing polyacrylamide gel.

S100 complementation and competition assays. Individual and total SR proteins were purified from calf thymus or HeLa cells as previously described (37, 38). The SR protein concentration was determined by comparing the intensity of Coomassie staining of an SDS-polyacrylamide gel electrophoresis (PAGE) gel with that of a bovine serum albumin standard.

Preparation of HeLa S100 splicing-deficient extracts, human β-globin ^{32}P-labeled substrate pre-mRNA, in vitro splicing conditions, and urea-PAGE analysis were previously described (37, 38). Only very low levels of residual SR proteins could be detected in S100 extracts by immunoblotting. S100 complementation assays were done in 25-μl reaction mixtures. Each reaction mixture contained approximately 0.1 pmol of ^{32}P-labeled pre-mRNA. Competitor RNAs and substrate pre-mRNAs were added prior to the addition of SR proteins and S100 extract. All reaction mixtures were prepared on ice and then incubated at 30°C for 2 h.

RESULTS

Sequence requirements for enhancer activity in vivo. cTNT exon 5 contains two purine-rich motifs separated by a short pyrimidine-containing region. A series of point mutations was generated within each of these three regions to define the specific sequence requirements for enhancer activity (Fig. 1A). The purine content of the purine-rich motif was not altered in any of the mutations, and mutations did not introduce sequences that might affect splicing such as stop codons or "pseudo 5′ splice sites." The exon 5 mutations were introduced into a minigene (Fig. 1B), and their effects on exon inclusion were tested by transient transfection into a fibroblast cell line (QT35) and primary skeletal muscle cultures as described previously (35). In QT35 cells, exon 5 is predominantly skipped (20 to 30% inclusion) in minigene mRNAs, while in primary skeletal muscle cultures it is predominantly included (70 to 80% inclusion) (35).

The results of the mutational analysis presented in Fig. 1A reveal several features of the splicing enhancer present in exon 5. First, the 5′ purine-rich motif (exon nucleotides 3 to 11; GAGGAAGAA) and not the 3′ purine-rich motif is primarily responsible for enhanced splicing. Note that the first two nucleotides of the exon are considered to be a component of the 3′ splice site rather than of the enhancer. The 5′ motif differs from the 3′ motif by only one nucleotide (GAGGAA GAC); however, the same nucleotide substitutions had a much greater effect in the 5′ motif (compare mutations B and J or A and H in Fig. 1A). Some mutations in the 5′ motif eliminated enhancer activity (mutations B and D), indicating that the 3′ motif may be unable to function on its own. Second, when the central pyrimidines of exon 5 were converted to purines to extend the motif, exon inclusion was significantly enhanced (mutations N, O, and P). Third, purine richness alone is not sufficient for enhancer activity, since substitutions that changed the sequence but not the purine content of the 5′ motif significantly affected the levels of exon inclusion. All substitutions in nucleotides 3 to 11 reduced enhancer activity, indicating that the natural sequence is one of the strongest enhancers for this context. Finally, enhancer mutations have the same general effects in both muscle and nonmuscle cell types, suggesting that the determinants of enhancer strength are ubiquitously recognized.

Enhancer strength determines the efficiencies of splicing and spliceosome assembly in vitro. The splicing enhancer is believed to promote exon inclusion by stimulating the splicing of exon 4 to exon 5 on the basis of previous in vivo and in vitro analyses (3, 35). We therefore tested the splicing efficiencies of exons 4 and 5 in vitro using six mutant pre-mRNAs that had increased or decreased levels of exon inclusion in vivo. The seven pre-mRNAs containing unmodified or mutant exon 5 were identical except for the nucleotide substitutions in exon 5 shown in Fig. 1A. The results show that in vitro splicing efficiency directly correlated with the level of exon inclusion in

