Influence of Polymerase II Processivity on Alternative Splicing Depends on Splice Site Strength*

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Transcription and pre-mRNA splicing are coordinated temporally and spatially, and both processes can influence each other. In particular, control of transcriptional elongation by RNA polymerase II has proved to be important for alternative splicing regulation. In this report we demonstrate that the efficiency of exon recognition by the splicing machinery is crucial for the elongation control. Alternative splicing of the fibronectin extra domain I (EDI) is because the polypyrimidine tract of its 3′-splice site occurs suboptimal. By mutating the polypyrimidine tract of EDI in two different positions, individually or in combination, and by disrupting its exonic splicing silencer, we managed to generate minigenes with increasing degrees of exon recognition. Improvement of exon recognition is evidenced by independence from the splicing regulator SF2/ASF for inclusion. The mutated minigenes were used to transfect human cells in culture and study the responsiveness of EDI alternative splicing to activation or inhibition of pol II elongation. Our results revealed that responsiveness of exon skipping to elongation is inversely proportional to 3′-splice site strength, which means that the better the alternative exon is recognized by the splicing machinery, the less its degree of inclusion is affected by transcriptional elongation.

Most human gene transcripts are alternatively spliced generating several different mRNAs that increase protein diversity (1). cis-Acting sequences and trans-acting factors regulate splicing. The sequences involved are mainly the 5′-splice site (5′-ss) or donor site, the 3′-splice site (3′-ss) or acceptor site that includes a pyrimidine-rich region called the polypyrimidine tract (PPT), and the branch point, located 18–40 nucleotides upstream of the 3′-ss. In metazoans, the PPT is essential for efficient branch point utilization and 3′-ss recognition (2). On the other hand, protein factors can act on regulatory sequences. When alternative splicing exists, the choice between the alternative sites depends on the relative quality of the constitutive signals. In addition, regulatory proteins can shift this balance and favor one site usage to the detriment of the other. This implies the presence of complex regulatory mechanisms.

Reactions involved in pre-mRNA processing such as capping, splicing, and 3′-end processing/polyadenylation are closely coupled to RNA polymerase II (pol II) transcription and can modulate each other, adding to the intricacy of these events (for reviews see Refs. 3–7). For example, we have demonstrated that promoter identity can affect alternative splicing (8, 9). This could be achieved by the recruitment of splicing factors and/or by modulating pol II elongation rate. Factors that increase elongation, such as certain transcriptional activators, stimulate skipping of the fibronectin (FN) EDI exon, whereas treatments with drugs that inhibit elongation like 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole (DRB), favor exon inclusion (10–12). More recent and direct evidence supports an in vivo control of splicing by pol II. The C4 point mutation of the Drosophila pol II largest subunit confers the enzyme a lower elongation rate (“slow” polymerase), and it was shown in Drosophila embryos carrying this mutation that resplicing of the Hax gene Ultrabithorax is stimulated. This provides the first evidence for the transcriptional control of alternative splicing on an endogenous gene (13).

The ability of activators to enhance elongation by pol II correlates with their ability to enhance exon skipping. EDI exon skipping occurs because the 3′-ss of the upstream intron (named EDI 3′-ss) is suboptimal compared with the 3′-ss of the downstream intron. Our model assumes that if the polymerase pauses anywhere between these two sites, only elimination of the upstream intron can take place. Once the pause is passed or the polymerase proceeds, there is no option for the splicing machinery to eliminate the downstream intron, which leads to exon inclusion (Fig. 1A, left). A highly processive elongating pol II would favor the simultaneous presentation of both introns to the splicing machinery, a situation in which the stronger 3′-ss of the downstream intron outcompetes the weaker EDI 3′-ss site, resulting in exon skipping (Fig. 1A, right). At the pre-mRNA primary sequence level, this model relies almost exclusively on the weakness of EDI 3′-ss. Therefore, the model can be tested by looking at the effects of pol II elongation rate on a transcript in which EDI 3′-ss has been strengthened (Fig. 1B).

In this study we strengthened EDI 3′-ss by mutating its polypyrimidine tract in two different positions, independently or in combination. These mutants not only provoked much
**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—pSVEDA/Gal5-HIV-2 has been described previously (12). This plasmid contains the α-globin/FN minigene reporter for alternative splicing of the EDI alternative exon of human FN (also named EDA and EIIIA) under the control of the HIV-2 promoter fused to five copies of the target site for the DNA-binding domain of Saccharomyces cerevisiae Gal4 transcription factor. pSVEDA/Gal5-HIV-2 WT contains the EDI alternative exon of human FN under the control of the HIV-2 promoter (9). None of these minigene reporter constructs contains the SV40 enhancer/origin region. Expression vector for Gal4 fusion protein Gal4-VP16 (410-490) and the control vector Gal4 (1-147) were described previously (14). g10 SF2/ASF wild type expression plasmid was described previously (15).

