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A Slow RNA Polymerase II Affects Alternative Splicing In Vivo

Short Article

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Summary

Changes in promoter structure and occupation have been shown to modify the splicing pattern of several genes, evidencing a coupling between transcription and alternative splicing. It has been proposed that the promoter effect involves modulation of RNA pol II elongation rates. The C4 point mutation of the *Drosophila* pol II largest subunit confers on the enzyme a lower elongation rate. Here we show that expression of a human equivalent to *Drosophila*'s C4 pol II in human cultured cells affects alternative splicing of the fibronectin EDI exon and adenovirus E1a pre-mRNA. Most importantly, resplicing of the *Hox* gene *Ultrabithorax* is stimulated in *Drosophila* embryos mutant for C4, which demonstrates the transcriptional control of alternative splicing on an endogenous gene. These results provide a direct proof for the elongation control of alternative splicing in vivo.

Introduction

Alternative splicing is a widely used mechanism to generate cell-type and developmental-stage specific protein diversity. The realization that a vast mammalian proteomic complexity is achieved with a limited number of genes demands a better understanding of alternative splicing regulation. For years transcription and pre-mRNA processing had been thought to be independent, until a series of biochemical, cytological, and functional experiments demonstrated that capping, splicing, and cleavage/polyadenylation reactions are tightly coupled to RNA polymerase II (pol II) transcription (for reviews see Maniatis and Reed, 2002; Bentley, 2002; Proudfoot et al., 2002; Neugebauer, 2002). The concept of a functional coupling between transcription and alternative splicing was established by findings that promoter identity affects alternative splicing of the fibronectin (FN) gene (Cramer et al., 1997; 1999). Recently, other labs extended these findings to the genes for the cystic fibrosis transmembrane regulator (Pagani et al., 2003), CD44, and the calcitonin gene related peptide (Auboeuf et al., 2002).

Studies on the effects of various transcriptional activators on the splicing of the FN EDI (extra domain I) exon show that rapid, highly processive transcription favors EDI skipping, whereas slower, less processive transcription favors inclusion. The extent of exon skipping correlates with the efficiency with which pol II transcripts reach the 3′ end of the gene, but not with the overall fold increase in transcript levels caused by activators (Kadener et al., 2001; 2002; Nogués et al., 2002). To explain this, a kinetic coupling model has been proposed, where the transcript elongation rate determines the outcome of competing splicing reactions that occur cotranscriptionally (Cáceres and Kornblihtt, 2002). Here we provide direct proof for the elongation mechanism in the transcriptional control of alternative splicing in human cells and demonstrate the existence of such control on an endogenous *Drosophila* gene that is key for development.

Results and Discussion

A Slow RNA Pol II Inhibits Skipping of the Fibronectin EDI Exon

To test the kinetic model with a direct tool, we transiently transfected a human hepatoma cell line (Hep3B) with reporter minigenes for alternative splicing together with vectors expressing an α-amanitin- resistant human pol II large subunit (Rpb1) carrying a single point mutation (R749H), equivalent to *Drosophila*'s C4 mutation (R741H). The C4 mutant pol II has a lower elongation rate in vitro (Coulter and Greenleaf, 1985) and is less efficient at reading through intrinsic elongation blocks, compared with the wild-type (WT) pol II (Chen et al., 1996). Transcription of the transfected minigenes by the mutant pol II was favored by inhibiting the endogenous enzyme with α-amanitin (Gerber et al., 1995). Our assay allowed high expression of the α-amanitin-resistant WT and mutant polymerases in Hep3B cells, compared to the endogenous pol II, both at the RNA (Figure 1A) and protein levels (Figure 1B, lanes 2 and 3). The molecular weight of the transfected large subunit is identical to that of the endogenous one (approximately 240 kDa). For this reason we included, as positive control, a plasmid ex-