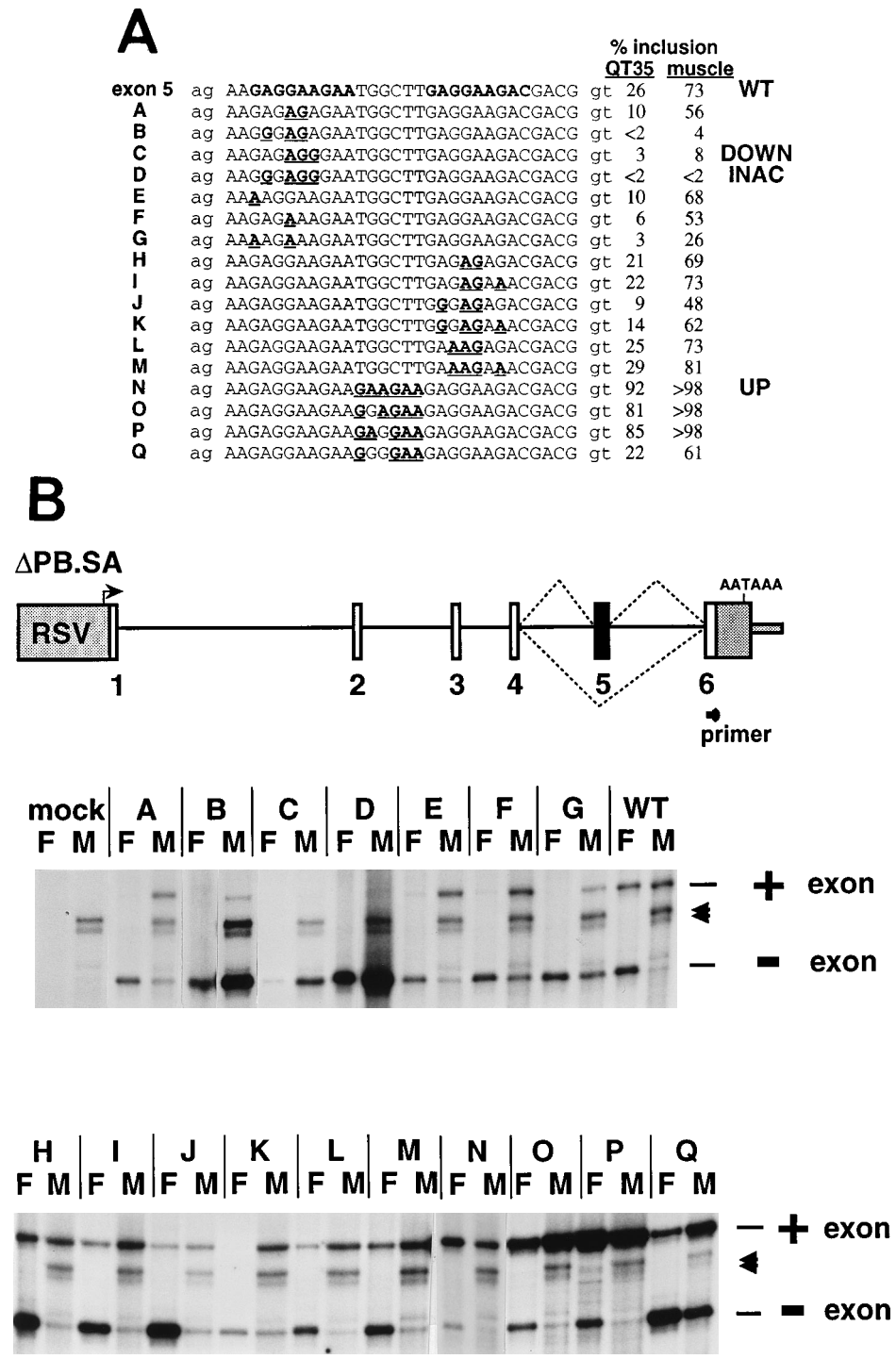


FIG. 1. Transient-transfection analysis of cTNT exon 5 mutants. (A) Exon mutations. The two purine-rich motifs in cTNT exon 5 are in boldface. Nucleotide substitutions in mutations A to Q are indicated by underlining and boldface type. Minigenes were transiently transfected into QT35 fibroblasts and primary skeletal muscle cultures, splicing was assayed by primer extension, and the level of exon inclusion was determined directly from the gel with a Betagen Betascope 610. The results are averaged from at least three independent transfections and are expressed as the percentages of spliced mRNA that include the alternative exon. Three mutations are designated according to their relative levels of exon inclusion in vivo (UP, higher than that of the unmodified exon; WT, the unmodified exon; DOWN, lower than that of the unmodified exon; INAC, complete exon skipping). (B) Primer extension analyses of exon mutants. Primer extension products from mRNAs that include (+) or exclude (−) the exon are indicated. Lanes: F, QT35 fibroblast; M, primary skeletal muscle. Primary skeletal muscle cultures express endogenous cTNT mRNAs that include exon 5 (indicated by the arrows). This band is a doublet due to two transcription start sites 4 nucleotides apart (4). Some lanes are overexposed to show extension products of both mRNAs. Lane-to-lane variations in RNA levels are due to differences in transfection efficiencies. Variation in RNA levels does not affect the levels of exon inclusion (data not shown).

vivo. Mutation N, which increased exon inclusion in vivo compared with inclusion of the unmodified exon, enhanced splicing of exons 4 and 5 in vitro. Similarly, mutations that decreased (B, C, G, and F) or eliminated (D) exon inclusion in vivo had corresponding effects on in vitro splicing efficiency. Therefore, the ranking of enhancer strengths was retained in vitro. The mutations that reduced splicing efficiency did not accumulate lariat-exon 5 or exon 4 intermediates, suggesting that enhancer mutations affected a step prior to the first transesterification reaction.

The same pre-mRNAs were used to determine which step of spliceosome assembly was affected by enhancer mutations (Fig. 2B). Mutations that increased or reduced the efficiency of exon inclusion in vivo similarly increased or reduced the levels of all three ATP-dependent complexes, including complex A, the first splicing complex that is detectable by this assay (40). Therefore, the enhancer in exon 5 is required for an early and rate-determining step of spliceosome assembly.

Enhancer strength correlates with binding affinity for titratable *trans*-acting factors. The results from in vitro analysis suggest that the enhancer mutations disrupt interactions with *trans*-acting factors that are required for an early step of spliceosome assembly. To determine whether the exon alone binds essential splicing factors, in vitro splicing of exons 4 and 5 was challenged with unmodified or mutant exon 5 competitor RNAs. The sequence of the competitor RNA containing the unmodified exon is shown in Fig. 3. Competitor RNAs are identical except for the nucleotide substitutions shown in Fig. 1A. The competition experiments whose results are shown in Fig. 3 demonstrated a direct correlation between the level of competition and enhancer strength. The N mutation (UP) (Fig. 3, lanes 2 to 4) was a more efficient competitor than the unmodified exon (WT) (lanes 5 to 7). A mutation that reduces exon inclusion in vivo (mutation C) (DOWN) was a less efficient competitor (Fig. 3, lanes 8 to 10), and the D mutation which inactivates that enhancer and leads to complete exon skipping in vivo (INAC) does not inhibit splicing in vitro (lanes 11 to 13). Splicing was not affected by the same molar amounts of RNA from either a heterologous constitutive exon which lacks an enhancer (HET) (Fig. 3, lanes 14 to 16) or three RNAs derived from internal positions of cTNT introns (data not shown). In addition, the UP and WT RNAs are not general inhibitors of splicing, since the identical competition reactions did not inhibit splicing of several substrates that lack an enhancer (data not shown). We conclude that titratable factors in HeLa nuclear extracts bind directly to exon 5 to mediate enhancer activity. Furthermore, the relative binding affinities of these factors for mutant enhancers directly correlate with enhancer strength.

