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A downstream splicing enhancer is essential for in vitro pre-mRNA splicing

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Abstract Splicing enhancers have previously been shown to promote processing of introns containing weak splicing signals. Here, we extend these studies by showing that also 'strong' constitutively active introns are absolutely dependent on a downstream splicing enhancer for activity in vitro. SR protein binding to exonic enhancer elements or U1 snRNP binding to a downstream 5′ splice site serve redundant functions as activators of splicing. We further show that a 5′ splice site is most effective as an enhancer of splicing. Thus, a 5′ splice site is functional in S100 extracts, under conditions where a SR enhancer is non-functional. Also, splice site pairing occurs efficiently in the absence of exonic SR enhancers, emphasizing the significance of a downstream 5′ splice site as the enhancer element in vertebrate splicing.

Key words: RNA splicing; U1 snRNP; SR protein; Splicing enhancer; Adenovirus L1

1. Introduction

Numerous studies have shown that splicing of pre-mRNAs containing weak 3′ splice sites, i.e. splice sites that do not conform to the 3′ splice site consensus sequence, requires splicing enhancer elements for activity (reviewed in [1–3]). Enhancer-dependent introns typically contain short polypyrimidine tracts that bind U2AF inefficiently and, thus, require auxiliary factors for the efficient recruitment of U2AF to the 3′ splice site. Inclusion of such exons is often subjected to regulation and has therefore been subjected to an intensive study. Splicing enhancers are usually located in the exon downstream of the affected intron. Exonic splicing enhancers typically bind members of the SR family of splicing factors (reviewed in [1,2]). However, U1 snRNP binding to a downstream 5′ splice site also functions as an enhancer, stimulating upstream intron removal, the so called exon definition model [4,5].

Here, we show that in vitro splicing of two constitutively active introns, the adenovirus 52,55K and *Drosophila* fushi tarazu (Ftz) introns, both require a downstream enhancer for activity. Our results show that SR proteins binding to an exonic splicing enhancer or U1 snRNP binding to a downstream 5′ splice site individually function as splicing enhancer factors activating the upstream intron removal. In fact, an exon with a downstream 5′ splice site obviates the need for an exonic SR binding splicing enhancer. Our results further suggest that a 5′ splice site is more effective compared to a SR enhancer in activating splicing. Thus, a 5′ splice site activates splicing in S100 extracts under conditions where a SR enhancer is non-functional. Collectively, our results lend further support for the model that initial pairing of a splice site preferentially occurs across the exon (the exon definition model [4,5]).

2. Materials and methods

2.1. Plasmids and transcript synthesis

Plasmids pGem2 V61 (here referred to as Ftz), IIIa and 52,55K have previously been described [6,7]. By PCR cloning the 49 nucleotide 3RE [8], a 28 nucleotide long, duplicated ASF/SF2 enhancer [8,9] or the 49 nucleotide β-globin enhancer minus sequence [8] was appended six nucleotides downstream of the 52,55K 3′ splice site, generating plasmids mini52,55K,3a (3RE), mini52,55K,3a (2ASF) and mini52,55K,3a (−3RE), respectively. Plasmid miniFtz,3a (−3RE) was generated by replacing the 52,55K first exon and intron with the corresponding sequence from pGem2-V61. All pre-mRNAs were transcribed with T7 RNA polymerase from PCR-amplified products, with or without a 5′ splice site appended at the 3′ end [10]. Suitable double-stranded DNA templates for in vitro transcription were generated by using appropriately designed oligonucleotides in PCR reactions [10]. Plasmid maps, DNA sequences and the composition of primer pairs are available on request or at www.bmc.uu.se/IMIM/res/GA.html.

2.2. Extract preparation and in vitro splicing

HeLa nuclear extract was prepared as previously described [6]. The cytoplasmic fraction was saved and used to prepare splicing deficient S100 extracts as described [11]. Nuclear extracts were fractionated by three steps of ammonium sulfate precipitation (ASP) to obtain the 40, 60 and 90 ASP fractions as previously described [12]. All fractions were dialyzed against buffer D [13]. The total splicing reaction volume was 25 μl and contained 5–25 fmol ^{32}P-labelled transcript, 10 μl nuclear or S100 extracts, 5 μl buffer D, extract fractions or HeLa SR proteins (0.5 μg), 2.6% polyvinylic alcohol, 12% glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl, 2 mM ATP, 20 mM creatine phosphate, 0.3 mM DTT, 2.5 mM $MgCl_2$ and 15 U RNasin. Reactions were incubated at 30°C, for 2 h. Splicing products were resolved on denaturing 8% polyacrylamide gels, followed by autoradiography.

