Molecular Cell, Vol. 19, 393–404, August 5, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.molcel.2005.06.035

A Polar Mechanism Coordinates Different Regions of Alternative Splicing within a Single Gene

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Summary

Alternative splicing plays a key role in generating protein diversity. Transfections with minigenes revealed coordination between two distant, alternatively spliced exons in the same gene. Mutations that either inhibit or stimulate inclusion of the upstream alternative exon deeply affect inclusion of the downstream one. However, similar mutations at the downstream alternative exon have little effect on the upstream one. This polar effect is promoter specific and is enhanced by inhibition of transcriptional elongation. Consistently, cells from mutant mice with either constitutive or null inclusion of a fibronectin alternative exon revealed coordination with a second alternative splicing region, located far downstream. Using allele-specific RT-PCR, we demonstrate that this coordination occurs in *cis* and is also affected by transcriptional elongation rates. Bioinformatics supports the generality of these findings, indicating that 25% of human genes contain multiple alternative splicing regions and identifying several genes with nonrandom distribution of mRNA isoforms at two alternative regions.

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Introduction

The realization that a vast mammalian proteomic complexity is achieved with a limited number of genes demands a better understanding of the mechanisms that generate multiple transcripts from a single gene and their regulation. These mechanisms include alternative transcriptional initiation, editing, alternative cleavage and polyadenylation, and alternative splicing (AS). Among these, AS is the major contributor to protein diversity because it usually involves internal, protein-encoding exons. Recent findings justify a renewed interest in AS. (1) The process is more a rule than an exception as it is now estimated to affect the expression of nearly 60% of human genes (Lander et al., 2001). (2) Mutations that affect AS regulatory sequences are a widespread source of human disease (Cáceres and Kornblihtt, 2002; Cartegni et al., 2002; Pagani and Baralle, 2004). (3) Alternative splicing regulation not only depends on the interaction of splicing factors such as serine-arginine-rich proteins (SR proteins) and heterogeneous nuclear ribonucleoproteins (hnRNP proteins) with their target sequences in the pre-mRNA (splicing enhancers and silencers) but it is also coupled to RNA polymerase II (pol II) transcription, as it happens with other pre-mRNA processing reactions (for reviews see Bentley, 2002; Maniatis and Reed, 2002; Neugebauer, 2002; Proudfoot et al., 2002; Kornblihtt et al., 2004).

The way transcription affects AS seems to be determined by promoter identity and occupation (Cramer et al., 1997; 1999; Auboeuf et al., 2002), which in turn may modulate pol II elongation rates. For example, transcription factors that increase elongation stimulate skipping of the extra domain I cassette exon (EDI) of fibronectin (FN) (Kadener et al., 2001), whereas treatments with drugs that inhibit elongation (like 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole [DRB]) (Nogués et al., 2002) or transcription by slow mutants of pol II (de la Mata et al., 2003; Howe et al., 2003) favor exon inclusion. These observations are consistent with chromatin immunoprecipitation experiments revealing stalling of pol II molecules upstream of the alternative EDI on minigenes with promoters that favor EDI inclusion (i.e., the FN promoter) compared to minigenes with promoters that favor EDI skipping (i.e., the α-globin [α-gb] promoter) (Kadener et al., 2002). EDI inclusion depends on the competition between a suboptimal 3′ splice site (3′ ss) at the upstream intron and a strong 3′ ss at the downstream intron. According to the prevailing model, 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's 3′ ss, resulting in exon skipping. When the upstream 3′ ss is strengthened by mutating its polypyrimidine tract,

EDI inclusion becomes constitutive and insensitive to changes in transcription elongation (Nogués et al., 2003).

Soon after the discovery of splicing it became evident that many genes contained more than one region of AS, a feature that undoubtedly multiplies the polypeptide encoding potential of a genome (Kornblihtt et al., 1984; Breitbart et al., 1985). The FN gene is a paradigmatic example (Sharp, 1994) as it contains three regions of AS that display cell type- and development-specific regulation. From 5′ to 3′, these regions are EDII (a.k.a. EDB or EIIIB), the already mentioned EDI (a.k.a. EDA or EIIIA), and IIICS (a.k.a. V). This complex pattern can eventually give rise to up to 20 mRNA isoforms in humans, 12 in rodents, and 8 in chicken (Kornblihtt et al., 1996). Although other genes with multiple regions of AS have been found, the prevalence of this phenomenon and a putative coordination between regions within the same gene have not been studied systematically. The existence of a coordinating mechanism was investigated here by transfecting human cells with minigenes carrying two alternative EDI regions in tandem, separated by 3400 bp spanning three constitutive exons and the corresponding introns. Mutations that affect AS of the proximal (with respect to the promoter) EDI deeply affect the splicing pattern of the distal one. On the contrary, the same mutations introduced in the distal EDI AS have much lower effects on the inclusion of the proximal one. The polar nature of the coordinating effect was found to be promoter specific in a way that strongly points to the involvement of transcriptional elongation in the underlying mechanism. Analysis of the endogenous FN gene in cultured cells from mutant mice with either constitutive or null inclusion of the EDI exon revealed coordination between EDI and IIICS AS regions, naturally separated by 5400 bp in the gene. Using heteroallelic mice and allele-specific RT-PCR we demonstrate that the influence of EDI on IIICS occurs in *cis*. Furthermore, bioinformatic evidence indicates that genes with multiple alternative regions are an important fraction of alternatively spliced genes and provides several examples with nonrandom combination of isoforms at two alternative regions of a gene, suggesting that the observed coordination might be a general phenomenon.

Results

Polar Effects in Alternative Splicing

To investigate a putative coordination between AS regions, we used reporter minigenes to transiently transfect cells in culture. Hybrid α-gb/FN minigenes were extensively used to assess the control of AS of the FN EDI exon (Vibe-Pedersen et al., 1984; Caputi et al., 1994; Cramer et al., 1999). We now constructed similar minigenes containing two alternatively spliced EDI regions, separated by three constitutive exons and their corresponding introns, under the control of the human FN promoter. The length of the EDI exon is 270 bp, a multiple of three. Transient expression of one of these constructs containing two wt EDI exons (pFN-EDI^{WT}/EDI^{WT}) in human hepatoma Hep3B cells was evaluated by RNA isolation followed by RT-PCR with pairs of primers that distinguish the proximal and distal EDI splicing events. Cells were cotransfected with small amounts (2 ng) of a plasmid expressing the SR protein SF2/ASF to stimulate EDI inclusion moderately. Under these conditions the inclusion ratios for the proximal and distal EDIs are 0.78 ± 0.04 and 0.24 ± 0.02, respectively (Figure 1A, top). EDI contains an exonic splicing enhancer (ESE), with the core sequence 5′-GAAG AAGA-3′, that is the target site for the SR proteins SF2/ASF and 9G8 (Cramer et al., 1999; Buratti et al., 2004). Disruption of the ESE at the proximal EDI exon (pFN-EDI$^{\Delta ESE}$/EDI^{WT}; Figure 1A, middle) not only abolishes its own inclusion but also provokes an 8-fold decrease in the inclusion of the distal EDI exon, located approximately 3400 bp downstream in the minigene. On the contrary, disruption of the ESE at the distal EDI exon (pFN-EDI^{WT}/EDI$^{\Delta ESE}$; Figure 1A, bottom) abolishes its own inclusion but has a much smaller effect on the inclusion of the proximal EDI exon. These experiments strongly indicate that both AS events are conditioned through a mechanism that displays polarity with respect to the promoter. The coordinating effect is observed both in the absence of SF2/ASF overexpression or in the presence of higher amounts of SF2/ASF (20 and 60 ng of cotransfected plasmid) (Figure 1B).