SRp40 and SRp55 but not SRp30b activate splicing of cTNT exon 5 in S100 extracts. The requirement for the enhancer in the earliest steps of spliceosome assembly suggested the possibility that SR proteins directly mediate enhancer activity and are the titratable factors in HeLa nuclear extracts detected as described above. This possibility is further supported by results demonstrating interactions between SR proteins and splicing enhancers in other genes (19, 20, 27, 28, 30). To address directly whether SR proteins participate in enhancer-dependent splicing of cTNT exon 5, we tested whether purified SR proteins activated splicing of wild-type pre-mRNA (E45SA) (Fig. 2A) in splicing-deficient S100 extracts. Individual SR proteins were prepared from calf thymus, their relative activities were determined with a human β-globin splicing substrate as previously described (38), and equal activities of SRp30b, SRp40, or SRp55 were added to S100 extracts. The results presented in Fig. 4 demonstrate that splicing of exon 5 was activated by SRp40 (lanes 5 and 6) and SRp55 (lanes 7 and 8) but not by SRp30b (lanes 3 and 4).

To determine whether SR proteins directly interact with the splicing enhancer, splicing of a human β-globin substrate in SRp30b- and SRp40-complemented S100 extracts was challenged by competitor exon RNAs. Although SRp30b had no activity when added to the wild-type cTNT pre-mRNA (see above), both SR proteins did promote efficient splicing of the β-globin pre-mRNA (Fig. 5, lanes 1 and 10). Splicing in SRp30b-complemented extracts was not significantly affected by competing enhancer RNAs (Fig. 5, lanes 2 to 9). SRp40 activity was specifically inhibited by RNAs containing UP or WT enhancer RNA (Fig. 5, lanes 11 to 14) and was only moderately affected by the inactive mutant and heterologous RNAs (lanes 15 to 18). We conclude that limiting amounts of SRp40 are titrated by UP and WT enhancer RNAs. Furthermore, competition by the UP mutation was stronger than competition by the WT enhancer (compare lanes 11 and 13 in Fig. 5), indicating that the relative affinity of SRp40 for enhancer mutants directly correlates with enhancer strength. In contrast, this assay demonstrates that binding of SRp30b to the enhancer is weak at best. It is formally possible that enhancer RNA inhibits splicing of SRp40-complemented S100 extracts indirectly by titrating a limiting splicing factor other than SRp40. We consider this possibility unlikely since it would require that SRp30b, which is not inhibited by enhancer RNA, complements S100 extracts by a mechanism different from that of SRp40.

At least four SR proteins exhibit sequence-specific binding to the splicing enhancer. The availability of enhancer mutants with a range of activities provided an experimental system for identifying functionally significant enhancer-binding proteins in HeLa nuclear extracts by UV cross-linking. Uniformly labeled WT RNA (Fig. 3) was incubated in HeLa nuclear extracts under standard splicing conditions, UV irradiated, and digested with RNase (see Materials and Methods). Proteins that formed UV-induced adducts to the labeled RNA were identified on a 10% denaturing polyacrylamide gel by autoradiography. A relatively large number of proteins UV cross-linked to the 47-nucleotide WT RNA (Fig. 6, lane 1). To identify sequence-specific interactions, binding was challenged by the series of enhancer mutant RNAs (Fig. 6, lanes 2 to 16). The binding of one protein migrating at 47 kDa demonstrated striking sequence specificity when challenged with UP, WT, DOWN, and INAC RNAs. The relative affinities of this protein for the enhancer mutants correlated exactly with enhancer activities: the UP mutation inhibited binding more efficiently than the WT exon (Fig. 6, lanes 2 to 4), the DOWN mutation competed less efficiently than WT (lanes 8 to 10), and the inactive mutant and heterologous RNAs did not compete (lanes 11 to 13 and 14 to 16, respectively). In addition to the 47-kDa band, a second band at 39 kDa showed significant but weaker competition by the enhancer mutants.

To determine whether the 47-kDa band corresponded to an SR protein, we performed a UV cross-linking and immunoprecipitation assay. Uniformly labeled WT RNA was incubated in HeLa nuclear extracts and UV cross-linked as described above, and UV-cross-linked proteins were immunoprecipitated with a monoclonal antibody that recognizes the six major SR proteins (22a). To determine the binding specificities of immunoprecipitated proteins for the enhancer RNA, reaction mixtures were challenged with competitor RNAs prior to UV cross-linking. Four immunoprecipitated bands were consistently detected in the absence of competitor RNAs (Fig. 7A, lane 1). Competition with enhancer mutants demonstrated that binding of each of these four proteins was sequence spe-