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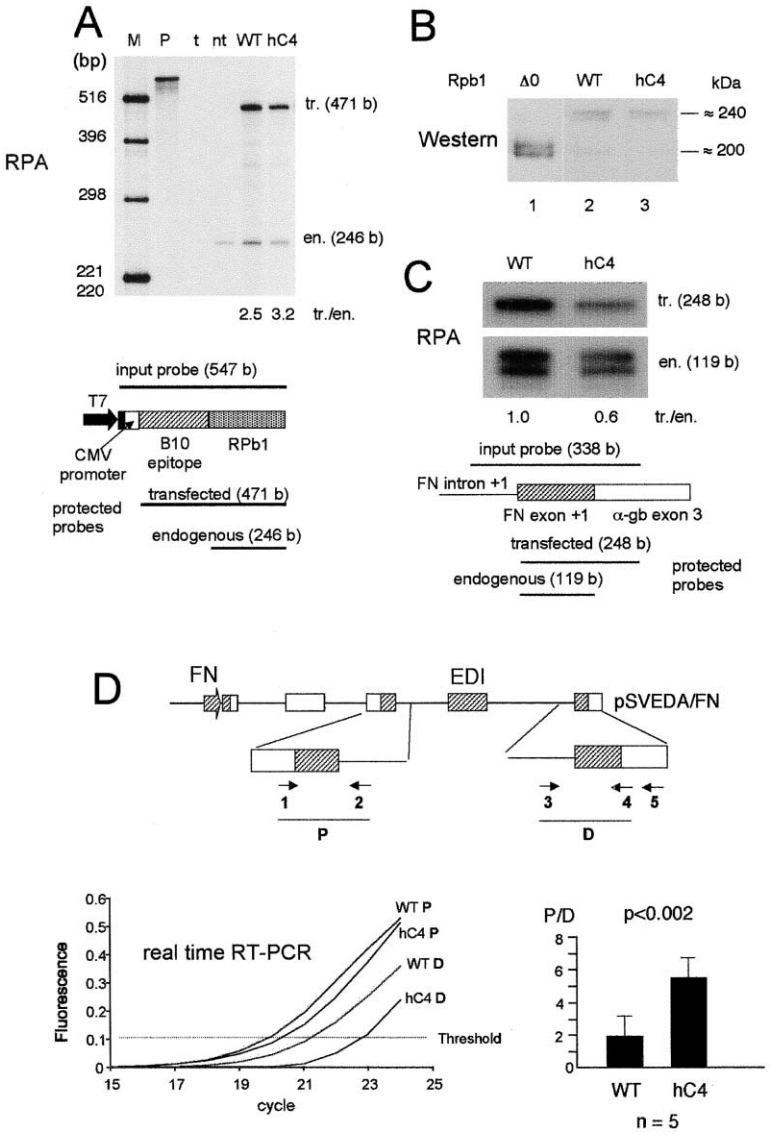

Figure 1. A Human Pol II Carrying a Mutated (R749H) Large Subunit Is Less Processive In Vivo

(A) RNase protection assays to quantify endogenous and recombinant human RNA pol II large subunit mRNAs expressed in Hep3B cells transfected with plasmids pAT7Rpb1αAm^r (WT) or pAT7Rpb1αAm^{r}R749H (hC4) and treated with α-amanitin. Diagrams for the input riboprobe (P) and protected probes are shown. M, molecular weight markers; t, control with yeast tRNA; nt, nontransfected cells; tr./en., ratios of transfected over endogenous radiactive bands.

(B) Western blots of total protein extracts from Hep3B cells transfected with pAT7Rpb1αAm^r (WT, lane 2), pAT7Rpb1αAm^{r}R749H (hC4, lane 3), or a plasmid expressing a truncated version of human Rpb1 that lacks the carboxy terminal domain (Δ0, lane 1). Blots were revealed with the sc-899 antiserum (Santa Cruz Biotechnologies), which recognizes the N terminus of human Rpb1.

(C) RNase protection assays to quantify endogenous FN mRNA and recombinant FN/α-globin mRNA expressed in Hep3B cells transfected with pSVEDA/FN and either pAT7Rpb1αAm^r (WT) or pAT7Rpb1αAm^{r}R749H (hC4), followed by treatment with α-amanitin as indicated in Experimental Procedures. Diagrams for the input and protected probes are shown below.

(D) Real time RT-PCR to quantify pre-mRNA accumulation at proximal and distal regions with respect to the transcription start site. Hep3B cells were transfected with pSVEDA/FN (top diagram) and either pAT7Rpb1αAm^r (WT) or pAT7Rpb1αAm^{r}R749H (hC4), and treated with α-amanitin as indicated in Experimental Procedures. 48 hr after transfection, nuclei were isolated, total RNA was prepared, and cDNA was synthesized using primers "2" or "5." PCR reactions containing the fluorescent dye SYBR green were performed in a DNA Engine Opticon System (MJ Research) with primers "1" and "2" for the proximal (P) region and primers "3" and "4" for the distal (D) region. The Opticon screen view (bottom, left) illustrates differences in threshold cycles obtained in the two regions with the WT and hC4 polymerases. P/D means ± SD ratios of five independent transfection experiments are shown (bottom, right).

pressing a truncated version of Rpb1 (Δ0, 200 kDa) in a transfection without plasmids expressing WT or hC4 full-length large subunits (lane 1). Both the truncated and full-length versions are expressed at levels higher than those of the endogenous Rpb1. In these conditions the mutant pol II only provokes a 40% inhibition of transcription (Figure 1C).

