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Factors Involved in the Activation of Pre-mRNA Splicing from Downstream Splicing Enhancers[1]

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The excision of introns with weak polypyrimidine tracts at their 3′ splice sites can be enhanced by sequence elements in the downstream exon or by a downstream 5′ splice site. The enhancers inside the exon do not conform to a strict consensus, but they are generally rich in purines. Here, we show that members of the family of SR proteins recognize these elements. Not only does SF2/ASF activate many different polypurine enhancers, but also at least one other SR protein, most likely SC35, is active as well. The degree of splicing activation varies with the polypurine enhancers and the SR proteins. Further, we show that the similar activation by downstream 5′ splice sites requires U1 snRNP, which is not the case with purine-rich enhancers. These results are consistent with a model showing that U1 snRNP binds to the 5′ splice site and SR proteins to exonic sequences upstream of the 5′ splice site. Both interact with U2AF at the 3′ splice site. This represents a molecular explanation for the exon recognition which is important for splice site selection in mammals.

Key words: exonic enhancer, pre-mRNA splicing, SR proteins, transacting factors, U1 snRNP.

One prerequisite for the precise excision of introns is the flawless identification of the authentic splice sites. In pre-mRNAs, the splice sites are characterized by their consensus sequences. However, these consensus sequences are very ambiguous in higher eukaryotes and especially in mammals: whereas cryptic splice sites that match the consensus are frequently found, weak splice sites that do not well match the consensus are sometimes used as authentic splice sites, depending on their sequence context (see below). Clearly, more information contributes to the strength of splice sites, and the factors reading the sequence information need to cooperate with each other to ensure that only authentic splice sites are used. Examples of essential splicing factors that participate in the selection of splice sites and interact with each other include U1 small nuclear ribonucleoprotein (U1 snRNP) which base-pairs with its RNA component to the 5′ splice site, as well as U2 auxiliary factor (U2AF) which binds to the polypyrimidine stretch at the 3′ splice site (*1-3*) and the SR proteins. Members of the SR protein family affect selection of both 5′ and 3′ splice sites, but their target sequences are not yet fully understood (*4-8*). The two most abundant proteins of the SR family, SF2/ASF and SC35, were identified by biochemical and immunological methods, respectively (*4, 9, 10*), and the whole family is characterized by its immunological relationship (*11*). Cloning of SR proteins revealed that they all contain a region rich in the dipeptide serine/arginine (*10-14*). This SR domain is also found in both subunits of U2AF (*15, 16*) and in U1 70k, an integral part of U1 snRNP (*17, 18*). The domain has been shown to be the effector domain of these factors (*15, 19, 20*) and it is necessary for protein-protein interactions between factors containing this motif (*21-23*). SF2/ASF and U1 70k cooperate in this way to position U1 snRNP at the 5′ splice site (*24*), and there is the possibility of an interaction spanning from U1 snRNP at the 5′ splice site to U2AF at the 3′ splice site, with either SF2/ASF or SC35 in between (*21*).

At least in the mammalian system, one important contribution for the usage of potential splice sites resides in the exon: mutations weakening the 5′ splice site often lead to skipping of the whole following exon, *i.e.*, not only the affected 5′ splice site but also the upstream 3′ splice site is inactivated. Likewise, an active 5′ splice site can activate an intrinsically weak upstream 3′ splice site (*25*). These observations led to the proposition that the exon is the first unit to be recognized during early spliceosome formation, and that all sequences between these committed exon units are subsequently excised as introns (*26*). The molecular basis of this "exon definition" has been shown to be an interaction between U1 snRNP, U2AF and at least one other factor (*27*).

Further, elements inside the exon are sometimes found to influence the choice of splice sites (see Ref. *28* and references therein). These elements generally contain polypurine stretches, and they enhance usage of weak

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Abbreviations: ERS, exon recognition sequence; snRNP, small nuclear ribonucleoprotein; U2AF, U2 auxiliary factor.

upstream 3′ splice sites (*28, 29*) by enhancing the binding of U2 snRNP (*30*). A detailed study showed that almost any stretch of mixed purines has an activating influence on a weak 3′ splice site provided that the stretch is long enough (*31*). A consensus sequence cannot easily be found, although repeats of AAG seem to be especially functional. We previously suggested the name "exon recognition sequence" (ERS) for this type of splicing element in order to emphasize that it induces the usage of surrounding splice sites and thereby the inclusion of the region as an exon into the nascent mRNA (*28*).

It was previously shown that SR proteins bind to two of these purine-rich elements and are necessary for the activation of splicing (*30, 32*). The ERS derived from the bovine growth hormone (BGH) gene is activated by SF2/ASF, but not by SC35 (*32*). Here, we extended the study to compare several previously reported ERS. We find that SF2/ASF is active in all cases, despite the considerable sequence divergence. In addition to SF2/ASF, the ERS elements are also activated by another SR protein, most likely SC35; the activation strength varies with both the ERS sequence and the SR protein. Further, whereas U1 snRNP is needed for the similar activation by a downstream 5′ splice site, it is not needed for activation by an ERS element. These observations identify the missing link between U2AF and U1 snRNP in exon definition, because the SR proteins interact with U1 snRNP, as shown in previous reports, and recognize exonic elements, thereby activating weak 3′ splice sites even without U1 snRNP.