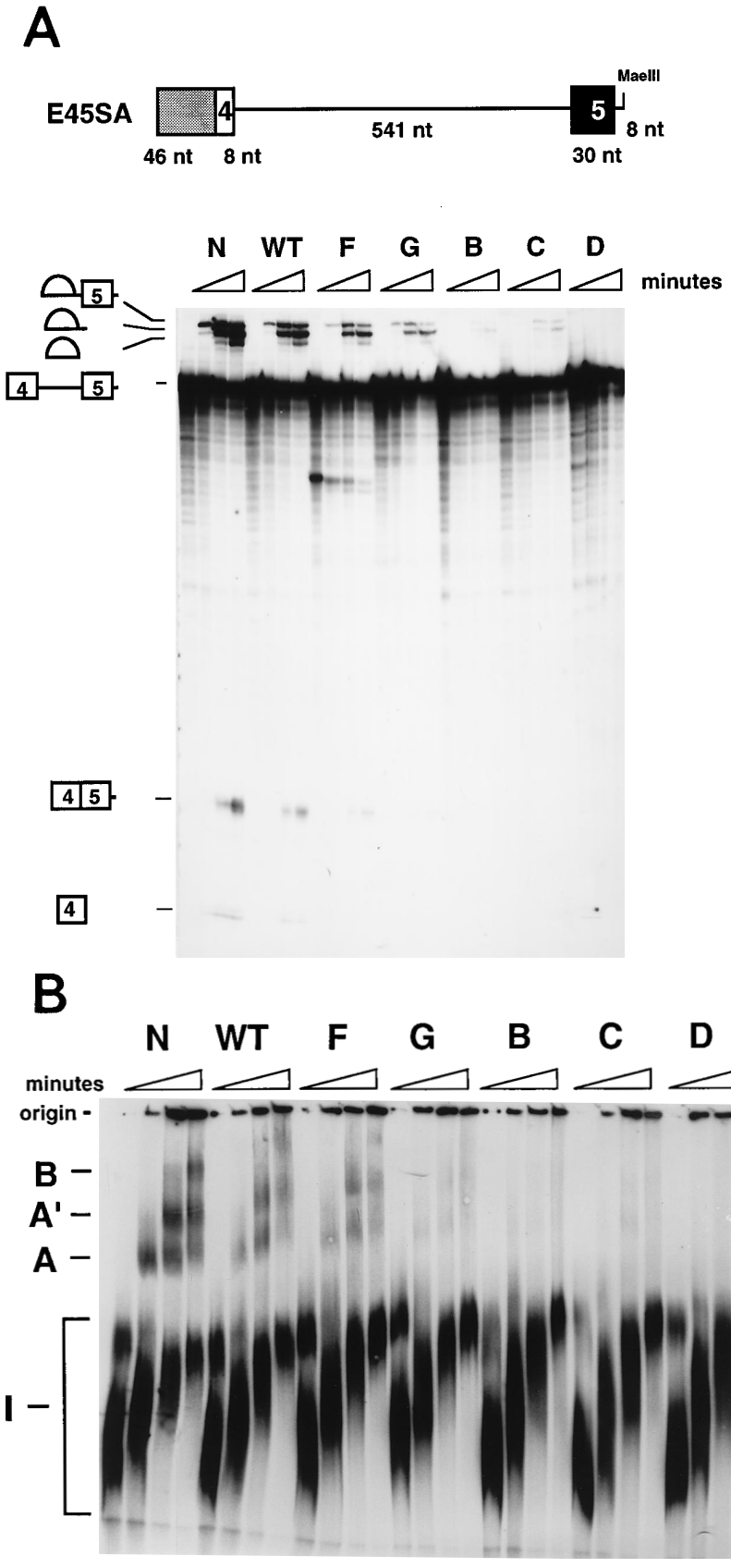


FIG. 2. In vitro analysis of cTNT exon 5 mutants. (A) Effects of exon 5 mutations on in vitro splicing of exons 4 and 5. A diagram of the E45SA RNA is shown at the top. All seven RNA substrates are identical except for the nucleotide substitutions listed in Fig. 1A. Reaction mixtures were incubated for 0, 30, 60, and 90 min. Splicing products and intermediates have been characterized previously (3) and are represented diagrammatically. The strong band below the F precursor has not been observed in repeated experiments. (B) Spliceosome assembly on the seven RNAs used for panel A was analyzed by native gel electrophoresis as described by Zillmann et al. (40). Reaction mixtures were incubated for 0, 5, 20, and 45 min. For panels A and B, all seven RNAs were synthesized from same cocktail and have identical specific activities.