To test that the mutant human pol II was less processive in vivo, we isolated RNA from nuclei of the transfected cells and determined the amounts of minigene pre-mRNAs accumulated at proximal and distal regions with respect to the transcription start site, by quantitative real time RT-PCR. cDNA was synthesized with a different primer for each region, followed by amplification of PCR products that span intron/exon junctions (Figure 1D), and therefore correspond to yet unspliced pre-mRNAs. DNA amounts resulting from contamination of RNA preparations with transfected template constructs were estimated in reactions in which reverse transcriptase was omitted, and substracted from total templates estimated by real time RT-PCR. Figure 1D shows that the mutant human pol II provokes a 2.7-fold increase in the accumulation of proximal (P) over distal (D) pre-mRNAs compared to the WT, which reveals that the introduced mutation provokes a lower elongation rate in human cells. In consequence, the mutant pol II will be referred to as "slow" pol II or human C4 (hC4).

Transcription by the slow pol II stimulates the inclusion of EDI by approximately 4-fold (Figure 2A), confirming the hypothesis of inverse correlation between elongation rate and EDI inclusion. However, inclusions of other two alternative exons (FN-EDII and exon 7B of the

heterogeneous nuclear ribonucleoprotein A1 [hnRNPA1-E7B] gene) are not affected by the slow pol II (Figures 2B and 2C). Whereas EDI splicing is mainly controlled by an exonic splicing enhancer (ESE) (Caputi et al., 1994), FN-EDII and hnRNPA1-E7B splicings are controlled by intronic elements (Lim and Sharp, 1998; Simard and Chabot, 2000). This difference might explain their unresponsiveness to the slow pol II. In addition, EDII unresponsiveness is consistent with the fact that its splicing is not affected either by promoter identity (Pagani et al., 2003) or by transcriptional regulators of pol II elongation (Kadener et al., 2001). In fact, unresponsiveness of the EDII exon is independent of the promoter that drives transcription (compare lanes 1 and 2 with lanes 3 and 4 of Figure 2B).

The Slow Pol II Favors the Use of Upstream 5′ Splice Sites in Adenovirus E1a Splicing

In our search for other differential splicing systems affected by pol II elongation we reasoned that adenovirus E1a was a good candidate because, similarly to EDI (Kadener et al., 2001), adenovirus splicing changes after template replication (Adami and Babiss, 1991). E1a splicing presents a single 3′ splice site (SS) and three alternative 5′SS, giving rise to three mRNA isoforms designated 13S, 12S, and 9S. Viral replication provokes a preferred use of the upstream 5′SS giving rise to higher proportions of the shorter isoforms. This has been attributed to changes in the state of the DNA template that could in turn reduce pol II elongation rates (Adami and Babiss, 1991). A reduction in elongation rate would allow more time to assemble splicing complexes at the upstream 5′SS, favoring their use compared to the downstream 5′SS. Consistently, a 3-fold increase in the proportion of shorter isoforms (9S/13S ratio) is observed when an E1a reporter minigene (Cáceres et al., 1994) is transcribed by the slow RNA pol II (Figure 2D, lanes 1 and 2). An extra band, marked with an arrow in Figure 2D, appears aleatorily in certain E1a RT-PCRs. The presence of this band, probably caused by the treatment with α-amanitin, does not affect the change in 9S/13S ratios observed with the slow pol II (lanes 3 and 4).

Effects of the slow pol II on EDI and E1a alternative splicings increase with the dose of α-amanitin (Figure 2E), reflecting that the more the host's cell pol II is inhibited, the more the transfected minigenes are transcribed by the recombinant slow pol II. Increasing amounts of the inhibitor do not affect splicing when transcription is carried out by the endogenous pol II (not shown). On the other hand, addition of both cycloheximide and α-amanitin to the transfected cells does not eliminate the slow pol II effect on splicing (data not shown), which indicates that this effect is not mediated indirectly by a product of de novo protein synthesis.

The Slow Pol II Effect and the Alternative Splicing Regulator SF2/ASF

Inclusion of EDI is stimulated by the binding of the splicing factor SF2/ASF to the sequence GAAGAAGAC present in the EDI's ESE, which stimulates the use of EDI's suboptimal 3′SS. In addition, EDI carries an exonic splicing silencer (ESS) downstream of the ESE (Caputi et al., 1994). Disruption of the ESE abolishes EDI inclusion and prevents its stimulation by the slow pol II (Figure 3A, lanes 1–4). On the contrary, disruption of the ESS provokes a 10-fold increase in EDI inclusion (lanes 1 and 5) and does not interfere with the slow pol II effect, which provokes a further 3-fold stimulation of EDI inclusion (lane 6). This indicates that mutating the polymerase cannot override the need for SF2/ASF.