EXPERIMENTAL PROCEDURES

Purification of SR Proteins from HeLa Cells—Approximately 5×10^9 cells, grown in suspension culture, were lysed by sonification in 30 ml of lysis buffer (*33*) and cleared by centrifugation at $8,000\times g$ for 20 min. Then 1.5 volumes of saturated ammonium sulfate was added to the supernatant, and the mixture was stirred for 1 h, and centrifuged as before. To the clear supernatant, a phenyl Sepharose slurry (1 ml settled bed volume) was added. The mixture was gently stirred for 10 min and centrifuged. The phenyl Sepharose was then packed in a column and step-eluted in 2 ml steps, starting with a buffer containing 20 mM Tris·HCl, 0.1 M KCl, 2 M $(NH_4)_2SO_4$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM *p*-aminophenylmethylsulfonyl fluoride, pH 7.9. The ammonium sulfate concentration was decreased by 0.2 M at each step, and SR proteins were eluted at 1.4 and 1.2 M. The corresponding fractions were dialyzed against buffer D (*34*) and concentrated by precipitation with 20 mM $MgCl_2$. The SR proteins were further fractionated by MonoQ chromatography using a linear gradient between 0.1 M KCl in buffer (20 mM 2-[*N*-morpholino]ethanesulfonic acid, 0.2 mM EDTA, 0.1% Triton X100 (pH 6.5)) and 0.4 M KCl in the same buffer, pH 6.5. SRp20 elutes at ≈0.2 M, SF2 at 0.28 M, and the other SR proteins (predominantly SC35) at 0.33 M KCl. Fractions were dialyzed against buffer D and concentrated by $MgCl_2$-precipitation The protein concentrations of the peak fractions were normalized on the basis of Coomassie blue staining.

Photo-Crosslinking—A *doublesex* (*dsx*) 3′ exon RNA spanning the sequences from the middle of the female-specific intron (the intron between exons 3 and 4) to the *Bam*HI site at the end of the downstream exon, transcribed by SP6 RNA polymerase, and the ERS RNA, identical to those used as competitor (*31*) but labeled with [α-^{32}P]-GTP, were ligated in the presence of a short bridging DNA oligonucleotide as described previously (*35*). The purified product (10^4 cpm) and the respective competitor RNAs were incubated in 5 μl of binding mixture containing 1.5 μl of HeLa nuclear extract (*31*) and 1.6 mM $MgCl_2$ at 30°C for 15 min. Then, 1 μl of 10 μg/μl heparin was added, and the mixture was further incubated on ice for 15 min. The sample was UV-irradiated with 900 mJ in a crosslinker (Stratagene), and digested with 5 U RNase T_1 for at least 20 min at 37°C. This mixture was subjected to SDS/10% PAGE.

Splicing Assay—Standard splicing assays contained $5\times$ 10^4 cpm of pre-mRNA (*31*) and 3 μl of HeLa nuclear extract (*31*) plus competitor RNA and other proteins as described in each individual experiment. The final conditions were always adjusted to 60 mM KCl, 0.12 mM EDTA, 1.6 mM $MgCl_2$, 0.5 mM ATP, and 20 mM creatine phosphate (*36*). Reaction mixtures were incubated at 30°C for 60 min. Samples were separated on denaturing PAGE and bands were identified according to Ref. *28*.

RNase H Digestion—The reaction mixture containing 80% nuclear extract, 40 ng/μl DNA oligonucleotide, 3.2 mM $MgCl_2$, 1 U/μl RNasin, and 0.4 U/μl RNase H (Takara) was incubated at 30°C for 30 min and then immediately used for standard splicing assays (3 μl of mixture per splicing reaction).

RESULTS

SR Proteins Activate the Exon Recognition Sequence Elements—*In vivo*, the weak splice acceptor of exon 4 of the *dsx* pre-mRNA is activated in a regulated fashion by an element inside exon 4. However, if a purine-rich ERS element, as found in heterologous pre-mRNAs, is placed near to the 3′ splice site of this exon, this activates the 3′ splice site and unregulated splicing is observed *in vitro* (*28*). Competitor RNAs containing only the ERS and minimal flanking sequences inhibit splicing, suggesting that *trans* active factors recognize the ERS (*28*).