**Generation of Point Mutations**—The point mutations G→T at position −9, A→T at position −5, and the double mutation G→T and A→T were generated by PCR-directed mutagenesis using complementary primers carrying the mutations as well as primers anchoring at the unique restriction sites of SnaBII (−1280 bp upstream of the mutation site) and XhoI (−120 bp downstream of the mutation site). Successive PCRs were performed with Pfu Turbo polymerase (Stratagene) until a 1.4-kbp product was obtained. This product was digested with SnaBII and XhoI and inserted into the minigene plasmids previously digested with the same enzymes. In this way, pSVEDA/Gal5-HIV-2 AT that carries the A→T mutation and pSVEDA/Gal5-HIV-2 DM that carries the G→T and A→T mutations were generated from the wild type plasmid (pSVEDA/Gal5-HIV-2 WT). In the case of plasmids in which the FN promoter drove transcription, the mutant constructs pSVEDA/FN GT (carrying the G→T mutation), pSVEDA/FN AT (carrying the A→T mutation), and pSVEDA/FN DM (carrying the G→T and A→T mutations) were generated from the wild type plasmid pSVEDA/FN WT. The resulting constructs were sequenced to confirm that they carried the designed mutations.

**Transfections**—Conditions for transfection of human hepatoma Hep3B cells with minigene constructs were described elsewhere (10). For RNAi functional knock down experiments, cells were co-transfected with 600 ng of the reporter minigene for FN EDI splicing and 500 ng of either one of the following siRNA duplexes (only sense sequence shown): SF2/ASF, 5’-CCAAGGACAUUGAGGACGdTdT-3’; Luc (GL3 luciferase), 5’-CUUACGUCUGAGACUCUCUGAUCdTdT-3’ (16). Cells were harvested 48 h post-transfection.

**Alternative Splicing Assays**—RNA preparation and reverse transcriptase reactions using oligo(T) as primer were described previously (10). Radioactive PCR amplification of cDNA splicing isoforms were performed using reported specific primers for EDI (9).

**RESULTS**

**Generation of Point Mutations to Strengthen the Polypyrimidine Tract of EDI 3’-Splice Site**—As a tool to assess the effects on alternative splicing, we used a minigene in which the human fibronectin EDI alternative exon and its flanking regions were embedded in the third exon of the human α-globin gene. By sequence inspection, the EDI 3’-ss appears weak compared...
with the 3′-ss of the intron downstream (intron +1), which seems to be the main cause of the EDI exon being alternative. In particular, the PPT falls apart from the consensus sequence (17, 18). Based on this information, we decided to mutate two purines found in the PPT at positions 9 and 5 (G and A, respectively) to T (see under “Experimental Procedures” for details). In this way, we generated two different point mutant constructs named GT and AT, and the double mutant named DM (Fig. 1C). These mutant minigenes were placed under the control of the Gal5-HIV-2 minimal promoter or the FN promoter.

**VP16 Effect on EDI Skipping Is Inverted in the Polypyrimididine Mutants**—The model in Fig. 1A predicts that if the 3′-ss of EDI is made stronger, treatments that increase pol II elongation rate should have smaller or null effects in promoting exon skipping of the EDI. We first assessed the effect of the elongation activator protein VP16 on the mutated constructs. As shown previously (10), Gal4-VP16 provokes exon skipping on transcripts of a construct driven by the Gal5-HIV-2 promoter (Fig. 2, A and B, lanes 1 and 2). As expected, constructs with the mutated PPT have a much higher (44- and 68-fold, respectively) basal exon inclusion (Fig. 2A, lanes 3 and 5), reflecting better exon recognition by the splicing machinery. Surprisingly, VP16 not only does not provoke exon skipping in the mutated transcripts (Fig. 2A, lanes 4 and 6) but enhances EDI retention.

**VP16 Has No Effect on EDI Inclusion in a Construct Where the ESS Has Been Disrupted**—To rule out that the observed results were caused by mutation effects other than a strengthening of EDI exon recognition, we tested a minigene in which the ESS has been disrupted. EDI carries an ESS downstream of the ESE (19). Disruption of this element provoked a 13-fold stimulation of basal EDI inclusion (Fig. 2B, lane 3) as a consequence of improving EDI exon recognition through a strategy different from PPT mutagenesis. Consistently, however, with the PPT results, VP16 failed to induce EDI skipping in the ESS mutant (Fig. 2B, lane 4).