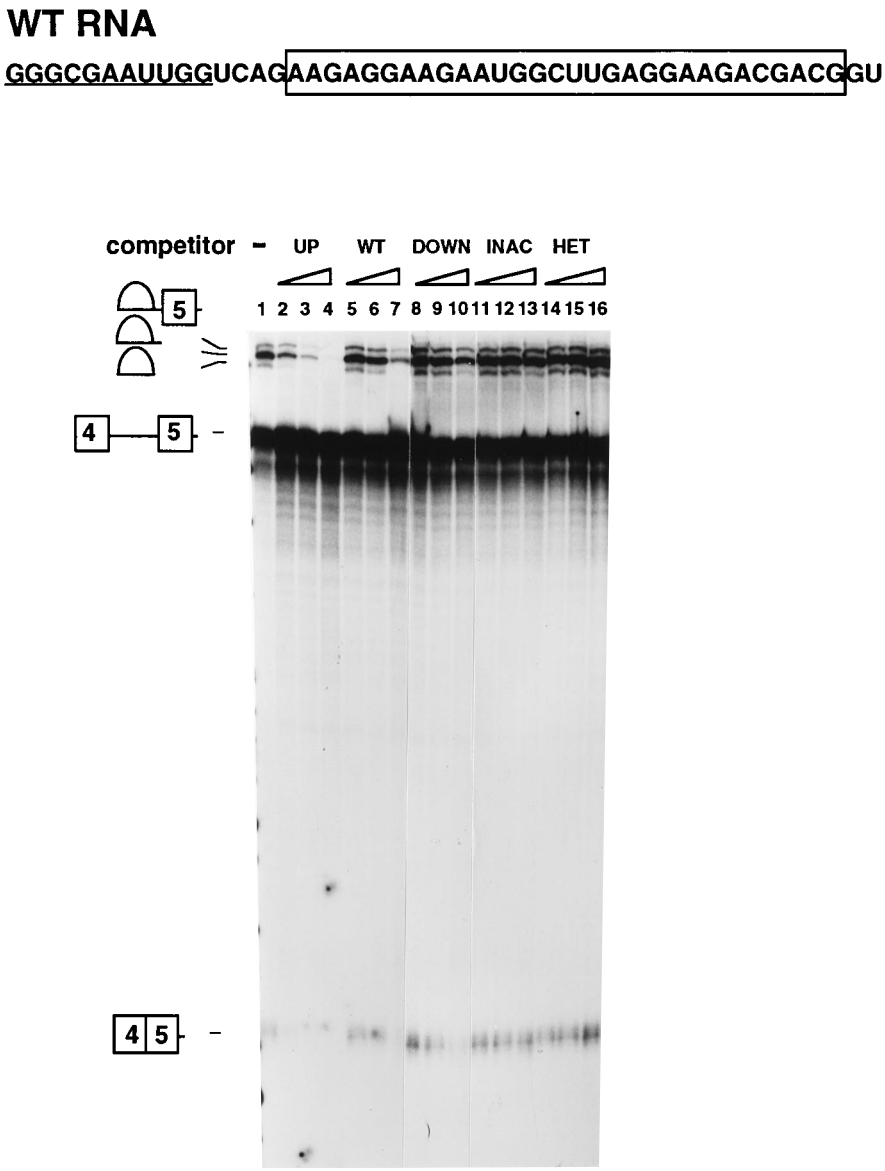


FIG. 3. Competition of in vitro splicing by exon 5 wild-type and mutant RNAs. Splicing of 0.01 pmol of E45SA substrate containing the unmodified exon in HeLa nuclear extracts was challenged with the indicated exon RNA competitors. The sequence of the WT competitor RNA containing the unmodified exon 5 is shown at the top. This RNA contains (5′ to 3′) 11 nucleotides from the Bluescript vector (underlined), the last 4 nucleotides of intron 4, the 30-nucleotide exon (boxed), and 2 nucleotides of intron 5. All competitor RNAs are identical except for the nucleotide substitutions listed in Fig. 1A. The mutants represented by the designations UP, WT, DOWN, and INAC are indicated in Fig. 1A. The HET RNA contains a 30-nucleotide constitutive exon (exon 2) from chicken skeletal troponin I substituted for exon 5. The substituted exon lacks an exonic enhancer; it is completely skipped in vivo (35) and is not spliced in vitro (22b). Splicing reaction mixtures were incubated for 45 min. Reaction mixtures contained 0.3, 1.0, or 3.0 pmol of competitor RNA.

cific (Fig. 7, lanes 2 to 5). The identities of the immunoprecipitated UV-cross-linked proteins were determined by size comparisons with purified HeLa SR proteins that were UV cross-linked to the WT RNA. As indicated in Fig. 7, the four immunoprecipitated proteins correspond in size to UV-cross-linked SRp30a and SRp30b (which comigrated on this gel), SRp40, SRp55, and SRp75 (data not shown). Furthermore, UV-cross-linked SRp40 comigrated with the 47-kDa protein and SRp30a and SRp30b comigrated with the 39-kDa protein detected in crude HeLa nuclear extracts (data not shown).

Sequence-specific binding of SRp30a, SRp30b, SRp40, SRp55, and SRp75 to the WT exon RNA was confirmed in UV cross-linking and competition assays with purified total HeLa SR proteins (Fig. 7B). Figure 7B, lane 1, shows binding of four protein bands corresponding in size to SRp30a and/or SRp30b, SRp40, SRp55, and SRp75. Binding of SRp20 is consistently found to be weak at best. The specificity of binding of these four bands is demonstrated by competition by the UP RNA (Fig. 7B, lane 3) but not the INAC RNA (lane 4). The results presented in Fig. 7A and B demonstrate sequence-specific binding of at least four SR proteins to the cTNT exon 5 enhancer. Furthermore, competitions with RNAs containing point mutations in the core enhancer demonstrate a direct correlation between enhancer strength and binding affinity.

UV-cross-linked SRp30a and SRp30b cannot be distinguished on the gels in Fig. 7A and B. To determine whether SRp30a binds to the enhancer in a sequence-specific manner, a UV cross-linking and immunoprecipitation assay was per-