As an initial assay to identify the factors binding to the ERS element, we tried photo-crosslinking to ERS RNA, but we could not obtain discernible signals that correlated well with the activating strength of the ERS used (data not shown). We therefore tried to narrow down the signals: we used truncated *dsx* pre-mRNA comprising only the second half of the female-specific intron and the downstream exon. This *dsx* 3′SS RNA forms an ATP-dependent, high-molecular-weight complex, putatively designated the A3′ complex (*37, 38*), and the formation of this complex correlated with the strength of the ERS tag (A. Watakabe, personal communication). We synthesized this *dsx* 3′SS RNA by ligating an unlabeled intron/exon portion to labeled ERS tags using T4 DNA ligase (*35*). When these substrates are used for photo-crosslinking, the label is transferred only to proteins that bind to the polypurine sequence, and this can be done under the stringent conditions, *i.e.* 1.7 μg/μl heparin. The crosslinking pattern at the ERS tag is the same in the presence or absence of ATP (data not shown), indicating that the crosslinking proteins are already active in the splicing commitment complex. The photo-crosslinks

are shown in Fig. 1. Good splicing enhancers, avian sarcoma leukosis virus (ASLV) and mouse immunoglobulin μ (IgM), are predominantly bound by proteins of 35 and 40 kDa. Analogous to the splicing of the corresponding *dsx* pre-mRNAs (*28, 31*), these signals can be competed by RNAs containing the ERS sequence, but not by non-specific RNA derived from the IgM M2 exon (lanes 2, 3, 5 and 7, 8, 10). Surprisingly, the mutated version of the HPRT tag which hardly activates splicing (*31*), does compete with the signals of the ASLV and IgM tags. Consistently, this HPRTm tag produced a similar signal of 35 kDa and a much weaker signal of 40 kDa. The main proteins binding to the HPRT tag, however, have molecular weights of approximately 50, 70, and 75 kDa.

For reasons outlined in the discussion, we speculated that the 35 kDa protein might be SF2/ASF or SC35. To test this idea, we prepared a fraction containing all SR proteins by ammonium sulfate precipitation, followed by phenyl Sepharose chromatography and magnesium chloride precipitation (see "EXPERIMENTAL PROCEDURES"). SR proteins prepared in this way can rescue the splicing of *dsx* ASLV pre-mRNA [see Ref. *28* for the list of the polypurine sequences], when this is suppressed by RNA containing only the ERS sequence (Fig. 2, lanes 1-4). If the pre-mRNA and the SR proteins are preincubated to allow stable complex formation, the splicing efficiency is only slightly increased, and remains dependent on the amount of competitor RNA added after preincubation (lanes 5 and 6). The same set of experiments conducted with RNA without the ERS tag did not show any splicing activation (Fig. 2, lanes 7-12). The faint band in lanes 7-12 migrating to a similar height as to the splicing products is most probably a product of nonspecific degradation, and its formation is not stimulated by SR proteins. Also note that the untagged pre-mRNA is severely degraded in the absence of exogenous SR proteins (Fig. 2, lane 7). We conclude that the SR proteins are the limiting factor for splicing of *dsx* ASLV pre-mRNA and they activate splicing through the ERS element. The interaction of the SR proteins with *dsx* ASLV pre-mRNA is not strong enough to commit the pre-mRNA to the splicing pathway, as reported for other pre-mRNAs (*39*).

Influence of Individual SR Proteins—It was previously shown that SF2/ASF, but not SC35 activates the ERS derived from the bovine growth hormone (BGH) gene (*32*). The reported polypurine sequences, however, are quite divergent (*28*), and the minimal requirement for splicing enhancement seems to be no more than a stretch of mixed purines (*31*). Therefore, we wanted to see whether SF2/ASF is the only factor contributing to the activation. To answer this question, we further purified the SR fraction by MonoQ column chromatography at pH 6.5 (Fig. 3). SF2/ASF elutes at 0.28 M KCl, and SC35 at 0.33 M. The two proteins were distinguished by their mobility in SDS PAGE (*8*) and by their activity in activating splicing of *dsx* BGH pre-mRNA (*31, 32*, and see below). SRp20 elutes in a very broad peak far ahead of SF2/ASF and it was too dilute to be efficiently concentrated. SRp20 could not be used in the assays described below. Other minor SR proteins cofractionate with SC35 (Fig. 3, fraction 16). The preparation selected for presentation in Fig. 3 was unusually rich in

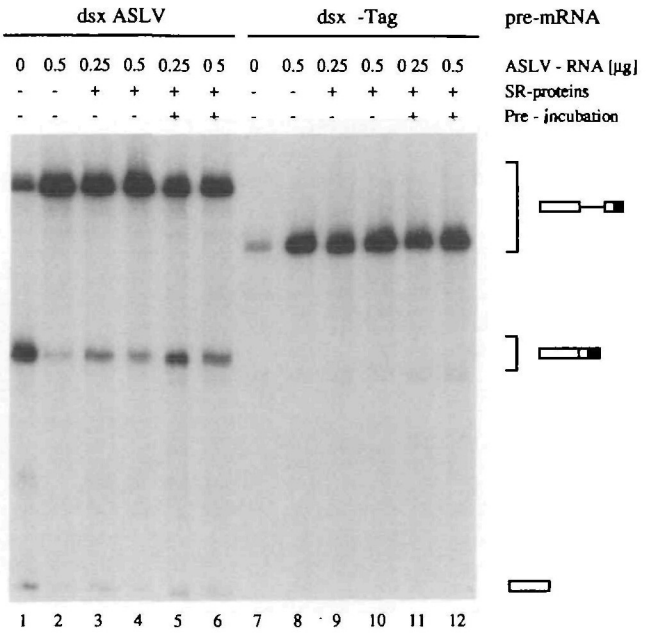

Fig. 2. **SR proteins rescue splicing against competition of ERS RNA.** *dsx* pre-mRNAs with or without ASLV ERS were subjected to the standard splicing assay containing variable amounts of ASLV RNA as competitor and 1.5 μl of SR proteins where indicated. "Preincubation" indicates that the pre-mRNA and the SR proteins were incubated for 10 min at 30°C before addition of the competitor RNA and the HeLa nuclear extract.