2.3. U snRNA depletion

Oligonucleotide-directed RNase H cleavage of U snRNPs in the 40 ASP was done exactly as described [14]. An aliquot of the cleaved extract was used to verify that the RNase H treatment effectively destroyed the designated U snRNA, the remaining of the extract was used for a splicing assay. Oligonucleotides 5′C and E15 [15] directed against the 5′ ends of U1 snRNA and U2 snRNA were used in the depletion reaction.

2.4. Western blot analysis

10 μl of 40, 60 and 90 ASP were separated by 12% SDS-PAGE under reducing conditions. Proteins were transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked in TBS buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk (w/v), washed in TBS and incubated with monoclonal antibody 2.73 ([16] 1:100 dilution in TBS) for 1 h with gentle shaking at room temperature. The membrane was washed three times in TBS, incubated with anti-mouse antibody conjugated with

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horseradish peroxidase (Amersham, diluted 1:5000 in TBS) for 1 h at room temperature, followed by three washes with TBS, each for 10 min. Proteins were visualized by chemiluminescence as described in the manufacturer's protocol (Amersham).

3. Results

Here, we have tested the significance of U1 snRNP and SR proteins as splicing enhancer factors. Splicing deficient S100 extracts contain low amounts of SR proteins, below the threshold concentration required to activate splicing [11]. Thus, by complementing S100 extracts with purified SR proteins or fractions enriched in U1 snRNP, the significance of a respective factor for splicing activation was tested.

In most of these studies, we used the adenovirus major late region 1 52,55K and IIIa introns as model substrates for strong and weak 3′ splice sites, respectively. The constitutively active 52,55K intron contains a prototypical 3′ splice site with a high pyrimidine content (18 pyrimidines out of 19 nucleotides), whereas the regulated IIIa intron is weak with a short polypyrimidine tract frequently interrupted by purines [6].

3.1. An U1 enhancer activates IIIa intron splicing

Appending the strong adenovirus major late first leader 5′ splice site [10] (we refer to this as an U1 enhancer) to the weak adenovirus IIIa second exon resulted in a 20–50-fold stimulation of splicing in HeLa-NE (Fig. 1A, transcript IIIa-U1, compare lanes 5 and 10). IIIa splicing without the U1 enhancer was barely detectable (Fig. 1A, lane 10). Based on results like this, we previously concluded [10] that U1 snRNP binding downstream of the weak IIIa 3′ splice site functions as a splicing enhancer stimulating IIIa intron removal. Later studies have shown that the SR protein ASF/SF2 can directly bind to a 5′ splice site [17]. We therefore re-investigated the importance of U1 snRNP as the IIIa-U1 splicing enhancer factor.

To characterize the factor causing activation of IIIa-U1 splicing, we fractionated HeLa-NE by ASP into three fractions, the 40, 60 and 90 ASP. As shown in Fig. 1A, the 40 ASP, but not the 60 or 90 ASP, activated IIIa-U1 splicing (lanes 3–5). Since most SR proteins are enriched in the 90 ASP fraction [12], this result argues against SR proteins as the enhancer factor stimulating IIIa-U1 splicing. This result was expected since we have previously demonstrated that SR proteins functions as repressor, not activator proteins of IIIa splicing [8,18]. None of the fractions was able to activate IIIa splicing (lanes 7–9).