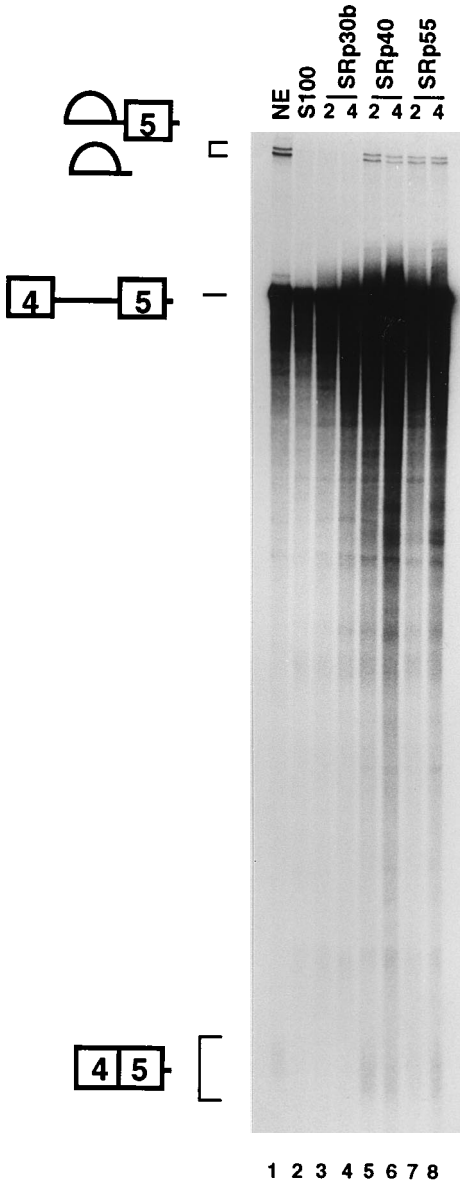


FIG. 4. SRp40 and SRp55 but not SRp30b activate splicing of exon 5. In vitro splicing reaction mixtures containing equal amounts of E45SA RNA and HeLa S100 extract were complemented with 2 or 4 μl of purified HeLa SR proteins (300 ng/μl). Gel-isolated SRp30b, SRp40, and SRp55 were demonstrated with a human β-globin substrate to have equivalent specific activities (data not shown). The splicing substrate, intermediates, and products are represented diagrammatically on the left. The lower levels of precursor RNA in lanes 2 to 4 are reproducible and are probably due to increased degradation of unassembled pre-mRNA.

formed with a monoclonal antibody specific for SRp30a (anti-SF2/ASF) (generously provided by A. Krainer, Cold Spring Harbor Laboratory). This antibody immunoprecipitated a doublet of the expected size for UV-cross-linked SRp30a (Fig. 7C, lane 1). Competition experiments demonstrated that the relative affinities of SRp30a for enhancer mutants directly correlated with enhancer strength (Fig. 7C, lanes 2 to 5). Therefore, this analysis indicates that SRp30a, SRp40, SRp55, and SRp75 present in HeLa nuclear extracts specifically bind to the enhancer in cTNT exon 5.

DISCUSSION

We have determined the specific nucleotide requirements for the function of an exonic splicing enhancer in vivo and in vitro and have used this system to identify splicing factors that specifically interact with the enhancer. We demonstrate that the activity of the cTNT exon 5 splicing enhancer resides primarily in nucleotides 3 to 11 of the exon. Enhancer mutants that increased or decreased the level of exon inclusion in vivo had parallel effects in vitro with respect to the efficiencies of both splicing and spliceosome assembly of exons 4 and 5. Importantly, in vitro competition assays with these mutants facilitated the identification of specific enhancer-binding factors present in HeLa nuclear extract. Results from UV cross-linking, immunoprecipitation, and competition studies demonstrate that four members of the SR protein family (SRp30a, SRp40, SRp55, and SRp75) bind directly to the cTNT exon 5 enhancer. The functional significance of SR protein-enhancer interaction was demonstrated in two ways. First, two SR proteins that bound to the enhancer activated splicing of exon 5 (SRp40 and SRp55), while an SR protein that did not bind to the enhancer did not activate splicing (SRp30b). Second, competition experiments demonstrated a direct correlation between enhancer strength in vivo and the relative binding affinities of SR proteins. Overall, these results demonstrate that a subset of the SR proteins bind to the enhancer of cTNT exon 5 and activate splicing of the upstream intron.

Our results indicate that the upstream repeat, GAGGAAG AA, is the major splicing enhancer in cTNT exon 5. Competition studies using RNAs containing point mutations in this sequence demonstrate a direct correlation between the level of competition and enhancer strength strongly suggesting that GAGGAAGAA is a binding site for all four SR proteins in the subset. SRp40 and SRp55 independently activate splicing in S100 extracts (Fig. 4). On the basis of the similar binding specificities of SRp30a and SRp75 shown in Fig. 7, we predict that these two proteins would also independently activate splicing. However, we cannot rule out the possibility that different sets of SR proteins bind different sequences within the exon (the upstream and downstream repeats, for example) and activate splicing by cooperative interactions.

Several exonic splicing enhancers have been recently characterized as purine rich, but the present study establishes the exquisite sequence specificity of enhancer activity. For example, point mutations within positions 3 to 11 of the exon decreased the activity of the enhancer in vivo and in vitro, even though the purine content was preserved. The splicing enhancers within several vertebrate exons typically require alternating G and A nucleotides to generate a repeating GAR pattern (where R is an A or a G), and oligomers of only A or G or $(A3G3)_6$ are inactive in vitro (29). Thus, a certain degree of sequence specificity was expected. The demonstration that single nucleotide changes had strong effects on enhancer activity argues for highly specific interactions between the enhancers and *trans*-acting factors. Indeed, we observed the interaction of only four of the six major SR proteins with the cTNT enhancer: SRp30a, SRp40, SRp55, and SRp75. In contrast, spliceosomal complexes assembled on a 115-nucleotide RNA containing the exonic enhancer of bovine growth hormone contain a different set of SR proteins: SRp20, SRp30a and/or SRp30b, and SRp40 (27). In addition, SRp30a but not SRp30b was shown to specifically bind to this same RNA fragment and activate splicing in vitro (28). Similarly, SRp30a and SRp30b significantly differ in binding specificities for a purine-rich sequence in the *dsx* splicing enhancer (20). Our results demonstrate that SRp30b neither binds to nor activates splicing of the cTNT exon 5