Constitutive versus Alternative Splicing

Figure 1B also shows that the sensitivity of the distal EDI inclusion to SF2/ASF overexpression is lower when the ESE of the proximal EDI is mutated. Because both wt EDI exons are targets for SF2/ASF, it is difficult to know whether the lower sensitivity of the pFN-EDI$^{\Delta ESE}$/EDI^{WT} construct is the consequence of the reduced binding of SF2/ASF to the mutated proximal EDI or a combined reduction in binding affinities at both sites caused by the coordinating effect. To elucidate this problem, we constructed tandem minigenes where inclusion of one of the two EDI exons was made constitutive by mutating its suboptimal 3′ ss (Nogués et al., 2003) instead of overexpressing SF2/ASF. In the absence of SF2/ASF overexpression and after transfection of Hep3B cells with pFN-EDI^{WT}/EDI^{WT}, inclusion ratios for the proximal and distal EDI exons are 0.32 ± 0.08 and 0.13 ± 0.01, respectively (Figure 2A). When the proximal EDI exon is made constitutive (pFN-EDI^{C}/EDI^{WT}), inclusion of the distal EDI exon increases by more than 7-fold (Figure 2B). However, when the distal EDI exon is made constitutive (pFN-EDI^{WT}/EDI^{C}), inclusion of the proximal EDI increases by only 1.8-fold (Figure 2C). This differential behavior again points independently to the existence of polarity in the coordinating effect.

Differential Dependence from SR Protein Binding

The above experiments indicate that the higher the inclusion of the proximal EDI, the higher the inclusion of the distal EDI. Because disruption of the proximal EDI ESE prevents SF2/ASF binding and affects distal inclusion (Figure 1), it appeared important to define whether the coordinating effect is caused by the ability of SF2/ASF to bind the proximal EDI ESE or to the inclusion of the EDI exon per se. For this, we expressed a tandem construct with a constitutive proximal EDI exon harbor-

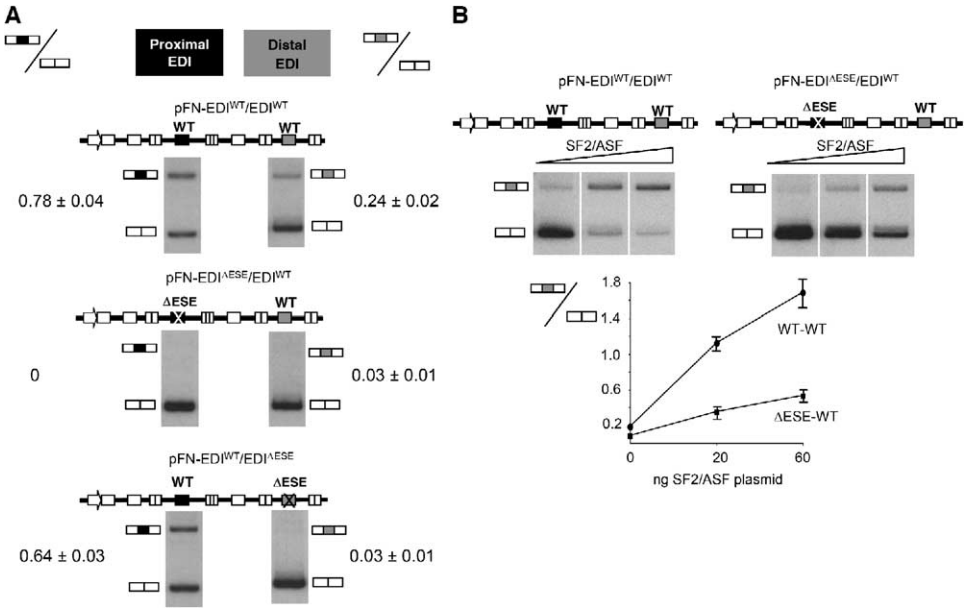

Figure 1. Polarity in the Coordination of Two Alternative Splicing Regions

(A) Effects of disrupting EDI's exonic splicing enhancer (ESE) at a proximal or distal alternative splicing regions located in tandem minigenes. Hep3B cells were transfected with 800 ng of plasmids pFN-EDI^{WT}/EDI^{WT} (top), pFN-EDI$^{\Delta ESE}$/EDI^{WT} (middle), or pFN-EDI^{WT}/EDI$^{\Delta ESE}$ (bottom) and 2 ng of g10SF2/ASF (Cáceres et al., 1997). Alternative splicing patterns of proximal (black) or distal (gray) EDI exons were assessed by radioactive RT-PCR with specific pairs of primers, followed by electrophoresis in native polyacrylamide gels as described in the Experimental Procedures. The data represent the average of at least three independent transfections of at least three 35 mm culture wells each. (A) n = 4. (B) n = 3. Plus minus (±) values and error bars represent the SD.

(B) Dose-response curve of SF2/ASF on alternative spicing of the distal EDI region when the proximal EDI region is wild-type or has a disrupted ESE. Hep3B cells were transfected with 800 ng of pFN-EDI^{WT}/EDI^{WT} (left) or pFN-EDI$^{\Delta ESE}$/EDI^{WT} (right) and increasing amounts of the expression vector for SF2/ASF (g10SF2/ASF). Alternative splicing was assessed as in (A).

ing a mutated ESE (pFN-EDI$^{C\Delta ESE}$/EDI^{WT}). Distal EDI inclusion levels were similar to those elicited by the construct with a constitutive proximal EDI and a wt ESE (pFN-EDI^{C}/EDI^{WT}), i.e., about 6-fold higher than those where both EDI exons are wt (Figure 2D), which indicates that the coordinating effect is not caused by binding of SR proteins to the proximal alternative exon but by the actual inclusion of the proximal EDI.

On the contrary, transfection with construct pFN-EDI^{C}/EDI$^{\Delta ESE}$ shows that the ESE at the distal EDI is required for its responsiveness to activation of inclusion caused by a constitutive proximal EDI (Figure 2E).

A Role for EDI Exon Definition

Our experiments open the question whether the coordinating effects are caused by the inclusion at the proximal region of any additional constitutive exon or specifically by the inclusion of the EDI exon. To answer this question we transfected Hep3B cells with a series of constructs with proximal configurations differing in the number of constitutive exons upstream of the distal EDI region and with or without proximal EDI sequences (Figure 3). First, a relevant observation is that inclusion levels of the distal EDI exon do not correlate with the number of upstream constitutive exons: constructs with six (Figures 3Aa, 3Ab, and 3Af–3Ah), three (Figure 3Ai), or one (Figure 3Aj) upstream constitutive exons have levels of distal EDI inclusion that are much lower than those of the construct with constitutive inclusion of the proximal EDI exon (Figure 3Ac). Second, the presence of the EDI sequence, not as a defined exon but embedded as a part of a hybrid proximal constitutive exon, is not sufficient to provoke high inclusion of the distal EDI (compare Figures 3Ad and 3Ae). This is independent of the position (compare Figures 3Ak and 3Aj). We then asked whether any of the proximal EDI flanking introns per se played a role in promoting distal EDI inclusion. Neither intron –1 nor intron +1 alone (Figures 3Af and 3Ah) were able to duplicate the effects observed in Figure 3Ac. On the other hand, constructs f and g (see Figures 3Af and 3Ag, respectively) provoke equally low levels of distal EDI inclusion, which rules out an intrinsic role for the mutations used in construct c to optimize the polypyrimidine tract of intron –1. This optimization of the polypyrimidine tract is therefore relevant only if it contributes to the process of inclusion of the proximal EDI exon. These results indicate that what affects the inclusion of the distal alternative exon is the process of exon definition at the proximal EDI. Exon definition is the mechanism by which splice sites are selected via interactions between splicing factors across an exon prior to spliceosome assembly and intron removal (Berget, 1995). Because well-defined exons other than EDI present in the FN minigenes do not seem to stimulate inclusion of the downstream alternative exon (Figures 3Ab and 3Ac–3Ak), we must con-