Based on our previous work [10], we predicted that 40 ASP would be enriched in U1 snRNP. Western blot analysis, using a monoclonal antibody specific for the U1-70K protein, confirmed that U1 snRNP was indeed selectively enriched in 40 ASP (Fig. 1B). To further show that it was U1 snRNP that was the enhancer factor, we used RNase H-oligonucleotide-directed depletion to functionally inactivate U1 snRNA in 40 ASP. As shown in Fig. 1C, incubating 40 ASP with increasing amounts of an U1 oligonucleotide during the RNase H treatment abolished the stimulatory effect of 40 ASP on IIIa-U1 splicing. In contrast, pre-treatment of 40 ASP with an U2-

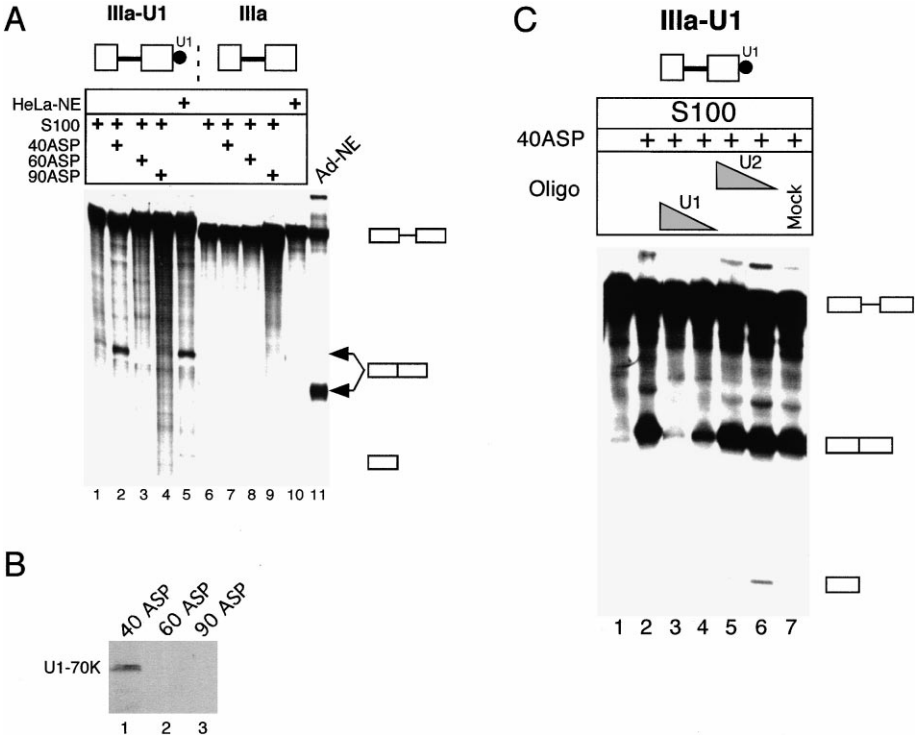

Fig. 1. Enhancer-dependent splicing of the adenovirus IIIa pre-mRNA. A: 40 ASP activates IIIa-U1 but not IIIa splicing in splicing deficient S100 extracts. HeLa-NE separated into three fractions (40, 60 and 90 ASP) by ASP was used to program splicing in splicing deficient S100 extracts. Ad-NE, a control showing IIIa splicing in nuclear extracts prepared from late adenovirus-infected cells [23]. B: Western blot analysis, demonstrating that U1 snRNP is enriched in the 40 ASP fraction. C: Oligonucleotide directed RNase H cleavage of 40 ASP showing that an intact U1 snRNA is required for the enhancer activity of 40 ASP. Mock, extract treated with RNase H in the absence of oligonucleotide. In A and C, the structure of transcripts is shown at the top with the position of pre-mRNA and splicing products marked on the side of each autoradiogram. $\bullet^{U1}$ denotes the presence of an U1 enhancer (the adenovirus major late first intron 5′ splice site [10]).