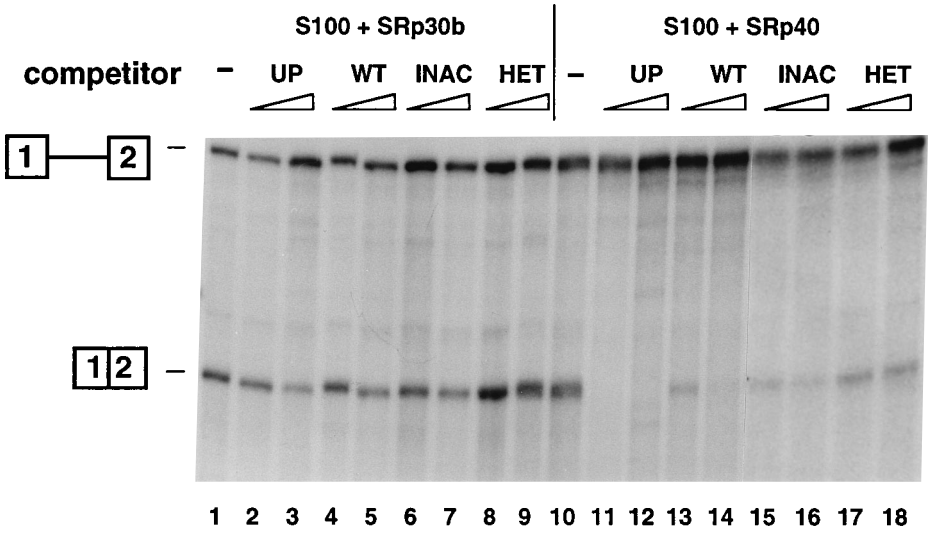


FIG. 5. Exon enhancer RNA inhibits splicing in SRp40- but not SRp30b-complemented S100 extracts. Splicing reaction mixtures contained ^{32}P-labeled SP64HβΔ6 RNA (25), HeLa S100 extract, and 600 ng (equal activities) of either purified HeLa SRp30b (lanes 1 to 9) or SRp40 (lanes 10 to 18). Splicing reaction mixtures contained 2.5 and 5 pmol (lanes 2 to 9 and 11 to 14) or 3 and 6 pmol (lanes 15 to 18) of competitor RNAs. Lanes 1 and 10 show results of reactions performed in the absence of competitor RNAs. The positions of the splicing substrate and products are indicated on the left.

enhancer and suggest that SRp20 also has a low affinity for this enhancer (Fig. 7B and data not shown). These observations strongly suggest that different enhancers are activated by different subsets of SR proteins.

Comparison of the primary amino acid sequences of SR proteins suggests an important element that may be required for enhancer-mediated splicing. All six SR proteins contain an NH_2-terminal RNA recognition motif (RRM) and a COOH-terminal serine-arginine (SR) domain. The four SR proteins that bind the cTNT enhancer also contain a divergent repeat of the RRM called the RRMH (37). This sequence is absent in both SRp20 and SRp30b, the two SR proteins that do not bind to the cTNT enhancer. We suggest that the RRMH may be important for binding the cTNT enhancer. Other than the presence or absence of the RRMH, the differences in the sizes of SR proteins are due to variation in the lengths of the SR

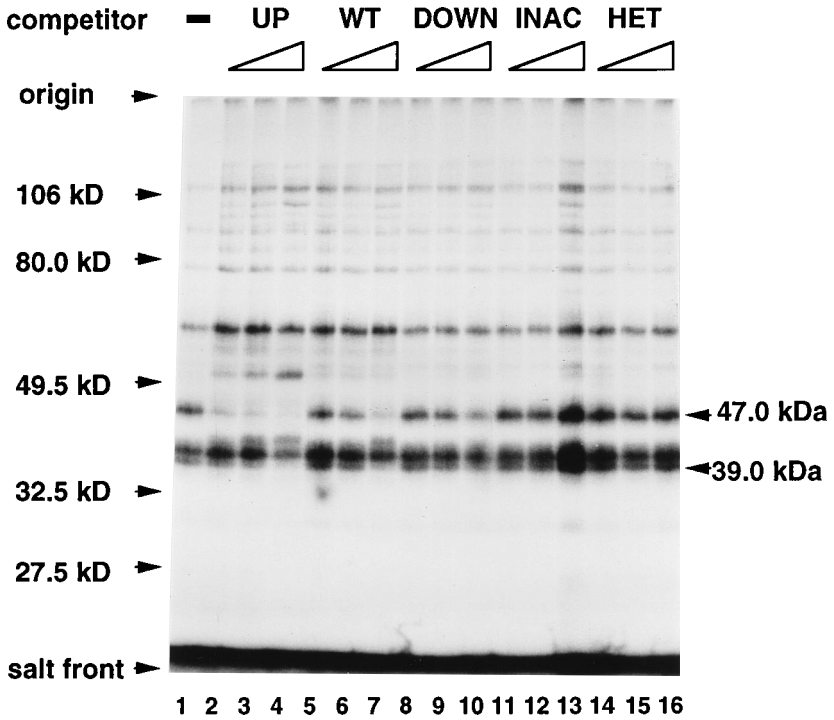
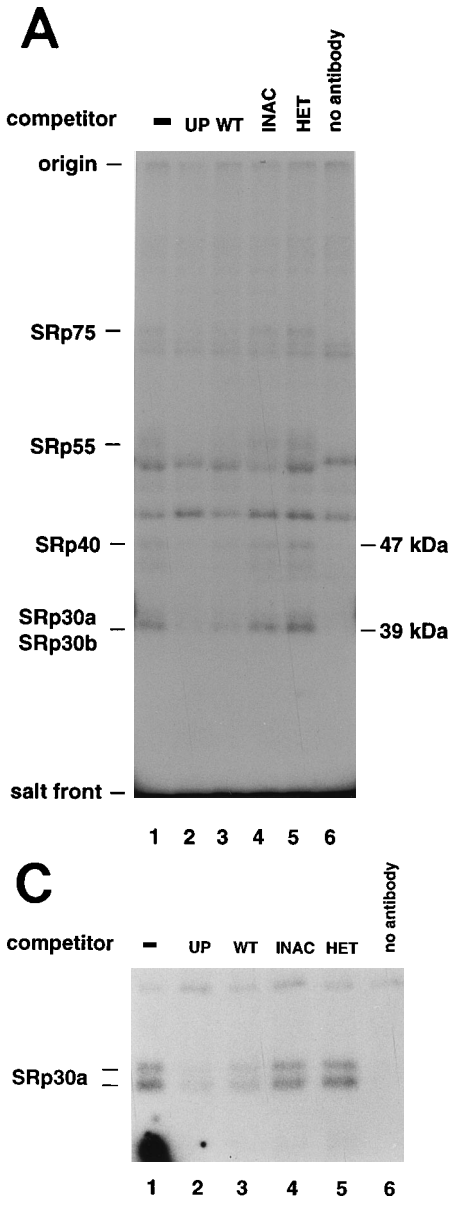