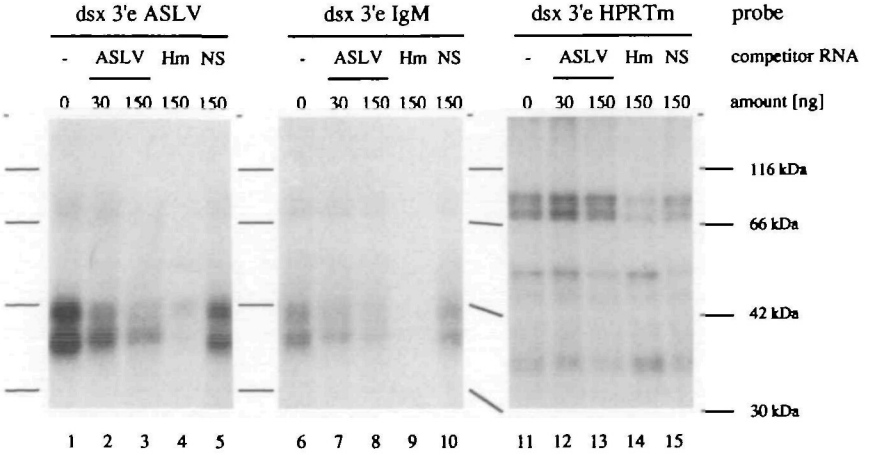

Fig. 1. **Photo-crosslinks to the ERS portion of the *dsx* 3′ exon constructs.** Unlabeled RNA containing the *dsx* 3′ exon sequence was ligated with labeled ERS RNAs as indicated in the figure. The product was incubated with nuclear extract, variable amounts of competitor RNA, and heparin as described in "EXPERIMENTAL PROCEDURES." The mixture was UV-irradiated, digested with RNase T_1 and separated by SDS/10% PAGE. Competitor RNAs: ASLV, HPRTm ("Hm"), and non-specific ("NS").

SRp20 and SRp55; these proteins are normally less readily visible. Still, this particular preparation showed no significant differences in splicing activation experiments as outlined below.

The chromatography fractions containing SF2/ASF and predominantly SC35, were individually tested for splicing activation of the *dsx* pre-mRNAs. In the case of the weak ERS sequences such as BGH, HPRT, and tropomyosin (TM) (see Ref. *28* for their sequences), this splicing activation could be well documented without adding competitor RNA. For *dsx* ASLV, *dsx* IgM, and *dsx* TM pre-mRNAs, 0.2, 1.0, and 0.2 μg of the respective ERS competitor RNAs was added to reduce the background splicing. The SR preparations were normalized to contain almost the same amount of SF2/ASF and SC35 as judged by Coomassie blue staining. The "whole SR" preparation was adjusted to contain the same amount of both SF2/ASF and SC35, *i.e.* more than double the amount of total protein. Figure 4 shows that SF2/ASF strongly activates all the ERS tags used here. The SC35 fraction also activates the ERS tags in varying degrees: although showing activation similar to that by SF2/ASF with the TNT and HPRT tags (lanes 11, 12 and 19, 20), it is significantly less active with the ASLV and BGH elements (lanes 3, 4 and 15, 16). The poor activation by the whole SR fraction in lane 14 was not reproducible and can not be explained. In conclusion, all purine-rich tags are strongly activated by SF2/ASF. In addition, at least one more SR protein is functional at the ERS elements. This protein is presumably SC35, since this is the most abundant protein in that fraction. To show this latter point more conclusively, we tried to express SC35 in *Escherichia coli*, but we could not obtain the full-length protein. Also, SF2/ASF expressed in *E. coli* is not active in our system. At higher concentrations it even inhibits splicing generally, perhaps due to the guanidine hydrochloride present in the preparation (*10, 12*).

Comparison of the Activation by ERS to the Activation by a Downstream Splice Donor—There is a functional relationship between the polypurine splicing enhancers and splice donors in that both can activate weak splice acceptors, if positioned at a suitable distance downstream of the splice acceptor (*25-27*). A 5′ splice site can substitute for