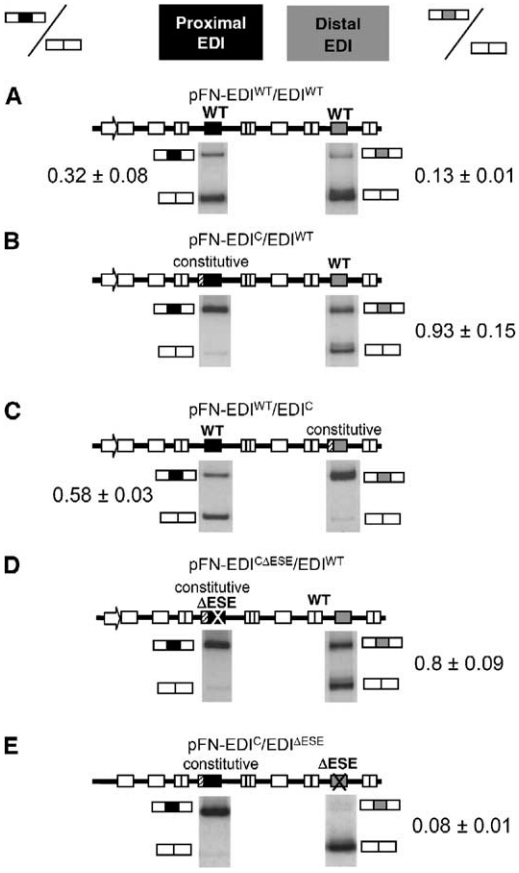

Figure 2. Constitutive versus Alternative Inclusion of the Proximal EDI

Effects of making constitutive the inclusion of proximal or distal EDI regions by optimizing the polypyrimidine tracts of their 3′ splice sites (Nogués et al., 2003). Hep3B cells were transfected with the indicated plasmids. Other conditions were as in Figure 1A. The data represent the average of at least three independent transfections of at least three 35 mm culture wells each. Plus minus (±) values and error bars represent the SD.

clude that the recruitment of specific splicing factors necessary to define EDI as an exon is regulating downstream alternative events.

The α-gb Promoter Abolishes Polarity

Experiments in Figures 1–3 were performed with constructs under the control of the human FN promoter. Because EDI splicing is affected by promoter identity (Cramer et al., 1997), we wanted to evaluate whether the coordinating effect and/or its polar behavior were also promoter sensitive. For this purpose we prepared tandem minigenes in which the human FN promoter was replaced by the human α-gb promoter. Stimulation of distal EDI inclusion was much lower when transcription was driven by the α-gb promoter: inclusion levels of the distal EDI exon increased only 1.6-fold when the proximal EDI exon was made constitutive (Figure 4A), compared to the 6- to 7-fold increase observed with the FN promoter (Figure 2). Most importantly, a similar degree of stimulation of inclusion at a wt proximal EDI (1.9-fold) is observed when the distal EDI is made constitutive (Figure 4A, bottom). These results indicate that under the control of the α-gb promoter, polarity disappears because both regions, proximal and distal, can influence each other at similar levels.

Inhibition of Pol II Elongation Recovers the Polar Effect

We have previously demonstrated that promoter swapping affects pol II densities along FN minigene templates (Kadener et al., 2002). Therefore, the promoter dependence points to a role for pol II elongation. Phosphorylation of the pol II CTD at Ser-2 by the kinase activity of P-TEF-b (positive transcription elongation factor b) stimulates transcriptional elongation. DRB, an inhibitor of the Cdk-9 kinase subunit of P-TEF-b, inhibits pol II elongation (Price, 2000). We reasoned that by inhibiting elongation, DRB should revert the lack of polarity observed with the "more elongating" α-gb promoter. Cells transfected with tandem minigenes under the control of the α-gb promoter were treated with DRB. Figure 4B shows that when transcription is driven by the α-gb promoter, inclusion levels of the distal EDI provoked by constitutiveness of the proximal EDI increase to 3-fold in the presence of DRB.

Few rounds of replication provoke a more compacted chromatin at transfected minigenes, accompanied by a decrease in pol II elongation which in turn provokes higher EDI inclusion levels (Kadener et al., 2001). The tandem minigenes used in this report contain the SV40 origin of replication, which becomes active in the presence of the SV40 large T antigen (T-Ag). When tandem minigenes under the control of the α-gb promoter were allowed to replicate by cotransfection with a plasmid expressing T-Ag, a constitutive proximal EDI exon (pα-gb-EDI^{C}/EDI^{WT}) provoked a 1.7-fold higher effect on the inclusion of the distal EDI exon, compared with the nonreplicated templates (not shown). So, two independent ways of inhibiting pol II elongation (DRB and T-Ag-mediated replication) revert the absence of polar effect observed with the α-gb promoter.

The Coordinating Effect Is Exon Specific

To investigate the exon specificity of the coordinating effect, we transfected Hep3B cells with tandem minigenes under the control of the FN promoter containing either wt or constitutive human EDI exons at the upstream alternative region and another FN alternative exon, EDII, at the downstream alternative region. Supplemental Figure S1A shows that when the EDI exon is alternative (pFN-EDI^{WT}/EDII^{WT}), the inclusion ratio for the EDII exon is 0.32 ± 0.05. When the proximal EDI exon is made constitutive (pFN-EDI^{C}/EDII^{WT}), EDII inclusion levels remain unaffected (ratio 0.28 ± 0.04), evidencing that unlike EDI, EDII splicing does not respond to changes in the inclusion ratio of an upstream EDI region. Although both EDI and EDII are alternative cassette exons of similar length (270 and 273 nt, respectively) and encode similar FN type III repeats, the *cis* determinants and *trans*-acting factors that regulate their inclusion are different (Muro et al., 1999).

To further explore the exon specificity, we made constructs in which the murine FN IIICS alternative region