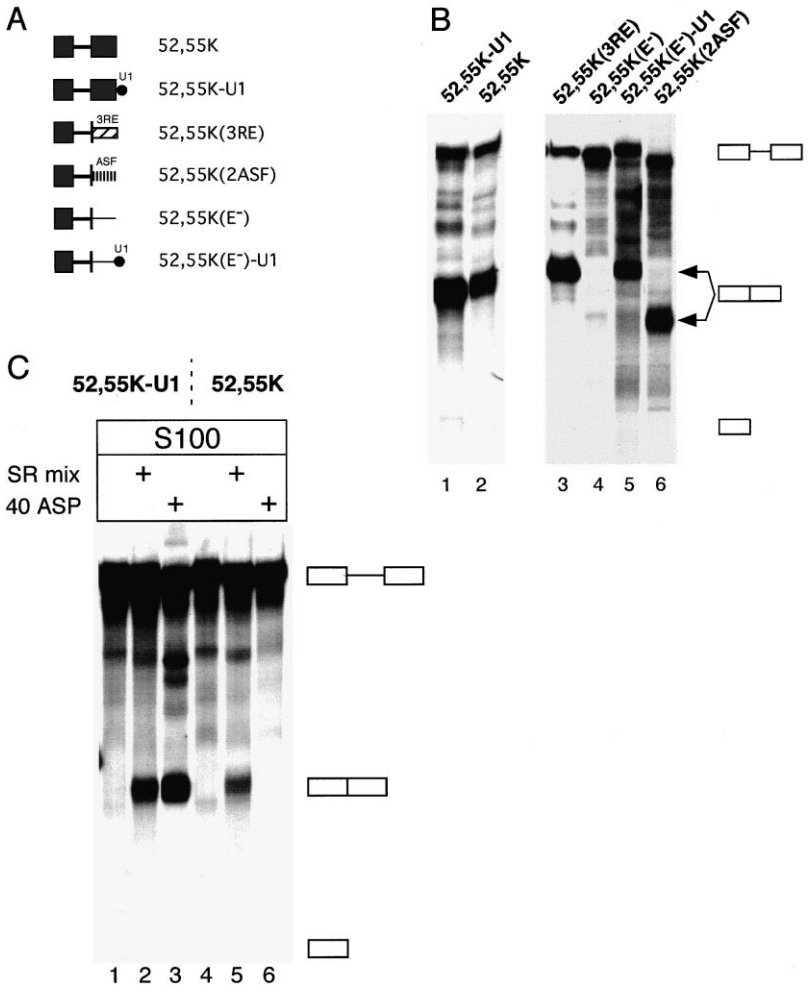

Fig. 2. A downstream splicing enhancer is essential for 52,55K splicing. A: The structure of 52,55K pre-mRNAs used in B and C is graphically depicted. $\bullet^{U1}$ denotes the presence of an U1 enhancer (the adenovirus major late first intron 5′ splice site [10]). 3RE and 2ASF are two types of exonic SR binding splicing enhancer sequences. E^- (thin line), an enhancer minus sequence that does not bind SR proteins [8]. B: Redundant function of a SR enhancer and an U1 enhancer in activating 52,55K splicing. The transcript shown in A was used to program splicing in HeLa-NE. C: SR proteins and 40 ASP differ in their capacity to activate 52,55K+/−an U1 enhancer in S100 extracts.

specific oligonucleotide had no effect on the 40 ASP activation of splicing. Collectively, these results demonstrate that U1 snRNP, with an intact U1 snRNA, is required for the enhancer activity of 40 ASP on IIIa-U1 splicing.

3.2. 52,55K intron splicing requires a downstream splicing enhancer

In contrast to IIIa splicing which is strongly activated by an U1 enhancer, splicing of the 52,55K intron is only marginally activated by an U1 enhancer in HeLa-NE (Fig. 2B, lanes 1 and 2). As shown in Fig. 2C, addition of 40 ASP to S100 extracts only activated 52,55K-U1 splicing. RNase H oligonucleotide depletion of U1 snRNA abolished the stimulatory effect of 40 ASP on 52,55K-U1 splicing (data not shown). In contrast, addition of purified HeLa SR proteins activated both 52,55K-U1 and 52,55K splicing in a S100 complementation assay (Fig. 2C). SR proteins function as splicing enhancer proteins typically by binding to exonic enhancer elements (reviewed in [1,2]). To determine whether 52,55K splicing requires a downstream splicing enhancer, we replaced the 52,55K second exon with a β-globin sequence that we previously have shown does not bind any of the classical SR proteins [8], thus, creating transcript 52,55K(E^-) (Fig. 2A). Remarkably, this transcript was completely inactive in splicing in HeLa-NE (Fig. 2B, lane 4). This result strongly suggests that splicing of a 'strong' constitutively active intron also requires