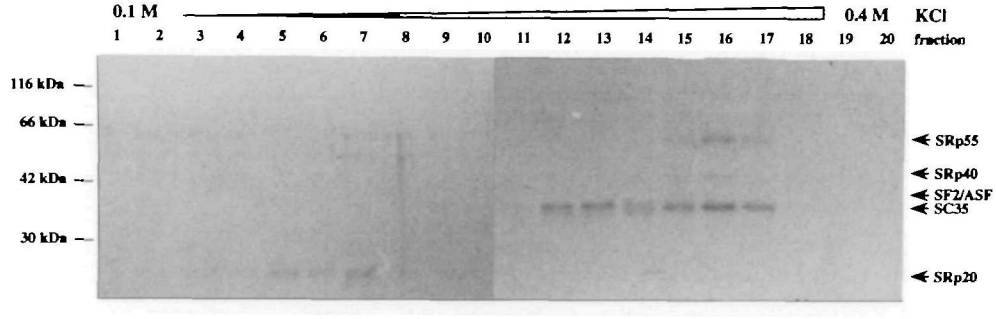

Fig. 3. **Fractionation of SR proteins by anion exchange chromatography.** SR protein were prepared from 5×10^9 Hela cells and separated on a MonoQ column as described in "EXPERIMENTAL PROCEDURES." Out of 25 μl, 2 μl of each fraction was separated by SDS/10% PAGE and stained with Coomassie Blue. Twenty consecutive fractions covering the whole KCl gradient are shown.

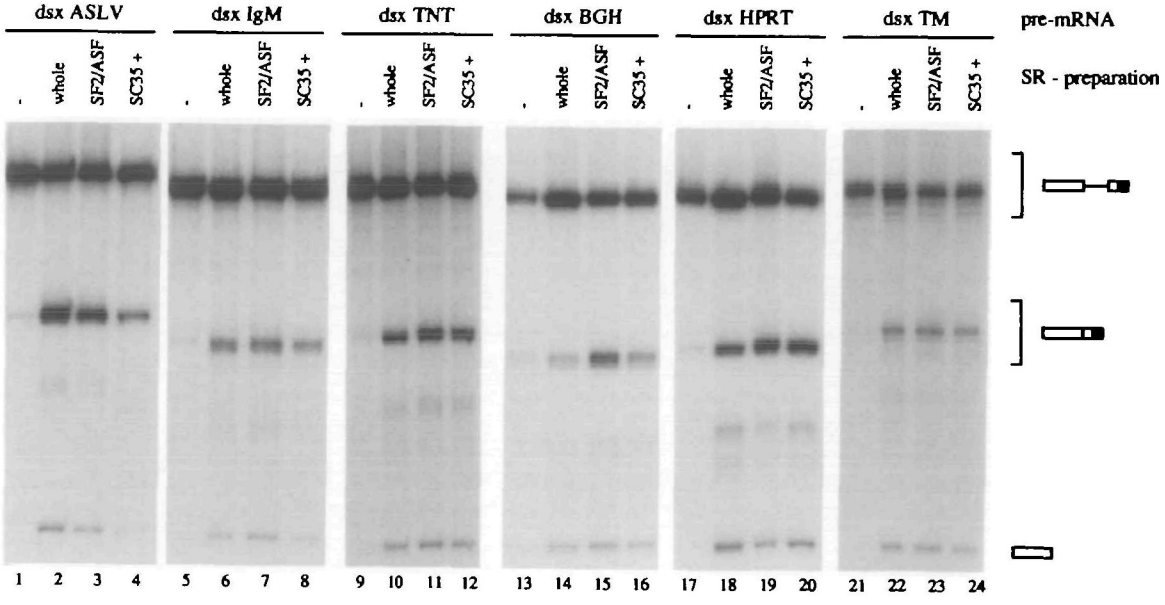

Fig. 4. **Splicing activation by individual fractions of the SR proteins.** *dsx* pre-mRNAs with various ERS tags were subjected to the standard splicing assay containing 1 μl of the whole SR preparation, of the SF2/ASF fraction or of the fraction consisting mainly of SC35. The fractions contained the same amounts of SF2/ASF and SC35 or both, as described in the text. For *dsx* ASLV, *dsx* IgM, and *dsx* TNT, the splicing activity without exogenous SR proteins was suppressed by adding 0.2, 1.0, and 0.2 μg, respectively, of the competitor RNA containing only the sequence of the tag used in the splicing assay.

an ERS (*28*). Furthermore, U1 snRNA is associated with ERS elements (*28, 40*). We therefore wanted to see whether the mechanisms of splicing activation by ERS elements and by 5′ splice sites are in fact identical, in which case the limiting factors should be the same.