We assumed that basal EDI inclusion levels in each con-
struct reflect indirectly 3′-ss strength. Thus, in this particular case, the ESS mutant would be stronger than the WT but weaker than the PPT mutants. These PPT mutants showed that these point mutations strongly enhanced exon recognition almost as if it were a constitutive exon. On the other hand, the ESS disruption perhaps had a more feeble effect as it acted indirectly on the 3′-ss as a way to strengthen this splice site. Nonetheless, the effects of the two strengthening strategies are qualitatively similar. Fig. 2C shows that the effect of enhancing exon skipping by VP16 correlates with the effectiveness of the 3′-ss recognition.

Inhibition of EDI Skipping by the Elongation Inhibitor DRB Is Smaller in the PPT Mutants—The positive transcription elongation factor (P-TEFb) complex is a dimer of CDK9 and cyclin T1/2. Its kinase activity (20) was involved in the stimulation of pol II elongation by activators. P-TEFb phosphorylates the carboxyl-terminal domain of pol II, and this phosphorylation converts the polymerase from a nonprocessive to a processive form (for a thorough review on P-TEFb action see Ref. 21). The fact that an inhibitor of this kinase, like DRB, prevents pol II elongation (22) strongly supports this idea.

Cells were transfected with wild type (WT) minigenes and with minigenes carrying the PPT mutations (GT, AT, and DM) under the control of the FN promoter (Fig. 3A) and were treated with DRB for the last 24 h of transfection. As foreseen, PPT mutations strongly provoked higher EDI inclusion levels also with this promoter. The AT, DM, and DM mutants showed 24-, 84-, and 142-fold increases in EDI inclusion, respectively (Fig. 3A, lanes 1, 3, 5, and 7). The mutation at position −9 (GT) appeared to be less effective than that at position −5 (AT) to promote exon recognition. DRB had a 3.2-fold effect on the WT construct (Fig. 3A, lanes 3 and 1), but this effect strongly decreased when the reporter minigene carries the PPT mutations (Fig. 3A, lanes 3–8). This supports our hypothesis that sensitivity to changes in pol II processivity correlates with the relative weakness of EDI 3′-ss compared with the 3′-ss located downstream of the alternative exon. Curiously, the effect of DRB is not completely abolished even in the DM constructs.

Fig. 3B shows that the stronger the 3′-ss is, the less the minigene is responsive to the elongation inhibitor DRB. As in Fig. 2C, we assumed that basal EDI inclusion correlates with 3′-ss strength. Apparently, at least when transcription is driven by the FN promoter, a plateau is reached, as the AT and DM mutants do not differ significantly in their behavior.

The SR Protein SF2/ASF Fails to Further Augment EDI Inclusion in the PPT Mutants—It has been shown that a 3′-ss is recognized and used depending on a combination between the branch point, the PPT, and splicing enhancer elements. Splicing enhancers can compensate for either a weak PPT or a weak branch point (23). When regulation of the splice sites selection is required, cells can modulate the production of different mRNAs by differentially expressing SR proteins that bind to splicing enhancer elements. In the β-tropomyosin pre-mRNA, for example, inappropriate exon skipping can be prevented by an excess of the SR protein SF2/ASF (24).

In the case of FN, SF2/ASF binds to the purine-rich sequence 5′-GAGAGAAGC-3′ found in the ESE of EDI. By doing so, it stimulates the use of the suboptimal EDI 3′-ss and consequently increases EDI inclusion (9). Thus, SF2/ASF binding functionally strengthens exon recognition in trans by improving splicing factor assembly on the intrinsically weak EDI 3′-ss. Therefore, SF2/ASF effect is somehow equivalent to make the 3′-ss closer to the consensus by mutagenesis.

We assessed the effect of SF2/ASF overexpression on the PPT mutants in the human FN minigene. It was previously shown that sensitivity to SF2/ASF was dependent on exon deformation (25). Whereas SF2/ASF enhanced EDI inclusion by more than 20-fold on the WT construct, it did so only by 5-fold on the GT mutant, and it had no effect at all on the AT and DM mutants (Fig. 4A). Once more, the point mutant GT appears to be weaker than the AT, which behaves like the DM as in Fig. 3A.

Overexpression of a protein could perturb normal cell behavior, as it may commit the transcription and translation machineries to work on its production to the detriment of other proteins of the cell. As a control of a different nature, we evaluated the SF2/ASF effect by specifically preventing its translation...
instead of overexpressing it. We did this through the RNAi technique. We have shown previously, by Western blot experiments, that co-transfection of RNAi against SF2/ASF completely abolishes SF2/ASF production (13) in transfected cells. Fig. 4B (lanes 1–4) shows that the WT and GT constructs are equally responsive to EDI inclusion inhibition by RNAi to SF2/ASF (~3-fold inhibition). The AT and DM mutants are less sensitive to SF2/ASF RNAi than the WT (Fig. 4B, lanes 5–8).