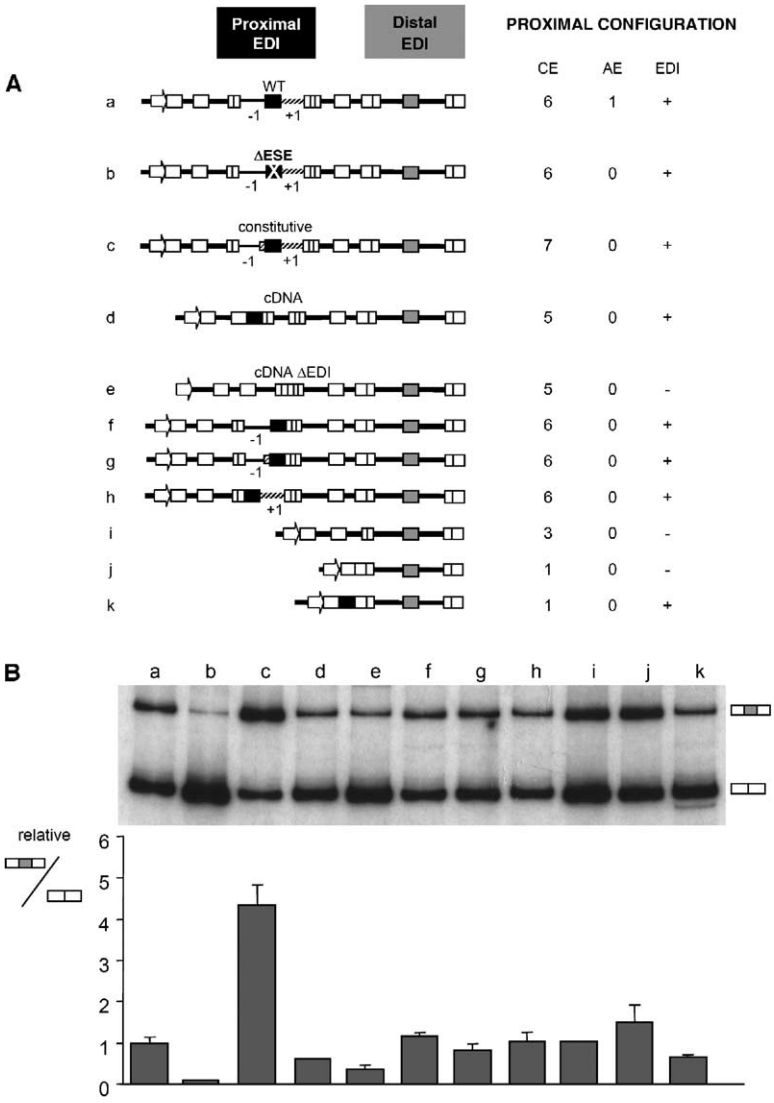

Figure 3. Effects of Proximal Configurations Differing in the Number of Constitutive Exons and in the Presence of the EDI Sequence on the Inclusion of the Distal EDI Exon

(A) Constructs a, b, and c, used as controls, are pFN-EDI^{WT}/EDI^{WT}, pFN-EDI$^{\Delta ESE}$/EDI^{WT}, and pFN-EDI^{C}/EDI^{WT}, respectively. In constructs d, f–h, and k, an upstream EDI sequence (black) is present not as a defined exon but embedded as a part of hybrid proximal constitutive exons. EDI flanking introns, named –1 and +1, are depicted by thin and dashed lines, respectively. CE, number of constitutive exons upstream of the distal EDI exon; AE, number of alternative exons upstream of the distal EDI exon.

(B) Hep3B cells were transfected with the constructs in (A). Alternative splicing at the distal EDI region was assessed as described in the Experimental Procedures.

The data represent the average of at least three independent transfections of at least three 35 mm culture wells each. (A) n = 4. (B) n = 3. Error bars represent the SD.

was placed downstream of the EDI region, which mimics the relative positions in the endogenous FN gene (see below). When the EDI exon is alternative (pFN-EDI^{WT}/IIICS), the IIICS120:IIICS0 ratio is 4.14 ± 0.76. When the proximal EDI exon is made constitutive (pFN-EDI^{C}/IIICS), there is a small but significant decrease in the IIICS120:IIICS0 ratio to 2.97 ± 0.21 (Supplemental Figure S1B).

Coordinated Alternative Splicing in the Murine Endogenous FN Gene

In the FN gene, EDI is the closest AS region upstream of IIICS (Figure 5A). The mouse IIICS region gives rise to three alternative mRNA isoforms generated from a single 5′ ss in combination with three alternative 3′ ss. These isoforms are IIICS120, IIICS95, and IIICS0, named according to the number of amino acids of the encoded protein segments. In order to validate our findings in a more physiological context, we investigated a putative coordination between the EDI and IIICS regions in the endogenous mouse FN gene. For this purpose we took advantage of mutant mice recently generated in one of our labs. Using gene targeting, Muro et al. (2003) developed mouse strains with either constitutive (EDA$^{+/+}$) or null (EDA$^{-/-}$) EDI expression. In vivo, the EDI and IIICS regions are separated by approximately 5400 bp, spanning six constitutive exons and their introns. We investigated IIICS AS by RT-PCR in mRNAs from embryo fibroblasts (MEFs) in culture, which were derived from the EDA$^{+/+}$ and EDA$^{-/-}$ mice. In EDA$^{-/-}$ MEFs, the IIICS120:IIICS0 ratio is nearly 5.5-fold higher than in EDA$^{+/+}$ MEFs (Figure 5B). The FN gene is in mouse chromosome 1. As good controls, we found no changes between EDA$^{+/+}$ and EDA$^{-/-}$ genotypes in alternative pre-mRNA splicing from genes located in other mouse chromosomes such as *CD44* (chromosome 2) and *FGFR2* (fibroblast growth factor receptor 2; chromosome 7). Endogenous EDII inclusion is identical in EDA$^{+/+}$ and EDA$^{-/-}$ MEFs (data not shown). However, we cannot interpret that the lack of change in EDII splicing reflects the polar mechanism (EDII is located upstream of EDI in the endogenous gene; Figure 6B) because

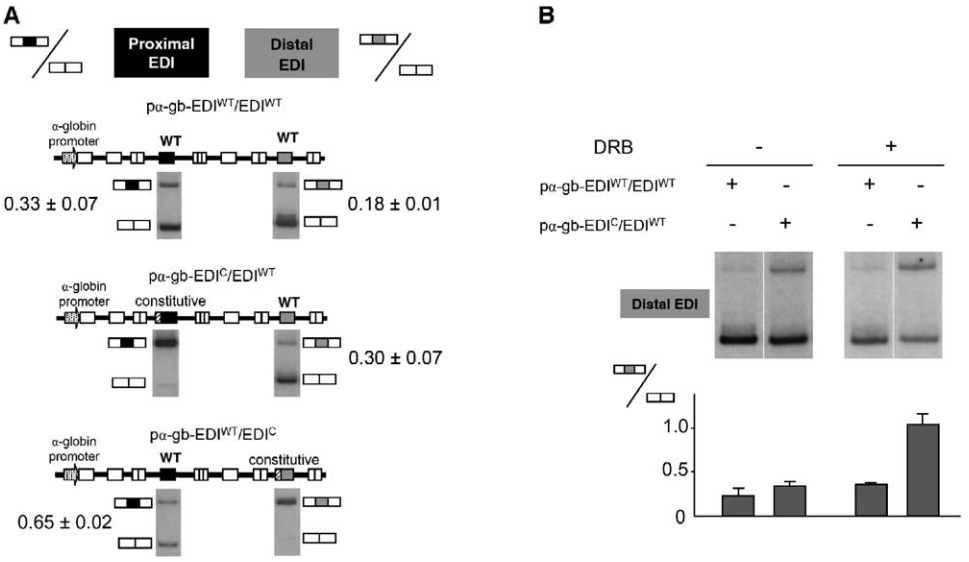

Figure 4. The Polar Effect Is Linked to Pol II Elongation

(A) Polarity is abolished when transcription is driven by the α-gb promoter. Hep3B cells were transfected with tandem minigenes under the control of the α-gb promoter (dotted) with a wt proximal EDI (pα-gb-EDI^{WT}/EDI^{WT}) or a constitutive proximal EDI (pα-gb-EDI^{C}/EDI^{WT}).

(B) The polar effect is recovered by DRB. Hep3B cells were transfected with tandem minigenes under the control of the α-gb promoter with a wt (pα-gb-EDI^{WT}/EDI^{WT}) or a constitutive (pα-gb-EDI^{C}/EDI^{WT}) proximal EDI exon. When indicated, 24 hr after the beginning of transfection cells were treated for further 24 hr with 50 μM DRB. In all cases, alternative splicing was assessed as described in Figure 1A and in the Experimental Procedures.