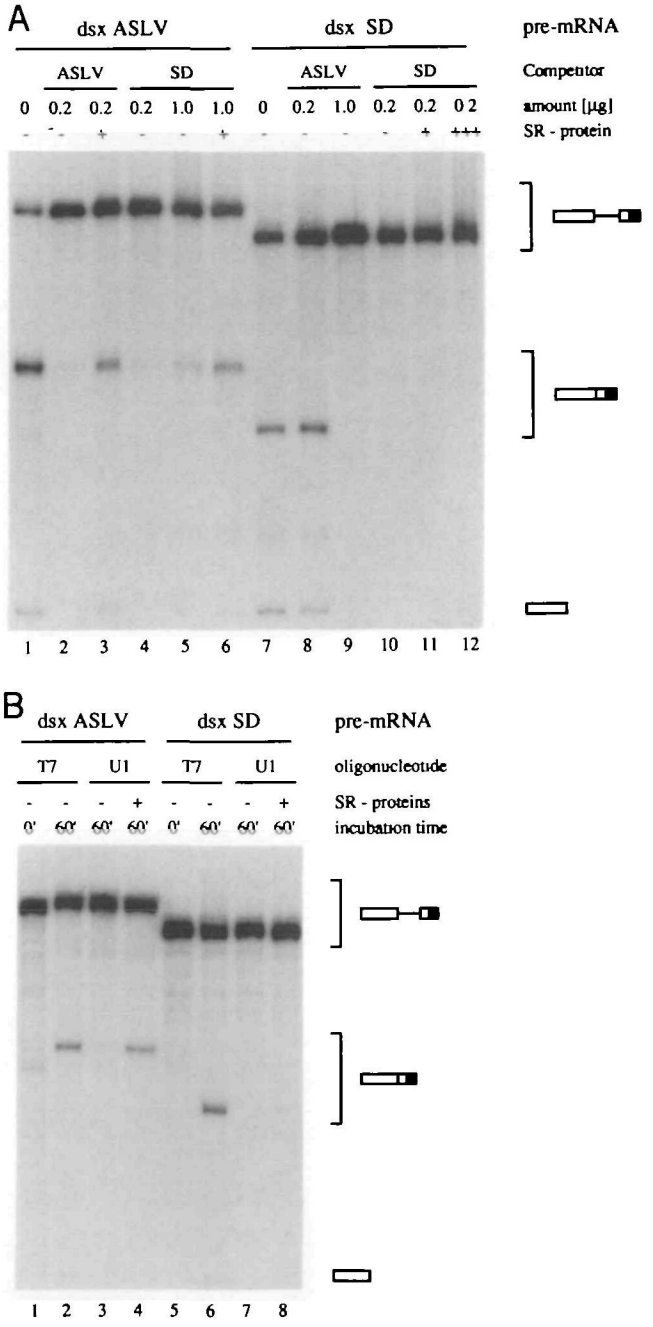

Fig. 5. **(A) Comparison of splicing activation by ERS- or by 5′ splice site sequences.** *dsx* pre-mRNAs activated by ASLV ERS or by an downstream splice donor ("SD") were subjected to the standard splicing assay containing the indicated amounts of the competitor RNA and 1 or 3 µl of the whole SR preparation. **(B) Inactivation of U1 snRNP by RNase H.** HeLa nuclear extract was incubated as detailed in "EXPERIMENTAL PROCEDURES" with RNase H and either a mock DNA oligonucleotide ("T7") or an oligonucleotide complementary to the first 9 nucleotides of U1 snRNA ("U1"). The extract was then used for the splicing assay in the presence of 1 µl of SR proteins where indicated.

We compared splicing in two cases where splicing of the *dsx* construct is driven either by a good ERS (*dsx* ASLV) or by a downstream splice donor (*dsx* SD). The construct *dsx* SD contains a perfect 5′ splice site, ACAGGTAAGTAC, inserted at the same site of the *dsx* construct as described for the other *dsx* ERS pre-mRNAs (*31*). The corresponding competitor RNA contains the downstream exon starting with position +2 in addition to the SD tag, because the tag alone is much shorter than the ERS sequences and cannot be efficiently transcribed by T7 polymerase. As shown in Fig. 5A, splicing of *dsx* ASLV can be inhibited by the SD RNA, but this competition is not complete even when a 5-fold increased amount of the competitor is used (lanes 4 and 5). SR proteins (whole fraction) restore splicing to a similar level as observed for the rescue of splicing against the ASLV competitor (lanes 6 and 3). In contrast, a 5-fold higher concentration of ASLV RNA is needed to inhibit *dsx* SD splicing completely (lanes 8 and 9), whereas the standard amount of SD RNA suffices (lane 10). Against competition by SD RNA, SR proteins cannot rescue splicing, even if added in 3-fold excess (lanes 11 and 12).

These data show that factors other than the SR proteins are limiting for the activation of *dsx* SD splicing. The most likely candidate for this limiting factor is U1 snRNP. If this is the case, the splicing of *dsx* ASLV seen in the presence of SD competitor RNA (Fig. 5A, lanes 4–6) would take place in the absence of, or at least at prohibitively low concentrations of U1 snRNP, which should be bound to the competitor RNA. To test this model further, we digested the 5′ end of U1 snRNA by RNase H, and examined splicing of *dsx* ASLV and *dsx* SD. Figure 5B shows that splicing of both *dsx* ASLV and *dsx* SD is inhibited in an extract treated with RNase H and an oligonucleotide complementary to the first 9 nucleotides of U1 snRNA (lanes 3 and 7), as compared to a mock oligonucleotide (T7 promoter sequence, lanes 2 and 6). Splicing of *dsx* ASLV, but not of *dsx* SD, can be restored by addition of SR proteins (lanes 4 and 8). This is analogous with the results of Fig. 5A and demonstrates that U1 snRNP is really the factor needed for splicing of *dsx* SD, but not of *dsx* ASLV.