DISCUSSION

Transcriptional modulation of alternative splicing was first suggested by promoter swapping experiments with minigenes transfected into mammalian cells (8, 9, 25, 26). More physiological evidence for the existence of co-transcriptional events controlling alternative splicing involved findings that transcription factors with different effects on pol II elongation, acting on a single promoter, had differential effects on alternative splicing (12). The use of a mutated large subunit of RNA polymerase II with lower processivity permitted a more direct demonstration that changes in transcription elongation rates modulate alternative splicing both on reporter minigenes transfected in human cells and on an endogenous gene in Drosophila embryos (13). Nevertheless, it is important to point out that not all alternatively spliced regions are subjected to transcriptional control. For example, inclusion levels of the alternatively spliced EDII exon of human fibronectin (also named EDB and EIIIB) are not affected by promoter swapping, transcription factor activation, or the slow polymerase but are stimulated by overexpression of the SR protein Srp40 (27). This indicates that control by SR proteins might or might not coexist with transcriptional control.

Responsiveness of alternative splicing to transcriptional elongation might not only depend on factors that are known to affect pol II elongation in general such as chromatin organization (10), histone acetylation (12), or carboxyl-terminal domain phosphorylation (DRB effect, see Ref. 12 and Fig. 3 in this paper) but also the gene pausing architecture, i.e. the number, strength, and distribution of transcription pauses along the DNA template (28) as well as the presence of long introns in the alternatively spliced regions (29).

In this report, we demonstrate that the efficiency of exon recognition by the splicing machinery is crucial for the elongation control. Previous studies have demonstrated that strengthening of EDI 3'-ss and 5'-ss of a mouse FN minigene increase exon recognition (30). By mutating the polypyrimidine tract of human EDI in two different positions, individually or in combination, and by disrupting its ESS, we managed to generate five constructs (including the wild type) with the following increasing degrees of EDI exon recognition: WT, ESS−, GT, AT, and DM. Improvement of exon recognition is evidenced both by increases in basal EDI inclusion/exclusion ratios and by independence from SF2/ASF for inclusion (Fig. 4). The mutated constructs were used to study responsiveness to activators (VP16) and inhibitors (DRB) of pol II elongation. Our results validate the model in Fig. 1A by demonstrating the hypothesis...
in Fig. 1B, and revealed that responsiveness of exon skipping to elongation correlates inversely with 3'-ss strength. In other words, the better the alternative exon is recognized by the splicing machinery, the less its degree of inclusion is affected by transcriptional elongation.

An inverse approach has been used recently in yeast to show a similar link between elongation and exon recognition. Alternative splicing is a very rare event in yeast. By mutating the branch point upstream of the constitutive internal exon of the DYN2 gene, Howe et al. (31) created an artificial cassette exon that becomes alternatively spliced. Skipping of this exon can be partially prevented when expressed in a yeast mutant carrying a slow pol II or in the presence of elongation inhibitors. This supports the hypothesis that what is important to the balance between exon skipping and exon inclusion is the relative rates of spliceosome formation and pol II processivity.

Regulation of alternative splicing by trans-acting factors has also been shown to depend on the presence of suboptimal splicing sites. TRA-2 protein is regulated in the male germ line of Drosophila through a negative feedback mechanism in which a specific TRA-2 isoform represses splicing of the M1 intron in the TRA-2 pre-mRNA. This repression depends on the presence of a suboptimal non-consensus 3'-splice site. Substitution of this 3'-splice site with a strong splice site resulted in TRA-2 independent splicing, implying that reduced basal splicing efficiency is important for regulation (32).

Fig. 2B shows that complete loss of regulation by pol II elongation is achieved by disruption of the exonic splicing silencer present in EDI exon. This silencer probably acts by stabilizing a pre-mRNA secondary structure that inhibits recognition of the neighboring splicing enhancer by SF2/ASF. Disruption of the ESS would alter secondary structure to better expose ESE sequences (33).

In this context it is possible to speculate that, from an evolutionary point of view, the appearance of ESEs not only compensated for mutations that weaken splice sites but also allowed fine-tuning in splicing regulation through combinatorial control by the abundance of SR proteins and the modulation of transcriptional elongation rates. This dual regulatory strategy seems to have an advantage in metazoans where, compared with other eukaryotes, introns are usually longer and have highly degenerate splice site and branch point recognition sequences.

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