Since overexpression of the slow pol II partially inhibits its transcription (Figure 1C), it may also cause partial depletion of SF2/ASF. Western blots in Figure 3B show that siRNAs specific for SF2/ASF completely abolish overexpression of SF2/ASF (lanes 4–6). Because of its T7 tag, recombinant SF2/ASF is recognized as a 36 kDa band, distinguishable from 33 kDa endogenous SF2/ASF. Even though the transfection efficiency is 10%–20%, inhibition of endogenous SF2/ASF levels by RNAi is also observed (lanes 1–3). RT-PCR analysis in the same figure indicates that specific depletion of SF2/ASF by RNAi indeed inhibits both basal (lanes 1–3) and SF2/ASF-stimulated (lanes 4–6) EDI exon inclusion. This eliminates the possibility the slow pol II effect on EDI splicing is an indirect consequence of SF2/ASF depletion.

Resplicing of Ubx Pre-mRNA Is Stimulated in *Drosophila* C4 Embryos

The use of transfected minigenes proved to be extremely useful to find the mechanism by which transcription controls alternative splicing. However, transfection experiments may be of questionable physiological relevance because minigenes are chimeric constructs where alternatively spliced regions are positioned at incorrect distances with respect to promoters. A physiological approach to the coupling between transcription and splicing should necessarily study an endogenous gene in its natural environment.

The *Drosophila* C4 mutation causes a synergistic enhancement of the phenotype elicited by mutations in the *Hox* gene *Ultrabithorax (Ubx)* (Mortin et al., 1988). In addition, flies carrying the C4 mutation in heterozygosis but being wild-type for both *Ubx* alleles show a mutant phenotype called "Ubx effect" that resembles the one seen in flies haploinsufficient for the Ubx protein (Greenleaf et al., 1980). These observations prompted us to investigate *Ubx* alternative splicing in C4 flies.

Ubx is a complex transcriptional unit with three long introns of 7.4, 14.6, and 51.5 kb. Alternative splicing generates up to six mRNAs that share the 5′- and 3′-terminal exons but differ in their inclusion of three units: the B element and internal microexons mI and mII (Figure 4A). *Ubx* most abundant isoforms (Ia, IIa, IVa, and Ib) follow specific spatio-temporal patterns of expression (Hatton et al., 1998). Splicing of either mI or mII to E5′ regenerates a 5′SS consensus sequence at the newly formed exon-exon junction. Thus, mI and mII are joined constitutively to E5′ in the nascent transcript and removed, in a subsequent step, along with the downstream intron by a mechanism known as resplicing. By varying *Ubx* intron lengths, Hatton et al. (1998) showed that resplicing is more efficient when the downstream intron is long enough to permit regeneration of a 5′SS before the next exon is transcribed. This predicts that slowing down pol II elongation rate would favor resplicing and therefore increase the relative abundance of