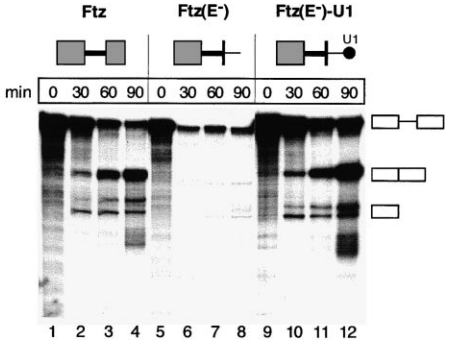

Fig. 3. A downstream splicing enhancer is required for Ftz splicing in HeLa-NE. Replacement of the Ftz second exon with an enhancer minus sequence (Ftz(E^-)) that does not bind SR proteins [8] abolishes Ftz splicing in vitro (lanes 5–8). Appending an U1 enhancer to the Ftz(E^-) pre-mRNA restored Ftz splicing (lanes 9–12).

a downstream splicing enhancer for activity. Importantly, appending an U1 enhancer to the 52,55K(E^-) transcript regained its splicing activity in HeLa-NE (52,55K(E^-)-U1: Fig. 2B, lane 5) or in S100 extracts supplemented with 40 ASP (data not shown). This result is significant, because it suggests that the β-globin sequence replacing the 52,55K second exon was not inhibitory for splicing. More likely, it was non-functional, because it does not have the capacity to bind any splicing enhancer factors. In agreement with this hypothesis, appending a characterized ASF/SF2 splicing enhancer [9], or the 3RE, which binds all of the classical SR proteins [8], effectively restored 52,55K intron splicing (Fig. 2B, lanes 3 and 6).

Collectively, these results demonstrate that a downstream splicing enhancer (SR or U1 enhancer) is obligatory for splicing the 'strong' constitutively active 52,55K intron.

3.3. *Drosophila Ftz intron splicing requires a downstream splicing enhancer*

To demonstrate that the requirement for a downstream splicing enhancer was not unique to 52,55K splicing, we tested the importance of the *Drosophila* Ftz second exon for its splicing activity. As shown in Fig. 3, Ftz intron splicing is very efficient with a conversion of almost 50% of input RNA (lane 1) to spliced product (lane 4) after 90 min of incubation. Substituting the Ftz second exon with the β-globin enhancer minus sequence used above (Fig. 2) completely abolished Ftz intron splicing (transcript Ftz(E^-)). Appending an U1 enhancer to the Ftz(E^-) transcript restored Ftz intron splicing, to wild-type levels (transcript Ftz(E^-)-U1). Again, demonstrating that the β-globin sequence did not function as an inhibitory sequence for splicing. This result strengthens our model that a downstream splicing enhancer is obligatory also for splicing of 'strong' constitutively active introns in HeLa-NE.

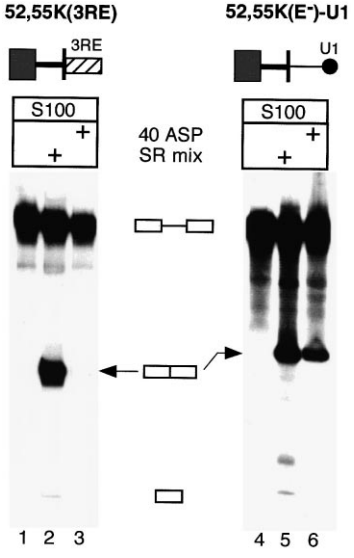

Fig. 4. An U1 enhancer is more effective compared to a SR enhancer in activating 52,55K splicing in vitro. Supplementing S100 extracts with SR proteins or 40 ASP, which is enriched in U1 snRNP, shows that an U1 enhancer, but not a SR enhancer, is activated by both enhancer factors. The structure of the pre-mRNAs used is depicted at the top of the figure and the position of pre-mRNAs and splicing products between the two autoradiograms.

We note that the Ftz(E^-) pre-mRNA was rapidly degraded if not spliced (Fig. 3, lanes 5–8), suggesting that the Ftz intron contains a destabilizing sequence that causes degradation of the pre-mRNA if it is not committed to splicing.

3.4. *An U1 enhancer is more versatile compared to an SR enhancer*

Neither an U1 enhancer nor a SR enhancer is functional in S100 extracts (Figs. 2C and 4), suggesting that S100 extracts contain sub-optimal concentrations of both SR proteins and U1 snRNP. This allowed us to design an experiment to test the importance of a respective enhancer for upstream intron splicing. As shown in Fig. 4, addition of 40 ASP activated 52,55K(E^-)-U1 splicing (lane 6), but not 52,55(3RE) splicing (lane 3). In contrast, supplementing S100 extracts with SR proteins activated both 52,55K(3RE) and 52,55K(E^-)-U1 splicing (lanes 2 and 5). We interpret this result to indicate that an U1 enhancer is more effective compared to a SR enhancer. It is activated both by SR proteins and U1 snRNP. In contrast, an SR enhancer is only activated by SR proteins. Based on this observation, and general considerations concerning the splice site definition (see Section 4), we propose that U1 enhancers are the prototypical enhancer element activating splicing of constitutively active introns.