FIG. 6. UV cross-linking of enhancer-binding proteins in HeLa nuclear extract. UV cross-linking of labeled WT RNA (Fig. 3) was challenged with 0.4, 1.0, and 4.0 pmol of the indicated competitor RNAs. Competitor RNAs are as in Fig. 3. Two bands at 39 and 47 kDa that show specific competition are indicated. The positions of molecular mass markers are shown on the left.



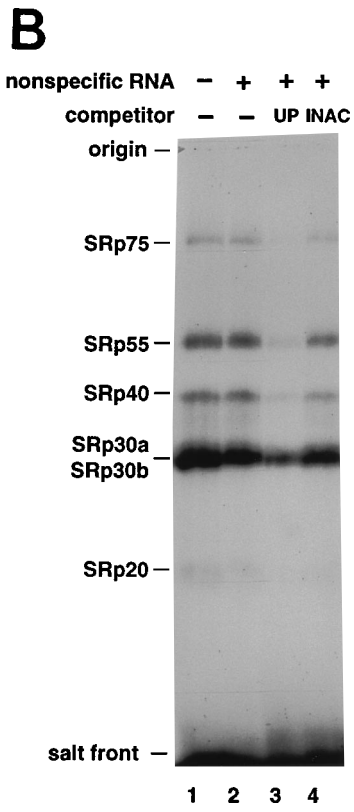


FIG. 7. Binding of four SR proteins to the enhancer is detected in HeLa nuclear extracts. (A) UV cross-linking and immunoprecipitation with an anti-SR protein antibody. Binding reaction mixtures contained labeled WT RNA and HeLa nuclear extract without (lane 1) or with (lanes 2 to 5) 4 pmol of competitor exon mutant RNAs prior to UV cross-linking. Reaction mixtures were immunoprecipitated following UV cross-linking. An unidentified band below 47 kDa is observed in some nuclear extract preparations. SRp30a and SRp30b comigrate on this gel. The sizes of all UV-cross-linked proteins are significantly increased because of residual covalently linked RNA fragments. Note that UV-cross-linked SRp55 and SRp75 are obscured by background bands in crude nuclear extracts and are not observed in Fig. 6. (B) UV cross-linking and competition studies using purified total HeLa SR proteins. Total purified SR protein (200 ng) was incubated with labeled WT RNA under standard splicing conditions in the absence of nuclear or S100 extracts. Lane 1 contains SR proteins in the absence of specific or nonspecific competitors. To reduce nonspecific binding, 4 pmol of a nonspecific RNA (HET) was mixed with the labeled substrate prior to the addition of SR proteins (lanes 2 to 4). Binding was challenged with 4 pmol of either UP (lane 3) or INAC (lane 4) mutant RNA. (C) Sequence-specific binding of SRp30a to the enhancer. Immunoprecipitation and competition reactions were performed as for panel A except with anti-SF2/ASF.

domains. Because the four SR proteins that bind the enhancer have SR domains that range in size from 44 to 314 amino acids, it appears that the length of this domain is not critical for function.

cTNT exon 5 is poorly defined and often skipped because of its small size and weak 5′ splice site (5, 35). Nevertheless, exon 5 is included in the mRNA of embryonic muscle tissue and primary cultures (4, 35). The enhancer is required for a default level of exon recognition in all cell types tested but is not required for preferential inclusion observed in embryonic striated-muscle cultures (35). We hypothesize that the exonic enhancer and a distinct regulatory element located elsewhere in the pre-mRNA are sites of the up-regulation of exon 5 inclusion in the embryo.

It has been proposed that exons are defined by factors bound to the 3′ and 5′ splice sites by communication across the exon (23). SR proteins bound to the enhancer may serve as a bridge to promote this communication during the process of exon definition. The demonstration of protein-protein interactions between SR proteins and U2AF65 and U1 70K, as well as the requirement for SR proteins to direct U1 and U2 SnRNP binding to the pre-mRNA (17, 19, 34, 39), supports this model. In this regard, the mechanism of enhancer-mediated splicing may be similar to that of the stimulatory effect of a 5′ splice site on a upstream 3′ splice site (14). In both cases, splicing to an upstream 3′ splice site is enhanced. It has recently been demonstrated that a novel exonic enhancer mediates U2AF binding to the 3′ splice site and is in a complex immunoprecipitated by an antibody to SRp30b, strongly suggesting that binding of SR proteins to exon enhancers promotes or stabilizes binding of U2AF (32). Unlike the splice site consensus sequences, exonic splicing enhancers serve as auxiliary elements that are not universally required for splicing. It remains to be determined whether there are significant mechanistic differences in

the recognition of exons that require exonic enhancers and those that do not. Specifically, SR proteins appear to be universally required for pre-mRNA splicing. It will be of interest to determine what roles SR proteins play in enhancer-dependent and enhancer-independent splicing.

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