DISCUSSION

Significance of tSR Proteins—Polypurine enhancers in downstream exons can activate weak upstream 3′ splice sites (see Ref. *28* and references therein). In this report, we investigated the proteins that bind to and activate such polypurine enhancers. Using photo-crosslinking experiments specific for the ERS portion of the pre-mRNA construct, we found dominant signals of 35 and 40 kDa which might correspond to activating proteins (Fig. 1). One possible candidate for the 35 kDa crosslink could be SF2/ASF, because (i) the molecular size is appropriate, (ii) this would explain why U1 snRNP is associated with the IgM ERS (*28*), since SF2/ASF and U1 snRNP bind to RNA cooperatively (*24, 41*), and (iii) it was previously shown for two ERS tags that SR proteins are necessary for the splicing activation (*30, 32*). Indeed, we find that SR proteins are the limiting factor in splicing of *dsx* ASLV pre-mRNA, and the ASLV tag is necessary for splicing activation by SR proteins (Fig. 2). This is valid not only for the ASLV tag, but also for five other previously reported sequences (Fig.

4), and it most probably holds true for every purine-rich ERS element.

SR proteins have been shown to be necessary for the activation of two exonic splicing enhancers: the one found in bovine growth hormone pre-mRNA (*32*) and the one in fibronectin pre-mRNA (*30*). They are also required in the regulated activation of the *tra* responsive element in *dsx* pre-mRNA, but in this case they do not recognize the exonic element on their own (*23, 42*). Here, we show that SR proteins also activate five other reported ERS elements (Figs. 2 and 4), and probably all other characterized purine-rich enhancers as well.

The SR family contains several proteins, and individual SR proteins overlap in many aspects of their function: they all complement the S100 extracts, they prefer usage of proximal splice sites and they commit pre-mRNAs to the splicing pathway (*7, 8, 39*). A sequence preference can be observed in these examples, since not all pre-mRNAs respond similarly to different SR proteins (*8, 39*). With ERS elements, only SF2/ASF has been shown to be active so far (*32*), and SC35 was shown to be inactive in two cases (*32, 43*). To investigate the contribution of more highly purified SR proteins, we fractionated the SR proteins by MonoQ column chromatography at pH 6.5, yielding almost pure SF2/ASF and SC35, of which the latter is contaminated by minor amounts of SRp40, SRp55, and SRp75. Using these protein preparations, we demonstrated that SF2/ASF is active in all the cases tested here, whereas the fraction containing mainly SC35 is active only with a subset of the ERS sequences, mainly TNT and HPRT (Fig. 4).

Our observation that the BGH ERS is activated most strongly by SF2/ASF is in good agreement with the previous report (*32*). The ASLV tag is also activated best by SF2/ASF, and this is in contrast to a very recent report showing that ASLV crosslinks mainly to SRp40; only mutations initially designed to inactivate this sequence shift the specificity to SRp30 (SF2/ASF or SC35) (*40*). We cannot explain this discrepancy. Perhaps binding and crosslinking does not always correlate with splicing activation. For example, SRp40 is found as the main SR protein in the earliest pre-spliceosome complex on *dsx* pre-mRNA, but it does not correlate with the splicing of the pre-mRNA (*44*).

The observation that other SR proteins are also involved in activation of polypurine sequences is new. We cannot rule out, in principle, that this activation is mediated by one of the minor SR proteins in the preparation of SC35, but we think that the activating protein is indeed SC35 for the following reasons: (i) the photo-crosslinking patterns for *dsx* ASLV, *dsx* IgM, and *dsx* TNT look very much the same (Fig. 1 and data not shown), but they respond differently to the individual SR fractions (Fig. 4). This can be most easily explained by the fact that SF2/ASF and SC35 give very similar signals due to their similar apparent molecular weights. (ii) The strong splicing activation of *dsx* TNT and *dsx* HPRT (Fig. 4, lanes 12 and 20) is not likely to be accomplished by a minor component of the SC35 fraction. The question can be resolved only by using recombinant SC35, but our attempt to prepare it from *E. coli* failed.

In summary, there are several proteins activating purine-rich enhancers in exons, and at least SF2/ASF does not seem to be very strict in its sequence requirements. This makes it possible that various purine-rich sequences without apparent consensus are recognized as splicing enhancers (*31*). It is reasonable that the sequence requirements for exonic enhancers should not be strict, because a general exonic splicing element with a fixed sequence would heavily constrain the coding capacity of the region. Further, we find that different SR proteins activate ERS elements in varying degrees. This might be of interest, because the concentration of individual SR proteins varies in different tissues (*8*).

Significance of U1 snRNP—Previously, it was shown that U1 snRNA associates with the ERS elements (*28, 40*), and U1 snRNP might be required for function of the ERS elements, *e.g.*, by enhancing the binding of the SR proteins through cooperative binding similar to that found at the 5′ splice site (*24*): the 5′ splice site is known to be a target for specific binding of SF2/ASF (*41, 45*), and U1 snRNP binds there strongly only together with SF2/ASF (*24*). A similar mechanism might work at the ERS to stabilize complex formation.