4. Discussion

Previous studies have shown that splicing enhancer elements are important regulatory elements controlling spliceosome assembly at weak-regulated 3′ splice sites containing sub-optimal splicing signals (reviewed in [1–3]). Here, we extend these studies by demonstrating that a downstream splicing enhancer also is obligatory for in vitro splicing of 'strong' constitutively active introns, here exemplified by the adenovirus 52,55K and the *Drosophila* Ftz introns. We further show that two types of elements function as splicing enhancers in *cis*-splicing. Thus, a downstream 5′ splice site, which binds U1 snRNP (U1 enhancer), or an exonic SR protein binding element (SR enhancer) can individually stimulate upstream intron splicing in vitro.

The mechanism of 3′ and 5′ splice site recognition across an exon operates with minimal sequence information in higher eukaryotes. Thus, multiple weak interactions between U2AF/U2 snRNP binding to the 3′ splice site, SR proteins binding to exonic splicing enhancer elements and U1 snRNP interacting with the downstream 5′ splice site have been proposed to be required for recognition of an exon in a pre-mRNA (reviewed in [1,2,3]). Our results suggest that U1 enhancers are more significant, compared to SR enhancers, as splicing activator elements. Two lines of evidence support this conclusion. First, efficient splice site pairing occurs in the complete absence of an exonic splicing enhancer element (the enhancer minus (E^-) transcripts, Figs. 2B, 3 and 4). Second, we show that an U1 enhancer is capable of activating splicing in S100 extracts under conditions where a SR enhancer is non-functional (Fig. 4). These findings are important since they suggest that U1 snRNP binding to the downstream 5′ splice site may in most cases suffice as a splicing enhancer for upstream intron removal. Thus, there appears to be no absolute requirement for SR enhancers in splicing of internal exons in vertebrate genes. Such a finding may be relevant since usage of an exonic SR enhancer requires co-evolution of the enhancer and

the amino acid sequence encoded by the exon. We propose that U1 enhancers are the prototypical splicing enhancer used in constitutive splicing. SR enhancers may serve their primary function as elements regulating processing of introns containing 'weak' splicing signals and unusual exons which are to long to allow direct pairing of splice sites across the exon (see [4]).

Current models suggest that SR proteins stabilize U2AF interaction with U1 snRNP by making simultaneous contact with U2AF35K and U1-70K (reviewed in [1,2]). The surprising finding that U1 snRNP activates splicing in S100 extracts (Figs. 1C, 2C and 4), which are depleted of functional concentrations of SR proteins [11], would, at a first glance, argue against this hypothesis by suggesting that SR proteins are dispensable for the activity of an U1 enhancer. However, splicing deficient S100 extracts contain low amounts of SR proteins (data not shown), below the threshold concentration required to activate constitutive splicing (Figs. 2C and 4). Thus, an alternative explanation would be that the RNA binding affinity of SR protein is functionally lower compared to the concentrations required for protein-protein interaction between SR proteins and U1 snRNP. Therefore, the sub-optimal concentrations of functional SR proteins in S100 extracts might be sufficient to activate splicing using an U1 enhancer, but fail to activate splicing through an SR enhancer. Also, SR proteins collaborate with U1 snRNP to form stable interaction with a 5′ splice site [19,20]. Thus, addition of an excess of U1 snRNP to S100 extracts might activate an U1 enhancer by efficiently recruiting the low amount of SR proteins in the extract to the 5′ splice site. Similarly, addition of an excess of SR proteins to S100 extracts might help to recruit the sub-optimal amount of U1 snRNP in the extract to an U1 enhancer. Such a model would explain why SR proteins activate an U1 enhancer (Fig. 2C). Although this hypothesis is attractive, we have not been able to experimentally prove that the sub-optimal amounts of SR proteins present in S100 extracts are efficiently recruited to an U1 enhancer (data not shown).