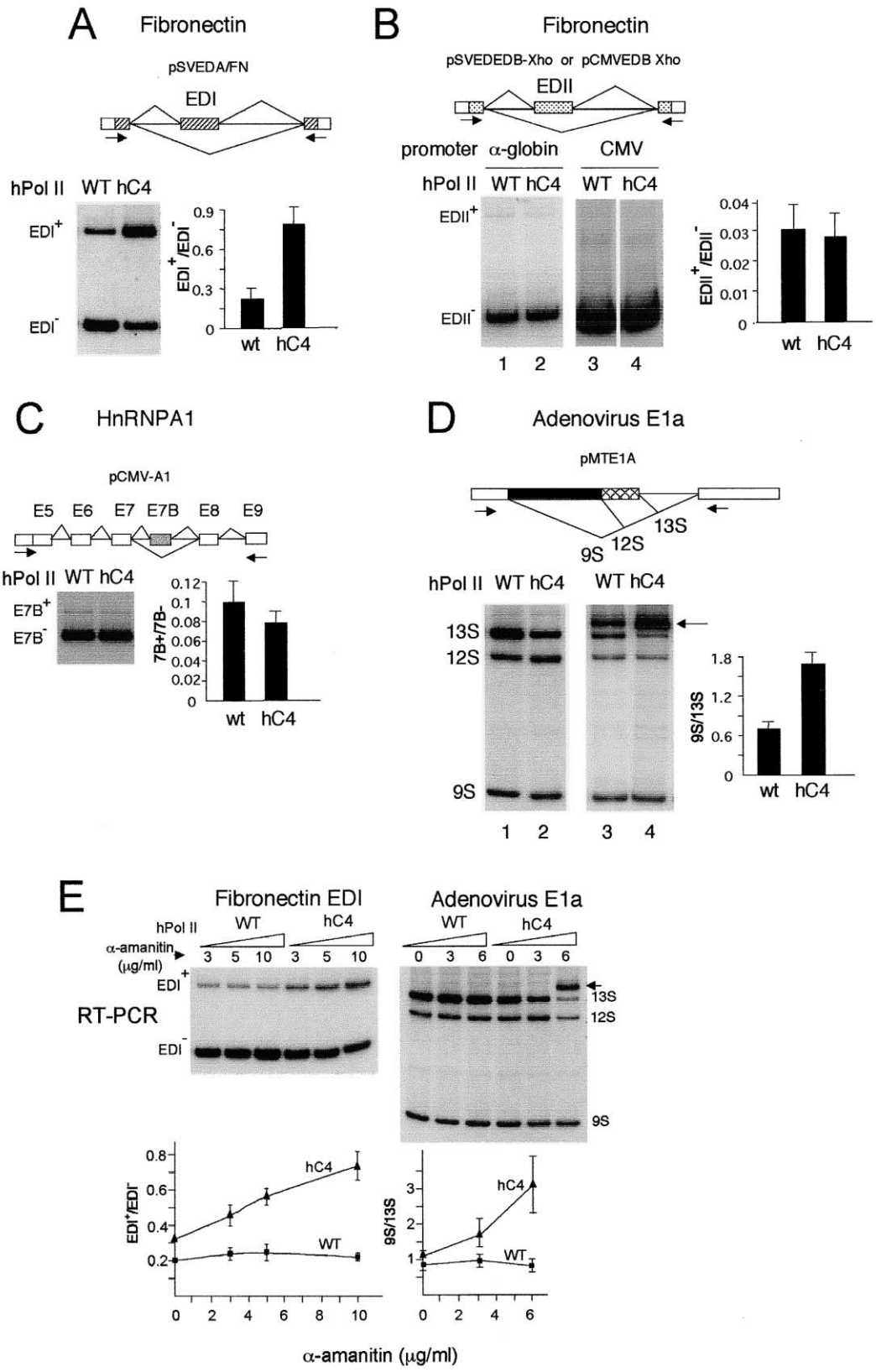

Figure 2. Effects of the Overexpression of WT and Slow (hC4) Pol II Largest Subunits on Different Alternative Splicing Systems

Hep3B cells were transfected with plasmids pSVEDA/FN (A), pSVEDEDB-Xho ([B], lanes 1 and 2), pCMVEDB-Xho ([B], lanes 3 and 4), pCMV-A1 (C), or pMTE1A (D). In each case cells were cotransfected with either pAT7Rpb1αAm^r (WT) or pAT7Rpb1αAm^{r}R749H (hC4) and treated

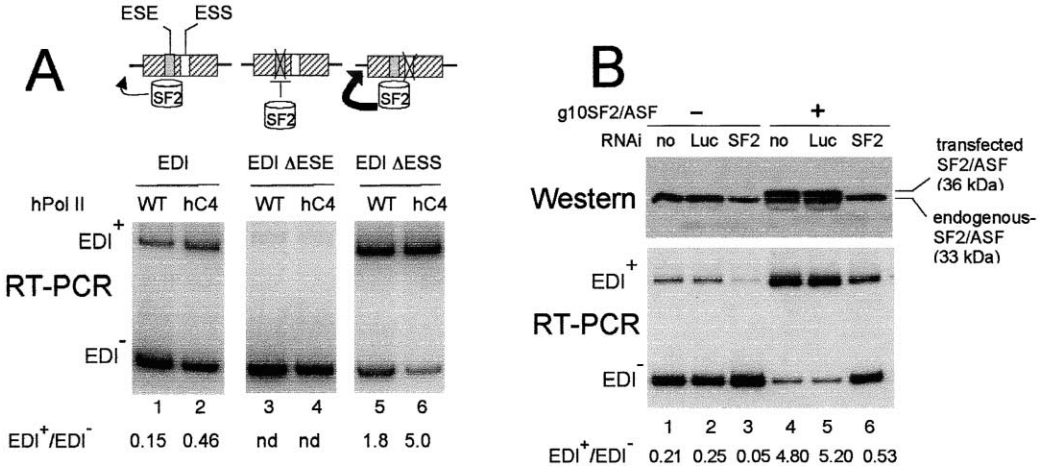

Figure 3. The Slow Pol II Effect and the Alternative Splicing Regulator SF2/ASF

(A) Effects of disrupting EDI's exonic splicing enhancer (ESE) or silencer (ESS) on the slow pol II effect. EDI minigenes carrying intact ESE and ESS (pSVEDA/FN, lanes 1 and 2), mutated ESE (pSVEDA/FNΔESE, lanes 3 and 4), and mutated ESS (pSVEDA/FNΔESS, lanes 5 and 6) were cotransfected into Hep3B cells with plasmids pAT7Rpb1αAm^r (WT) or pAT7Rpb1αAm^{r}R749H (hC4), followed by treatment with α-amanitin and alternative splicing assessment by RT-PCR as described in Experimental Procedures.

