Annu. Rev. Genet. 1998. 32:279–305
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ALTERNATIVE SPLICING OF PRE-mRNA: Developmental Consequences and Mechanisms of Regulation

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KEY WORDS: SR proteins, U2AF, PTB, *Sex lethal*, *doublesex*

ABSTRACT

Alternative splicing of pre-mRNAs is a powerful and versatile regulatory mechanism that can effect quantitative control of gene expression and functional diversification of proteins. It contributes to major developmental decisions and also to fine tuning of gene function. Genetic and biochemical approaches have identified *cis*-acting regulatory elements and *trans*-acting factors that control alternative splicing of specific pre-mRNAs. Both approaches are contributing to an understanding of their mode of action. Some alternative splicing decisions are controlled by specific factors whose expression is highly restricted during development, but others may be controlled by more modest variations in the levels of general factors acting cooperatively or antagonistically. Certain factors play active roles in both constitutive splicing and regulation of alternative splicing. Cooperative and antagonistic effects integrated at regulatory elements are likely to be important for specificity and for finely tuned differences in cell-type-specific alternative splicing patterns.

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0066-4197/98/1215-0279$08.00

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INTRODUCTION

Alternative splicing is used to enhance the information contained within a gene and to control its expression. It is frequent in metazoans as diverse as the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster*, and our own species. Some alternative splicing events appear to be constitutive, with mRNA variants coexisting at constant ratios in the same cells, whereas others are regulated in response to developmental or physiological cues. The consequences range from switching expression of a protein on and off [e.g. by including or excluding stop codons, as in *Sxl* (18)], to structural and functional diversification of protein products [by including or excluding elements as small as a single amino acid, for example in Pax-3 and Pax-7 (137)]. Hence, alternative splicing is a versatile process that can be integrated with other regulatory mechanisms to generate complex genetic switches, to modulate cellular responses to developmental and physiological signals, to fine tune the function of regulatory factors, and to diversify the biochemical inventory within and between cells. This review examines the current state of knowledge about alternative splicing regulation in the context of developmental control. The first part is an overview of the importance of alternative splicing as a strategy for developmental regulation. Part two deals with the mechanisms that control developmentally specific alternative splicing decisions, focusing on emerging general themes.

DEVELOPMENTAL ROLE OF ALTERNATIVE SPLICING

A Complex Regulatory Hierarchy Based on Alternative Splicing

The regulatory pathway that specifies sexual identity and controls dosage compensation in *Drosophila* illustrates the power and versatility of alternative

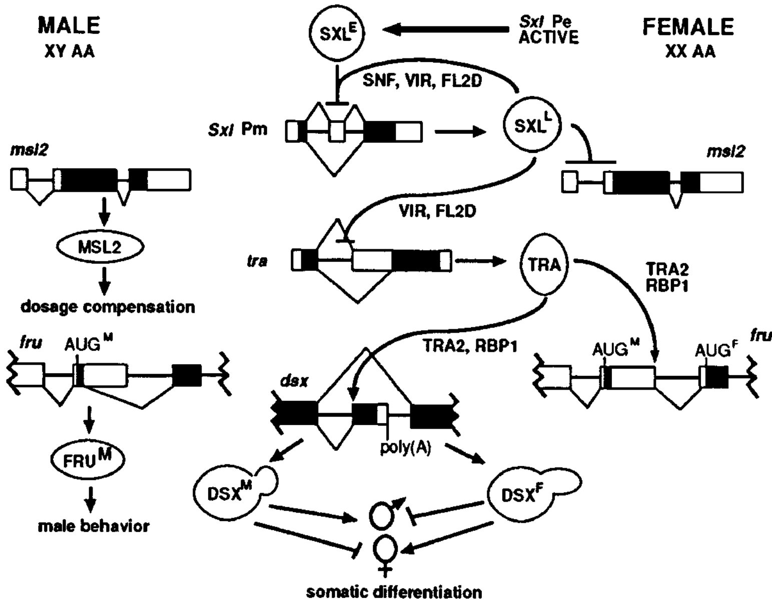

Figure 1 Regulation of alternative splicing in the *Drosophila* sex-determination pathway. The transcript from the early *Sxl* promoter (Pe) is activated only in females and is processed to exclude the male-specific exon. This produces an early burst of SXL protein that blocks splicing of the male exon in transcripts from the late promoter (Pm), which is active in both sexes; the positive feedback loop thus initiated maintains expression of active SXL in females. TRA2, SNF, VIR, FL2D, and RBP1 are non-sex-specific factors described in the text. SXL also represses translation of *msl2* mRNA (reviewed in 49). *Shaded areas* represent open reading frames.

splicing (Figure 1) (for detailed reviews, see 29, 84). The primary target of the sex-determination signal is the female-specific switch gene *Sex-lethal* (*Sxl*), which coordinately controls sexual differentiation and dosage compensation. The presence of two X chromosomes in diploid flies signals female identity and triggers expression of Sex-lethal protein (SXL) early in development. SXL is an RNA-binding protein that controls three known alternative splicing decisions. In its own RNA, SXL represses inclusion of a default exon that aborts translation, thus initiating a positive feedback loop that maintains functional SXL expression in females and constitutes a memory of the sex-determination signal, which is transient. SXL also blocks splicing of an intron in the 5′ untranslated region of *male-specific-lethal-2* (*msl2*) pre-mRNA (49); the presence of this intron interferes with translation of MSL2 protein in females, thus preventing the hyperactivation of X-linked transcription, which accounts for most dosage compensation in males. Finally, SXL blocks the more upstream of two competing