The data represent the average of at least two independent transfections of three 35 mm culture wells each. (A) n = 4. (B) n = 2. Plus minus (±) values and error bars represent the SD.

EDII proved to be unresponsive to changes in EDI inclusion levels, even when put downstream of EDI in the minigenes (Supplemental Figure S1A).

Pol II Elongation and the Effects on the FN Endogenous Gene

Similarly to what happens with the transfected minigenes (Figure 4B), treatment of MEFs with the inhibitor of pol II elongation DRB enhances the effects of proximal EDI configuration on the IIICS120:IIICS0 ratio at the endogenous FN gene (Figure 5C). Conversely, we reasoned that activation of elongation by promotion of histone acetylation should have opposite effects. Figure 5C also shows that this is the case, as treatment of MEFs with trichostatin A (TSA), a potent inhibitor of histone deacetylation, inhibits the effects of EDI constitutive inclusion on the IIICS120:IIICS0 ratio.

Allele-Specific Effects

Because EDI^{+} FN was demonstrated to have growth-factor activity (Manabe et al., 1999), the differences observed in IIICS splicing could be caused by differences in the metabolic status or growth rate determined by the presence of EDI^{+} FN in EDA$^{+/+}$ conditioned medium. To rule out this possibility, we assessed IIICS AS in an MEF culture derived from a mouse heterozygous for the constitutive and null alleles (EDA$^{+/-}$ strain). The identification of allele-specific RT-PCR bands in the polyacrylamide gel electrophoresis allowed us to estimate a IIICS120:IIICS0 ratio 3-fold higher in mRNAs transcribed from the EDA^{-} allele compared with the mRNA transcribed from the EDA^{+} allele (Figure 5E). These results confirm the differences in IIICS splicing observed in Figure 5B and, coming now from a single cell culture, rule out a differential cell environment as the cause for those differences. Furthermore, the allelic specificity indicates that coordination between EDI and IIICS ASs occurs in *cis*, which is coherent with the polar nature observed with minigene transfections. The endogenous coordination is consistent with the tendency observed in transfections with minigenes where IIICS was put downstream of EDI (Supplemental Figure S1B).

Bioinformatics

To evaluate the generality of our findings, we searched human databases for the frequency of multiple AS and evidence for coordination. The number of alternatively spliced regions per gene was determined by alignment to the longest isoform of all possible isoforms, thereby satisfying the conditions described in the Experimental Procedures. Regions present in all isoforms were considered constitutive, whereas regions present in a fraction of isoforms were considered alternative.

The fraction of alternatively spliced genes (in the above definition) in the human genome depended on the required EST coverage, and it ranged from 40% (if only variants supported by protein data were considered) to 60% if ESTs were considered as well (Figure 6A). However, the estimated number of alternative regions per alternatively spliced gene (Figure 6B) remained similar, independently of the estimation of the number of alternatively spliced genes: 52%–72% of al-

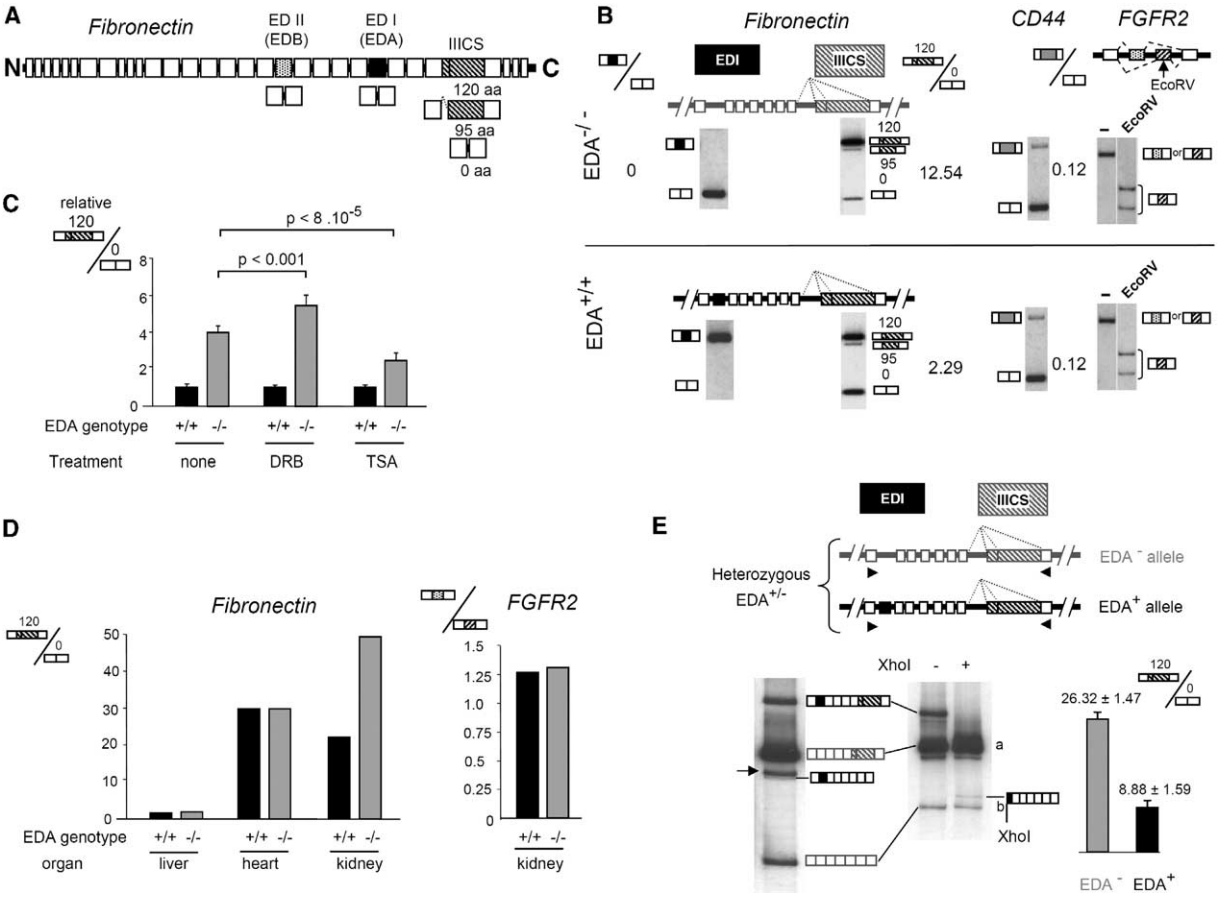

Figure 5. Coordinated Alternative Splicing in the Endogenous FN Gene

(A) Variations of the mouse FN primary structure. The top scheme represents the longest FN polypeptide showing the repetitive domain structure. EDII (dotted), EDI (black), and IIICS (dashed) alternative splicing regions and their variants are depicted.

(B) IIICS splicing is affected in MEFs by the inclusion of the upstream EDI exon. Alternative splicing patterns at the EDI and IIICS regions of transcripts from the endogenous FN gene in embryo fibroblasts from mutant mice with null ($EDA^{-/-}$) or constitutive ($EDA^{+/+}$) expression of the EDI (EDA) exon. Splicing patterns of *CD44* and *FGFR2*, whose genes are located in other chromosomes, are not affected by EDI inclusion.

(C) DRB enhances whereas TSA inhibits the polar effect exerted by changes in upstream EDI configuration on IIICS splicing. MEFs were treated with 50 μM DRB or 5 ng/ml TSA for 24 hr prior to total RNA preparation.