We find that U1 snRNP is necessary for splicing enhancement of *dsx* SD which is driven by a downstream 5′ splice site. *dsx* ASLV is activated independently of U1 snRNP: first, competition with the RNA containing a 5′ splice site does not completely abolish splicing, even at very high concentrations, and splicing can be almost fully restored by adding purified SR proteins (Fig. 5A). Second, digestion of the 5′ end of U1 snRNA, which makes binding of U1 snRNP to the RNA impossible, inhibited splicing of *dsx* ASLV and it could be restored by addition of SR proteins. In both experiments, splicing of the *dsx* SD construct was also inhibited, but could not be restored by addition of exogenous SR proteins, even at threefold higher concentrations. It is, of course, possible that small amounts of U1 snRNP are left free or functional in these assays, or that trace amounts of U1 snRNP are present in the SR protein fraction. If this residual U1 snRNP were necessary at the ASLV elements, it would have to bind to that sequence more strongly than to the SD element, because SD RNA can inhibit splicing of *dsx* ASLV pre-mRNA only at very high concentrations (Fig. 5A, lanes 8 and 9). Then, however, ASLV RNA should easily inhibit *dsx* SD splicing, and this is not the case (Fig. 5A, lanes 4 and 5). We conclude that U1 snRNP is not needed for activation of the ASLV element, whereas activation by a downstream 5′ splice site requires U1 snRNP. Our results on the dependence on U1 snRNP contain another twist: since *dsx* ASLV is spliced presumably without the participation of U1 snRNP, how is the 5′ splice site of this construct activated? Again, it can be argued that competition for or inactivation of U1 snRNP is not likely to be complete or that the SR protein might contain trace amounts of U1 snRNP, but it can be held that the same low level of U1 snRNP should be sufficient to splice *dsx* SD pre-mRNA. We have no idea how the 5′ splice site is identified in these cases, but the [U4/U6·U5] tri-snRNP has been shown to bind independently to oligonucleotides containing a 5′ splice site (*46*). Furthermore, other recent reports show that certain, but not all, pre-mRNAs can be spliced *in vitro* in the absence of U1 snRNP (*47, 48*).

In conclusion, the binding of the SR proteins to the ASLV tag, but not to the downstream 5′ splice site is strong enough to enhance splicing of the weak upstream 3′ splice site independently of U1 snRNP. Splicing activation *via* the

downstream 5′ splice site is dependent on U1 snRNP, and most likely on the SR proteins, because ASLV RNA does inhibit splicing of the *dsx* SD pre-mRNA, albeit only at high concentrations, and because efficient binding of U1 snRNP has been shown to be dependent on SF2/ASF (*24*). Still, the specific binding of SF2/ASF to 5′ splice sites (*45*) is not strong enough to activate an upstream splice acceptor without the participation of U1 snRNP (this report).

Proteins Competing in Trans—SR proteins are probably not the only determinant of the strength of a given polypurine tag: even the *dsx* constructs which are not very efficiently spliced in the standard splicing assay still respond well to exogenous SR proteins, as shown with *dsx* BGH, *dsx* HPRT and, to a lesser extent, with *dsx* TM (Fig. 4, lanes 13-24).

This observation can be explained by antagonizing factors which bind preferentially to weak ERS sequences. A major candidate for such an inhibitory factor would be hnRNP A1, since it is well established that SF2/ASF and hnRNP A1 antagonize each other (*5, 6, 49, 50*), and this is also true in the case of splicing activation by the ERS derived from the bovine growth hormone gene (*32*). The target sequence for hnRNP A1 is thought to be the 5′ and 3′ splice sites (*51, 52*), but notably, the reported high-affinity binding sequences of hnRNP A1 contain uridines in a purine-rich context (*52*), resembling the features of weak ERS sequences (*31*). In good agreement with this idea, we found that the splicing activation by SR proteins can be counteracted by hnRNP A1 with every *dsx* construct used in this work (data not shown). However, the extent of inhibition does not correlate with the weakness of the ERS sequences. Other possible inhibitors include the proteins of 50, 70, and 75 kDa that bind to HPRTm (Fig. 1, lanes 11-15).

Conclusion—The 5′ and 3′ splice sites of mammalian introns interact with each other in the earliest characterized splicing complex, the commitment complex (*38, 53*). The interaction between U1 70 k, SF2/ASF or SC35, and U2AF seems to be a clamp which brings the two splice sites at either side of the intron into proximity (*21, 40*). Here, we show that not only SF2/ASF, but also at least one other SR protein—most probably SC35—recognizes a variety of sequences which are typically exonic. Furthermore, they bind to exonic elements such as the ASLV tag on their own (this report), whereas binding to a 5′ splice site requires cooperation with U1 snRNP (*24* and this report). Therefore, the "SR bridge" seems more likely to span the exon and thereby to represent the molecular basis of "exon definition" (*26*). In this case, the SR proteins would be the unidentified factor postulated in the initial description of the interaction between U2AF and U1 snRNP at a downstream 5′ splice site (*27*). The antagonists, hnRNP A1 and others, might be necessary to set a threshold for the binding of the agonists, to avoid the possibility that the initial complex fortuitously forms on cryptic splice sites, and is then stabilized by cooperativity.

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