(B) Effect of RNAi to SF2/ASF on EDI alternative splicing. Hep3B cells were cotransfected with pSVEDA/FN (all lanes), a plasmid expressing T7-tagged-human SF2/ASF (g10SF2/ASF, lanes 4–6), and siRNAs luciferase (negative control, lanes 2 and 5) or human SF2/ASF (lanes 3 and 6). Total RNA was prepared 48 hr later and EDI splicing was assessed by RT-PCR as described (bottom). Western blots of total protein extracts from transfected cells, probed with an antibody to SF2/ASF (mAb103, Hanamura et al., 1998), are shown in the top panel.

shorter mRNA isoforms. This is in fact what we find when we measure the relative levels of *Ubx* mRNA isoforms in fly embryos during development (Figure 4B). Eggs were collected in 1 hr intervals. At 2.5 ± 0.5 hr after egg laying (AEL), C4 embryos have 3-fold lower Ia/IIa ratios than WT embryos. This difference is reduced in embryos collected at 6.0 ± 0.5 hr AEL and disappears in two late time windows tested (18.0 ± 0.5 and 20.0 ± 0.5 hr AEL). Isoform IVa is virtually undetectable in the two early windows but is the most abundant in the two late windows. Noticeably, IVa is specific to the fly central nervous system, which has not yet developed in embryos of the two early windows. Ia/IVa ratios show no significant differences between WT and C4 flies at the 18.0 ± 0.5 hr window but Ia/IVa becomes significantly lower in C4 flies in the 20.0 ± 0.5 hr window. Thus, C4 flies show a clear reduction in the relative amounts of the long isoform Ia in favor of the shorter, respliced, isoforms IIa first and IVa later. It will be important to investigate to what extent this alteration in splicing is causative for the display of the *Ubx*-like phenotype.

The kinetic mechanism shown here might explain the promoter effect on alternative splicing. However, it is important to point out that not all alternative splicing regions are subjected to the processivity control (Figures 2B and 2C). Both the pol II pausing architecture and the relative strengths of the splice sites that are being presented to the splicing machinery by the transcription process must be crucial for the degree of responsiveness to elongation rates. On the other hand, the kinetic control does not exclude a concomitant role for recruitment to the elongating pol II of *trans*-acting factors reported to couple transcription and splicing (Bentley, 2002).

Consistently with our findings, Howe et al. (2003) found that exon skipping in yeast is inhibited when transcription is slowed by RNAPII mutants or when cells are treated with inhibitors of elongation. Minigenes with a yeast two-intron gene with a constitutive internal exon were mutated to reduce recognition of the intron 1 branchpoint and therefore create an alternative cassette exon. Inclusion of this artificial cassette exon is increased in yeast mutants carrying a slow pol II and inhibited by overexpression of the elongation factor TFIIS.

Our results indicate that the kinetic control is achieved differently in genes with optional cassette exons (exon skipping) like EDI from genes with alternative 5′SS or resplicing, such as E1a or *Ubx*. Unlike exon skipping, resplicing per se allows removal of each intron as soon as it is transcribed, avoiding competition between splice sites that are distant in space and in time of transcription (Hatton et al., 1998). This explains why a slow elongation rate inhibits exon skipping in EDI splicing but favors resplicing in *Ubx*. In our model, EDI exon skipping occurs because the 3′SS of the upstream intron is suboptimal

with α-amanitin as indicated in Experimental Procedures. Positions of primers for RT-PCR are indicated by arrows under each diagram. Histograms display means ± SD of ratios between radioactivity in the indicated RT-PCR bands of at least three independent transfections. (E) Dose-response of α-amanitin on alternative splicing of FN EDI and adenovirus E1a in cells expressing the slow pol II. Hep3B cells were cotransfected with pSVEDA/FN (left) or pCMV-A1 (right) and pAT7Rpb1αAm^r (WT) or pAT7Rpb1αAm^{r}R749H (hC4). 24 hr after transfection the indicated amounts of α-amanitin were added and cells were incubated for a further 24 hr before RNA preparation. Curves display means ± SD of ratios between radioactivity in the indicated RT-PCR bands of at least three independent experiments.