(D) FN IIICS120:IIICS0 ratio is higher in kidney from newborn $EDA^{-/-}$ mice compared with newborn $EDA^{+/+}$ mice. The effect is gene specific because it does not affect alternative splicing of *FGFR2* transcripts. No changes in IIICS120:IIICS0 ratio are detected in liver or heart.

(E) Allele-specific RT-PCR of RNA from embryo fibroblasts of heterozygous $EDA^{+/-}$ mutant mice to assess IIICS alternative splicing patterns. In the right panel, the EDI^{+}/IIICS0 product was digested with XhoI to generate a shorter fragment distinguishable from the EDI^{-}/IIICS95 variant (arrow in the left panel). a:b ratios are identical (approximately 26.5) with or without XhoI digestion.

The data represent the average of RNA isolations from at least 12 35 mm culture wells each. (B) n = 18. (C) n = 12. (E) n = 24. Plus minus (±) values and error bars represent the SD.

ternatively spliced genes contained only one alternative region, the lower value coming from ESTs and the upper value from proteins, with the mRNA-based value falling in between (62%); 20%–29% contained two regions; 6%–12%, three regions; and 1%–6%, more than three regions. Thus, approximately 25% of human genes contain more than one alternatively spliced region, a significant fraction to deserve a close look at a possible coordination. Accordingly, we collected all pairs of adjacent alternatively spliced regions present in the EDAS database (http://www.belozersky.msu.ru/edas/). For each pair, we collected all EST sequences spanning both regions and compiled contingency tables in which rows and columns correspond to variants of the proximal and distal regions, respectively, and the cells contain the number of ESTs spliced by the given combination of variants. Columns and rows with less than two ESTs total were ignored. This resulted in tables for 630 genes. We then applied the Fisher's Exact test (http://www.unc.edu/~preacher/fisher/fisher.htm) to these tables in order to identify cases of coordinated AS. Most tables were statistically nonsignificant, mainly due to a very small number of ESTs covering both regions. In 60 genes the variants were correlated with a significance of <0.1, which would be expected from a random distribution. However, in several cases, the dependencies were significant even after the Bonferroni correction for multiple testing. Figure 6C shows five

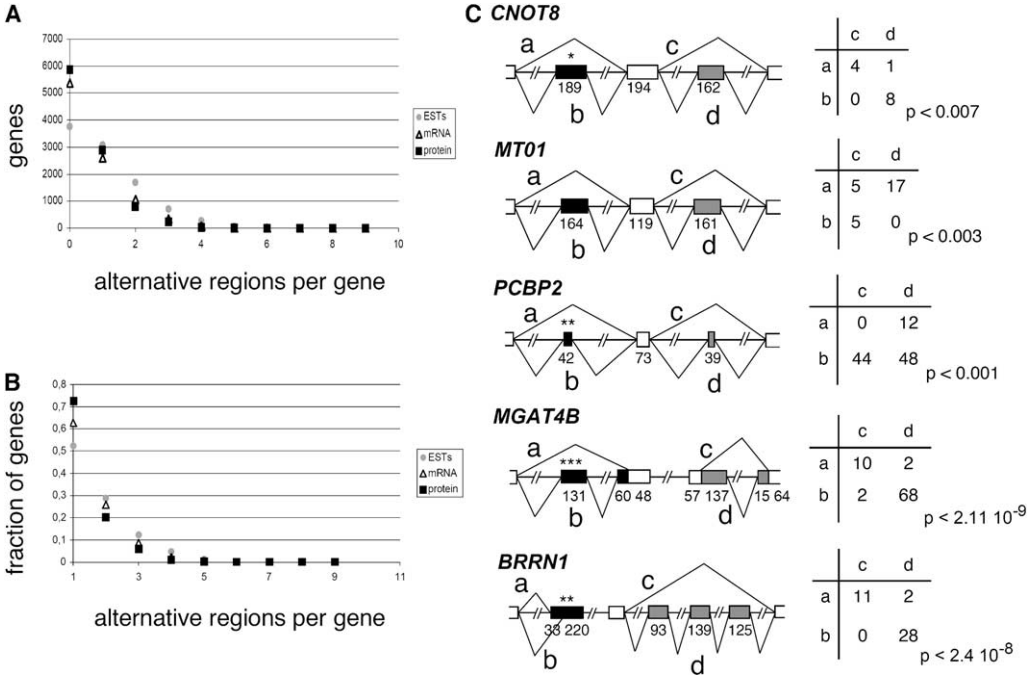

Figure 6. Bioinformatics Evidence for Coordination in Multiple Alternative Splicing

(A) Number of alternative regions per human gene.

(B) Fraction of human genes with the given number of alternative regions among all genes with alternatively spliced coding regions. Squares, triangles, and circles denote variants supported by proteins, mRNAs, and ESTs, respectively (see the Experimental Procedures).

(C) Bioinformatic evidence for coordination between different alternative splicing regions within the same gene. Examples of human genes with two consecutive alternative splicing regions with two alternative patterns for each region (a or b for the upstream one, c or d for the downstream one). The tables show the number of ESTs for each possible combination found in the EDAS database. *Exon located in the 5′ untranslated region. **Exons located in coding regions that do not introduce premature stop codons. ***Exon located in the coding region that introduces a stop codon in the penultimate exon in a position that does not elicit NMD.

gene examples with nonrandom combinations significant at the level of <0.007. In three cases (*CNOT8*, *PCBP2*, and *BRRN1*), the length difference between variants corresponded to a multiple of three. The upstream alternative exon of *CNOT8* is in the 5′ untranslated region. Those of *PCBP2* and *BRRN1* are in the coding region but do not introduce any premature stop codons. In the case of *MGAT4B*, inclusion of the upstream alternative exon introduces a premature stop codon at the penultimate exon of the gene but at a distance shorter than 50 nt from the 5′ splice site. These observations exclude the possibility that the absent or underrepresented combinations in four examples were eliminated by nonsense-mediated decay (NMD).

Coordinated splicing events could just reflect cell-type-specific factors acting on both regions in each transcript. Supplemental Table S1 rules out this possibility as it shows that except for *BRRN1*, there is little tissue specificity in the distribution of ESTs for the different combinations.

Coordinated Alternative Splicing in the PCBP2 Gene

We chose one of the bioinformatic examples in Figure 6C, *PCBP2*, to see if the biased EST distribution correlated with the relative proportions of the different mRNA isoforms detected by RT-PCR and whether this was affected by pol II elongation. We found a tight correlation between the EST data and the relative abundances of the different *PCBP2* isoforms. In three different cell lines (Hep3B, Cos7, and HeLa) the most abundant isoform is b-d, followed by b-c and a-d (Supplemental Figure S2). The isoform lacking both alternative exons, a-c, is only detectable at longer gel exposures and represents less than 1% of the *PCBP2* mRNAs, which explains the absence of ESTs for this isoform in the databases. The EST data indicate that when the *PCBP2* upstream alternative exon is excluded there is a 100% inclusion of the downstream alternative exon. However, when the upstream exon is included, inclusion of the downstream exon falls to approximately 52%. Therefore, an increase in the inclusion levels of the upstream alternative exon (caused, for example, by a delay in Pol II elongation) should provoke a decrease in the inclusion of the downstream alternative exon. This is observed in the three cell lines in which treatment with DRB causes a reduction of about 50% in the inclusion of the downstream alternative exon.