Our results lend further support to the model that splice sites are initially paired by splicing factor interaction across the exon [4]. Thus, supplementing S100 extracts with a fraction enriched in U1 snRNP stimulates 3′ to 5′ splice site interaction across the exon, but did not stimulate 5′ to 3′ splice site interaction across the intron (Figs. 1A, 2C and 4). The observation that a downstream 5′ splice site activates upstream intron splicing in the absence of an exonic SR enhancer further emphasizes the significance of splice site pairing across the exon, through factor interaction directly with the 3′ and 5′ splice sites. We also show that splicing of the *Drosophila* Ftz intron requires a downstream splicing enhancer (Fig. 3), i.e. splice site definition according to the exon definition model. At a first glance, this observation appears to contradict the proposal that introns, not exons, constitute the unit for splice site pairing in lower eukaryotes (reviewed in [5]). However, *Drosophila* appears to be intermediate, with some genes using exons as the unit for splice site definition [5,21]. In fact, the Ftz intron has been suggested to exhibit properties similar to that of introns of higher eukaryotes (discussed in [22]). Our data show that Ftz intron splicing operates via exon definition in HeLa-NE (Fig. 3).

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References

[1] Fu, X.D. (1995) RNA 1, 663–680.
[2] Manley, J.L. and Tacke, R. (1996) Genes Dev. 10, 1569–1579.
[3] Reed, R. (1996) Curr. Opin. Genet. Dev. 6, 215–220.
[4] Robberson, B.L., Cote, G.J. and Berget, S.M. (1990) Mol. Cell. Biol. 10, 84–94.
[5] Berget, S.M. (1995) J. Biol. Chem. 270, 2411–2414.
[6] Kreivi, J.P., Zerivitz, K. and Akusjärvi, G. (1991) Nucleic Acids Res. 19, 2379–2386.
[7] Rio, D.C. (1988) Proc. Natl. Acad. Sci. USA 85, 2904–2908.
[8] Kanopka, A., Mühlemann, O. and Akusjärvi, G. (1996) Nature 381, 535–538.
[9] Tacke, R. and Manley, J.L. (1995) EMBO J. 14, 3540–3551.
[10] Kreivi, J.P., Zerivitz, K. and Akusjärvi, G. (1991) Nucleic Acid Res. 19, 6956.
[11] Krainer, A.R., Conway, G.C. and Kozak, D. (1990) Cell 62, 35–42.
[12] Zahler, A.M., Lane, W.S., Stolk, J.A. and Roth, M.B. (1992) Genes Dev. 6, 837–847.
[13] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[14] Lamond, A.I. and Sproat, B.S. (1994) in: RNA Processing, a Practical Approach (Higgins, S.J. and Hames, B.D., Eds.), Vol. 135, pp. 103–140, IRL press, Oxford.
[15] Black, D.L., Chabot, B. and Steitz, J.A. (1985) Cell 42, 737–750.
[16] Billings, P.B., Allen, R.W., Jensen, F.C. and Hoch, S.O. (1982) J. Immunol. 128, 1176–1180.
[17] Zuo, P. and Manley, J.L. (1994) Proc. Natl. Acad. Sci. USA 91, 3363–3367.
[18] Kanopka, A., Mühlemann, O., Petersen-Mahrt, S., Estmer, C., Öhrmalm, C. and Akusjärvi, G. (1998) Nature 393, 185–187.
[19] Kohtz, J.D., Jamison, S.F., Will, C.L., Zuo, P., Luhrmann, R., Garciablanco, M.A. and Manley, J.L. (1994) Nature 368, 119–124.
[20] Jamison, S.F., Pasman, Z., Wang, J., Will, C., Luhrmann, R., Manley, J.L. and Garcia-Blanco, M.A. (1995) Nucleic Acids Res. 23, 3260–3267.
[21] Mount, S.M., Burks, C., Hertz, G., Stormo, G.D., White, O. and Fields, C. (1992) Nucleic Acids Res. 20, 4255–4262.
[22] Talerico, M. and Berget, S.M. (1994) Mol. Cell. Biol. 14, 3434–3445.
[23] Kreivi, J.P. and Akusjärvi, G. (1994) Nucleic Acids Res. 22, 332–337.