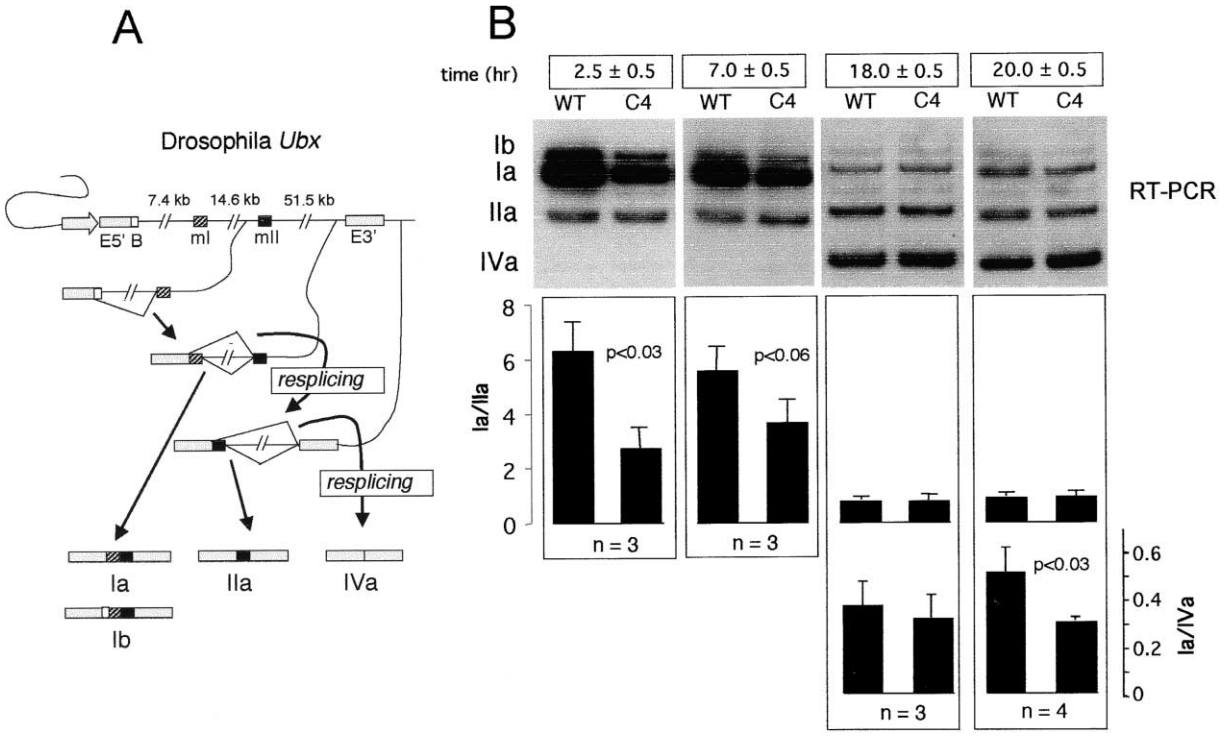

Figure 4. *Ubx* Resplicing Is Affected in Drosophila's C4 Embryos

(A) Diagram of *Drosophila Ubx* gene and the generation of alternative splicing isoforms Ia, IIa, Iva, and Ib. E5′, 5′ exon; mI and mII, microexons I and II; E3′, 3′ exon; B, B element.

(B) Radioactive RT-PCR to evaluate the relative abundance of *Ubx* mRNA isoforms in *Drosophila* embryos collected at different times during development. Histograms display means ± SD of ratios between radioactivity in the indicated RT-PCR bands of at least three independent (n) embryo collections for each time window.

compared to the 3′ splice site of the downstream intron. If the polymerase pauses or slows down 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 but to eliminate the downstream intron, which leads to exon inclusion. A highly processive or fast elongating pol II, or the absence of internal pauses, would favor the simultaneous presentation of both introns to the splicing machinery, a situation in which the stronger 3′ splice site of the downstream intron outcompetes the weaker 3′ splice site of the upstream intron, resulting in exon skipping. In the case of E1a, slow transcription or pausing would allow more time for recruitment of splicing components to the more distal 5′ splice sites relative to the 3′ splice site, favoring the generation of shorter isoforms. In addition, our results suggest new clues for the control of resplicing, which might function as a general mechanism for the stepwise removal of very long introns.