Discussion

By expressing minigene constructs with different configurations at two regions of AS (Figures 1–4), we demonstrate here that there is a tight coordination between two regions of AS in the same gene. The

coordinating mechanism involves specific pre-mRNA sequences acting preferentially in a 5′-to-3′ polarity. When two alternatively spliced EDI regions are put 3400 bp apart in the same minigene, mutations that either inhibit (Figure 1) or stimulate (Figure 2) inclusion of the proximal EDI correlate directly with inclusion levels of the distal EDI exon. However, similar mutations applied to the distal EDI exon have much smaller effects on the proximal one. Results in Figure 3 indicate that exon definition of the proximal EDI stimulates inclusion of the distal one. It was previously shown that the EDI exon is governed by exon definition because optimization of either its 3′ or 5′ splice sites enhances inclusion (Muro et al., 1998). The activity of EDI's ESE in the context of a particular RNA secondary structure (Buratti et al., 2004) is also a determinant of its inclusion. How does EDI exon definition affect downstream AS? We propose a mechanism in which recruitment of general and specific splicing factors necessary for the definition of one alternative region acts cooperatively on the recruitment of factors that act in similar ways (in the case of EDI-EDI) or antagonistically (in the case of EDI-IIICS) at a distant alternative region through physical interactions between both regions. This cooperative effect displays polarity when slow elongation provokes a delay in the emergence of the distal alternative region, allowing more time for recruitment and exon definition at the proximal alternative region.

The physiological importance of the reported coordination was investigated in two naturally occurring events on the endogenous genes for mouse FN (Figure 5) and human *PCBP2* (Supplemental Figure S2). In the case of FN, we looked at IIICS splicing patterns in mutant mice defective in regulated EDI splicing. In these mice, constitutive inclusion of the EDI exon correlates inversely with inclusion of the IIICS120 segment. Chauhan et al. (2004) looked at coordinated splicing between the EDI and IIICS regions in several organs from the same mutant mice used in the present study. They found absence of coordination in heart, liver, spleen, lung, and brain in 1-day-old newborn and 4-month-old adult mice. However, in kidney, although there is no coordination in the adult mice, the $EDA^{-/-}$ newborn mice showed an increase in the IIICS120:IIICS0 ratio with respect to the $EDA^{+/+}$ mice, in agreement with our results obtained with mouse embryo fibroblasts. Whether the absence of coordination in adult and newborn organs other than kidney compared to cultured embryo fibroblasts is a consequence of cell-type or developmental specificities remains to be determined. In any case, we confirmed that changes in IIICS splicing observed in $EDA^{-/-}$ newborn kidney are gene specific because AS of the *FGFR2* transcripts is identical in both EDA genotypes (Figure 5D). A putative upregulation of IIICS120 inclusion in EDI null embryos would compensate for the absence of EDI^{+} FN isoforms. In fact, both EDI and IIICS120 protein segments contain binding sites for the $\alpha4\beta1$ integrin (Liao et al., 2002), which means that overexpression of one of the alternative segments could compensate for the functional defect caused by the absence of the other one. This would be consistent with the fact that homozygous EDI null mice are viable, although they have a shorter lifespan, abnormal wound healing (Muro et al., 2003), and reduced motor-coordination abilities and vertical exploratory capacity (Chauhan et al., 2005).

Bioinformatic evidence indicated that in approximately 40% of genes with AS, the process takes place at multiple regions (Figures 6A and 6B). This means that about a quarter of all human genes have more than one region of AS. The in silico evidence for coordination between two regions within a single gene is also striking. For example, in the BRRN1 gene (Figure 6C) the two variants at the upstream region differ by the inclusion of a 33 nt segment as a consequence of the use of a common 5′ ss in combination with two alternative 3′ ss. Inclusion of the upstream 33 nt segment (variant "a") is tightly coordinated with exclusion of the 3 downstream alternative exons (variant "c"): 11 of 13 ESTs with variant a also contain variant c. Conversely, exclusion of the 33 nt segment (variant "b") seems to be the determinant for inclusion of the three downstream alternative exons (variant "d"): all ESTs (28) found to contain variant b also contain variant d. The fact that the length of the upstream differential segment is a multiple of three and that its inclusion does not introduce any premature stop codons rule out preferential degradation by NMD as an explanation for the virtual absence of "a-d" and "b-c" combinations. Similar considerations can be applied to the *PCBP2* example. In the case of *CNOT8*, NMD must be ruled out because the 189 nt alternative exon is in the 5′ untranslated region.

We searched in the available human databases for the existence of ESTs spanning the EDI and IIICS FN regions. Unfortunately, and probably due to the long distance between both regions, no ESTs simultaneously spanning both regions were found.

Concerning the coordinating mechanism elicited by the EDI exon, it should be first noticed that EDI's length (270 nt, a multiple of three) precludes any involvement of NMD as the cause for the observed coordination both at the minigene and endogenous gene levels. Second, two mechanistic aspects should be considered either separately or in conjunction: the influence of one splicing event over another one and the polar behavior. Activation of distal EDI inclusion does not seem to be caused by the binding of SR proteins to the proximal EDI's ESE because it survives disruption of the ESE as long as the EDI exon is included constitutively (Figure 2). It could be speculated that inclusion of the proximal EDI affects distal AS by priming spliceosome assembly together with specific splicing factors, ready to act upon distal precursor sequences as soon as they are transcribed. This "priming" could occur in both directions. In fact, although at a lower extent, a constitutive distal EDI is able to increase the inclusion of a proximal alternative EDI (Figure 2). The 5′-to-3′ direction of transcription is what makes it polar. A similar, but not identical, mechanism takes place in the human thrombopoietin gene, where elimination of an intron generated by suboptimal splice sites within exon 6 depends on the splicing of constitutive upstream introns (Romano et al., 2001). The thrombopoietin mechanism depends on the actual process of splicing of the upstream constitutive introns, which suggests the existence of a priming effect of the splicing machinery by an upstream constitutive intron that permits the recognition of downstream suboptimal splice sites.

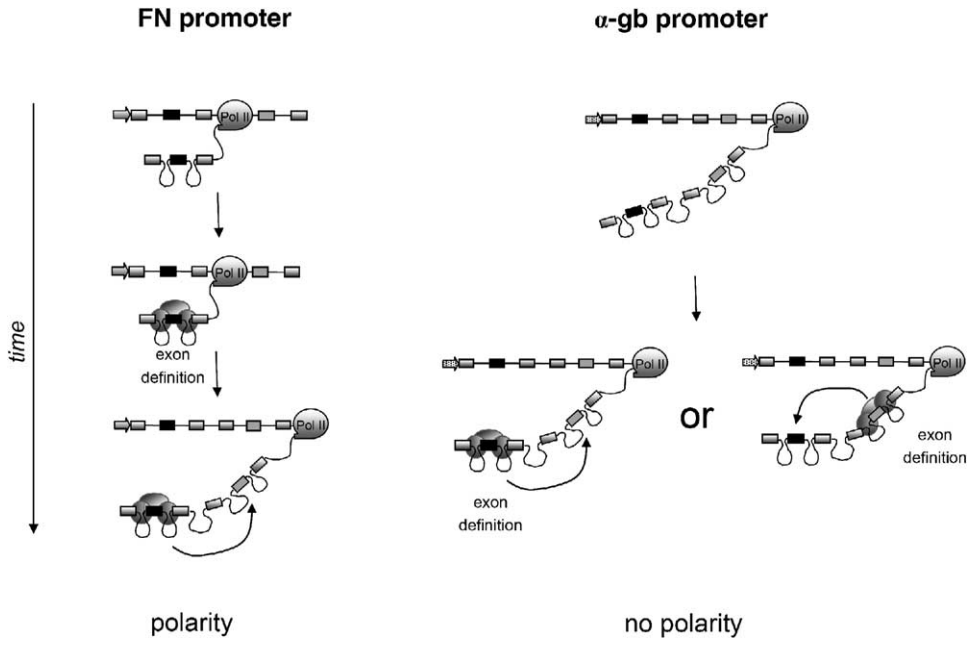

Figure 7. Model for the Role of Promoters Provoking Different Pol II Elongation Rates on Alternative Splicing Polarity

Low elongation rates or internal pauses (FN promoter) allow a temporal window of opportunity for splicing complexes to assemble at the proximal EDI before the distal EDI is transcribed. As pol II proceeds, the exon definition complexes at the proximal EDI stimulate distal EDI inclusion in a polar way. High processivity or lack of internal pauses (α-gb promoter) allows both proximal and distal EDIs to be exposed simultaneously to the splicing machinery, which results in the absence of polarity.

The following findings argue strongly that the polarity of the coordinating effect described here is a consequence of the cotranscriptionality of splicing and is linked to pol II elongation. (1) The 5′-to-3′ polarity is coincident with the polarity of transcription. (2) The allelic specificity in the heterozygous EDA$^{+/-}$ mutant mice (Figure 5) is consistent with a coordinating mechanism in *cis*. (3) The polar effect observed with the FN promoter disappears when transcription is driven by the α-gb promoter (Figure 4A). Chromatin immunoprecipitation (ChIP) analysis showed that the α-gb promoter provokes higher pol II elongation than the FN promoter (Kadener et al., 2002). (4) Inhibition of pol II elongation by DRB (Figure 4B) or by template replication elicited by T antigen (not shown) reverts the absence of polar effect observed with the α-gb promoter. (5) DRB and TSA, which have opposite effects on pol II elongation, also have opposite effects on the influence of EDI configuration on the IIICS region at the endogenous FN gene in MEFs.

Although the observed allele specificity is consistent with a cotranscriptional mechanism, other posttranscriptional options cannot be ruled out. For example, changes in pre-mRNA secondary structure or stability might persist after transcription is finished and affect preferentially the generation of certain isoform combinations.

Results reported here might explain the generation of complex patterns of AS such as those of the mouse CD44 gene. This gene has ten alternative exons (v1–v10) arranged in a central tandem array. Unrestricted combination of these exons could produce, in theory, up to 1024 isoforms (2^{10}). However, using a single-molecule profiling technique known as digital polony exon profiling, Zhu et al. (2003) showed that some exon combinations were more common than others, with the 15 most abundant isoforms accounting for 95% of all *CD44* transcripts with at least one v exon. Most importantly, and in striking agreement with our findings on polarity, inclusion of any given alternative exon seems to provoke a cascade inclusion of all exons 3′ to it.

The model in Figure 7 explains why polarity is observed with the FN promoter, which provokes low pol II elongation rates, but not with the α-gb promoter, which provokes higher pol II elongation.

Our findings suggest a new role for AS. The adaptive value of a particular alternatively spliced segment might not exclusively reside in its intrinsic protein-encoding potential but in its regulatory role on the inclusion of downstream alternative segments with protein-encoding functions. On the other hand, involvement of pol II elongation adds a new level of regulatory complexity. Two AS regions could function autonomously under certain conditions but become interdependent and coordinated upon stimuli affecting pol II elongation through, for example, cell-specific promoter occupation or epigenetic modifications affecting DNA methylation and chromatin configuration.

Experimental Procedures

Bioinformatics

The data about AS of human genes were taken from the EDAS database (http://www.belozersky.msu.ru/edas/). The EST data were taken from UniGene (http://www.ncbi.nlm.nih.gov/UniGene/). This

database contains genes with at least one intron in the coding region and that are covered by at least 20 ESTs. Protein, mRNA, and EST sequences that mapped to a genomic sequence without introns in the coding region were ignored. To avoid artifacts, only exons supported by ESTs from at least two clone libraries were considered.

All possible alternatively spliced isoforms generated by exons and introns with the given reliability were constructed. Isoforms containing only short open reading frames (less than half of an average length of all RefSeq proteins for that gene), multiple upstream ATG codons (more than 2), or introns located more than 55 nucleotides downstream of the stop codon were filtered out. The latter two conditions were based, respectively, on the ribosome-scanning model for the translation initiation in eukaryotes (Kozak, 2000) and on the model of surveillance for mis-spliced transcripts by nonsense-mediated decay (Lewis et al., 2003).

Plasmids

Construction of the different AS reporter minigenes is described in the Supplemental Experimental Procedures.

Transfections

Conditions for transfection with minigene constructs were described elsewhere (Kadener et al., 2001).

Alternative Splicing Assays

RNA preparation and reverse transcriptase reactions using oligo dT as primer were previously described (Kadener et al., 2001). Conditions for radioactive PCR amplification of cDNA splicing isoforms were as described below.

Regions in Tandem Minigenes

Proximal EDI (human), same conditions of those for EDI minigenes (Cramer et al., 1999), using primers pSV5′j (Caputi et al., 1994) and 3′NJ1 (5′-ATACTCGCCAGCGTGCGCGAG-3′); distal EDI (human), 30 cycles of 45 s at 94°C, 60 s at 59°C, and 60 s at 72°C in 1 mM MgCl2 using primers pSV5′j (Caputi et al., 1994) and 3′polyA (5′-CAAAGACCACGGGGGTACGGG-3′); EDII (human), 30 cycles of 30 s at 95°C, 60 s at 58°C, and 60 s at 72°C in 1.5 mM MgCl2 using primers 23-glob (5′-TTCAAGCTCCTAAGCCACTG-3′); IIICS (mouse), 30 cycles of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C in 1.5 mM MgCl2 using primers 5′-IIICSsec (5′-CCACTGCCTG CTGGTGAAAC-3′) and p1W/IIICS3′ (5′-CACAGAAGCCAGGAACT TGTC-3′).

Distal EDI PCR for All Constructs in Figure 4

Common distal PCR consisted of 30 cycles of 45 s at 94°C, 60 s at 55°C, and 30 s at 72°C and was performed on 3 μl of the RT reaction, with 1μM primers dosfor3 (5′-GTGGAGTATGTGGTTAGTG-3′) and 3′polyA.

Regions in Mouse Endogenous Genes

See the Supplemental Experimental Procedures.

All PCR reactions contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 2 μCi [α-32P]dCTP, and 1.5 U of Taq DNA polymerase. RT-PCR products were electrophoresed in 6% acrylamide and detected by autoradiography, and the amount of radioactivity in the bands was measured in a scintillation counter (Cerenkov method).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, two figures, and one table and are available with this article online at http://www.molecule.org/cgi/content/full/19/3/393/DC1/.

Acknowledgments

We thank D. Cazalla, A. Colman-Lerner, and A. Mironov for helpful discussions and M. Okawa, R. Nurtdinov, V. Buggiano, M. Muñoz, M. de la Mata, and M. Blaustein for valuable help. This work was supported by grants to A.R.K. from the Fundación Antorchas, the International Centre for Genetic Engineering and Biotechnology, and the Agencia Nacional de Promoción de Ciencia y Tecnología of Argentina; to F.E.B. from the Fondazione per la Ricerca Biologica; and to M.S.G. from the Ludwig Institute of Cancer Research, the Russian Fund of Basic Research, and the Russian Academy of Sciences. J.P.F. is recipient of a fellowship and A.R.K. is a career investigator from the Consejo Nacional de Investigaciones Científicas y Técnicas of Argentina. A.R.K. and M.S.G. are international research scholars of the Howard Hughes Medical Institute.

Received: December 21, 2004
Revised: March 14, 2005
Accepted: June 30, 2005
Published: August 4, 2005

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