Experimental Procedures

Plasmids

Alternative Splicing Reporter Minigenes

pSVEDATot contains the human FN EDI (also named EDA) alternative exon under the control of the human α-globin promoter (Caputi et al., 1994). pSVEDA/FN contains the human FN EDI alternative exon under the control of the human FN promoter (Cramer et al., 1999). pSVEDA/FNΔESE and pSVEDA/FNΔESS are similar to pSVEDA/FN but bear disruptions of the exonic splicing enhancer (ESE) and exonic splicing silencer (ESS), respectively (Cramer et al., 1999). pSVEDEDBXho contains the human FN EDII (also named EDB) alternative exon under the control of the human α-globin promoter (Muro et al., 1999). pCMVEDB-Xho is a variant of pSVEDEDB-Xho in which the α-globin promoter was replaced by the CMV promoter. pCMV-A1 expresses the mouse hnRNPA1 7B alternative exon under the control of the CMV promoter (Simard and Chabot, 2000). pMTE1A contains adenovirus E1a alternative splicing region under the control of the mouse metallothionein I promoter (Cáceres et al., 1994).

Pol II Expression Vectors

An expression vector (pAT7Rpb1αAm^r) (Nguyen et al., 1996) for an α-amanitin resistant variant (N792D) of human RNA pol II (hRpb1) large subunit was modified to introduce a second point mutation in hRpb1 codon 749 (CGT to CAG). The resulting plasmid (pAT7Rpb1αAm^{r}R749H) expressed a hRpb1 with an amino acid change (R749H) that is equivalent to the *Drosophila* C4 mutation (R741H) (Coulter and Greenleaf, 1985). Δ0, a variant of pAT7Rpb1αAm^r with a deletion of the region encoding the 52 heptads of the carboxy terminal domain (CTD), has been previously described (McCracken et al., 1997).

Transfections

Conditions for transfection with minigene constructs were described elsewhere (Kadener et al., 2001). When indicated, minigenes were cotransfected with pAT7Rpb1αAm^r or pAT7Rpb1αAm^{r}R749H. α-amanitin was added to the cells at 5 μg/ml, except when otherwise indicated, 24 hr after transfection. Cells were harvested 24 hr later.

For RNAi knockdown experiments, cells were cotransfected with 500 ng of the mFN EDI splicing reporter minigene and 500 ng of

either one of the following siRNAs duplexes (only sense sequence shown): SF2/ASF 5′-CCAAGGACAUUGAGGACGUdTdT-3′; Luc (GL3 luciferase) 5′-CUUACGCUGAGUACUUCGAdTdT-3′ (Elbashir et al., 2001). Cells were harvested 48 hr posttransfection.

Pol II Processivity Assay

RNA was prepared from nuclei isolated from Hep3B cells transfected with pSVED/FN and either pAT7Rpb1αAm^r or pAT7Rpb1αAm^{r}R749H, and treated with α-amanitin. Quantification of pSVED/FN unspliced transcripts accumulated at proximal or distal regions (Figure 1D) was carried out by real time RT-PCR using a DNA Engine Opticon System (MJ Research). Proximal region (P): Primer "2" (5′- GCATTCAGACACCCAAGAAC-3′) was used for cDNA synthesis and primers "1" (5′-TTCTCTGCACAGCTCCTAAG-3′) and "2" were used for PCR. Distal region (D): Primer "5" (5′-GGTATTTG GAGGTCAGCA-3′) was used for cDNA synthesis and primers "3" (5′-TTGGAACTACGTTTATTTTCC-3′) and "4"(5′-GCGGCCAGGGG TCACGAT-3′) were used for PCR. Amplifications were performed in 4 mM MgCl2, 1:30000 SYBR Green (Molecular Probes Inc.) with an initial incubation at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, and 45 s at 72°C.

Alternative Splicing Assays

RNA preparation and reverse transcriptase reactions using oligo dT as primer were previously described (Kadener et al., 2001). Radioactive PCR amplification of cDNA splicing isoforms was performed using reported specific primers for EDI (Cramer et al., 1999), EDII (Kadener et al., 2001), hnRNPA1 (Simard and Chabot, 2000), and E1a (Cáceres et al., 1994). *Drosophila Ubx* isoforms were detected by PCR with the following primers: 5′-TGGAATGCCAATTGCAC CATC-3′ and 5′-GTCTGGTAGCGGGTGTATGTC-3′. PCR conditions: 30 cycles of 45 s at 94°C, 60 s at 56°C, and 30 s at 72°C.

RNase Protection Assays

A riboprobe template for human Rpb1 was obtained by subcloning into pBluescriptKS a 506 bp PCR DNA fragment encompassing the B10 epitope-Rpb1 boundary of the pAT7Rpb1αAm^r construct (Nguyen et al., 1996). The resulting plasmid was linearized with EcoRI and a 547 base anti-sense, ^{32}P-labeled riboprobe was obtained by transcription with T7 RNA polymerase. RPA conditions were described previously (Kadener et al., 2001).

Fly Stocks

Homozygous stocks carrying the RNA pol II 215 C4 allele were obtained from the Bloomington Stock Centre. Embryos were collected on apple juice plates for approximately 1 hr and then allowed to develop at 25°C for variable times.

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