$3'$ splice sites in *transformer* (*tra*) pre-mRNA, thus allowing expression of TRA protein in females. TRA is also a splicing regulator that, together with the constitutive TRA2 protein, controls two splicing decisions required for somatic sexual differentiation. TRA activates the more upstream of two competing $3'$ splice sites in the pre-mRNA for the bifunctional switch gene *doublesex* (*dsx*); this results in incorporation of a female-specific terminal exon rather than the default exon used in males. The alternative *dsx* mRNAs encode structurally and functionally distinct transcription factors (DSX^F in females and DSX^M in males); these proteins establish appropriate gene expression patterns for most somatic sexual characters in females and males, respectively. TRA also activates the more downstream of two competing $5'$ splice sites in the *fruitless* (*fru*) pre-mRNA (62), thus preventing the expression of male-specific FRU proteins, which are transcriptional regulators required for male courtship behavior and for differentiation of a male-specific abdominal muscle.

Modulation of Transcription Factors by Alternative Splicing

Alternative splicing is used in each branch of the *Drosophila* sexual identity pathway to control the expression or functional properties of transcriptional regulators or chromatin-associated proteins that influence transcription. The ability of *dsx* to function as a bifunctional switch is noteworthy because it results from the production of alternative isoforms. DSX^M and DSX^F share identical amino-terminal regions comprising 397 amino acids but differ in sex-specific carboxyl-terminal regions of 152 (DSX^M) or 30 (DSX^F) amino acids. They contain the same DNA-binding domain (40) and exhibit identical DNA-binding specificities in vitro (14, 30, 40). Existing data suggest that the DSX proteins bind to the same target sequences in vivo but exert different effects on transcription when bound, probably as a result of protein-protein interactions mediated by the sex-specific carboxyl-terminal regions (reviewed in 80).

DSX represents only one way in which alternative splicing generates transcription factor isoforms with different functional properties. Sequence elements required for DNA binding, dimerization, cooperativity, transcriptional activation, subcellular localization, and ligand binding are frequently separable and can be substituted, deleted, or modified independently by alternative splicing. The diversity of known strategies and the developmental consequences of this regulation have been reviewed recently (80). Among well-studied cases, alternative splicing modulates DNA-binding affinity and specificity, produces transcriptional effectors and antagonists from the same gene, and generates isoforms with different capacities for protein interactions that control activity or contribute to combinatorial regulation of gene expression. Documented consequences span the range from profound differences in function that control major developmental decisions (as in *Drosophila* sex determination) to subtle

but selectively advantageous fine-tuning of function during development [as with the six isoforms encoded by the homeotic gene *Ultrabithorax* (17, 127)].

Effects on Diverse Proteins and Developmental Processes

Modular organization is not unique to transcription factors. Accordingly, alternative splicing generates functionally diverse isoforms for virtually every type of protein involved in metazoan development, cell function, and physiology. Other proteins of particular interest from the point of view of developmental regulation and morphogenesis are receptors for signaling molecules [e.g. fibroblast growth factor receptor, alpha and beta subunits of integrins (43, 94, 117)], extracellular matrix and cell adhesion molecules [e.g. fibronectin, CD44 (73, 116)], cytoskeletal proteins [e.g. BPAG1, tropomyosins (78, 145)] and RNA-binding proteins [e.g. hnRNP A1 and SR proteins (48, 87, 114)], for which alternative splicing can generate functional diversity with the potential to influence cell fate, patterning, or the construction of tissues and organs. Control of programmed cell death includes the use of alternative splicing to generate apoptosis-promoting and apoptosis-suppressing isoforms of BCL-X in mammals (16) and CED-4 in *C. elegans* (115), as well as soluble isoforms of the mammalian Fas/APO-1/CD95 death signal receptor that antagonize activation by the Fas ligand (27). Alternative splicing also influences cell function by generating structural diversity among products of terminal differentiation; prominent examples in both vertebrates and invertebrates are tissue-specific isoforms of structural and regulatory muscle proteins (8; reviewed in 112) and of central nervous system components, including ion channels (e.g. 79) and receptors (e.g. 37, 140).

Alternative Splicing and Human Health

Some human genetic diseases and developmental defects have been correlated with disruptions of alternative splicing control in particular genes. For example, a form of aniridia is associated with a C for T substitution at position −3 of the acceptor splice site for alternative exon 5a in the *Pax-6* gene; alternative splicing of this exon changes the DNA sequence recognition properties of the protein (39). Mutations that disrupt the ratios of alternatively spliced protein isoforms from the Wilm's tumor locus *WT1* have been implicated as a cause of abnormal urogenital development in Denys-Drash syndrome (20). In other situations, the function of a *trans*-acting splicing regulatory factor may be affected, with repercussions on processing of one or more RNAs. The *trans*-dominant effect of CUG trinucleotide expansion in the myotonic dystrophy protein kinase RNA has been attributed recently to increased activity of CUG-binding protein (CUG-BP), which is required for inclusion of a stage-specific exon in cardiac troponin T (96). Disfunction of a *trans*-acting factor may also underlie aberrant

splicing of the glutamate transporter EAAT2 RNA in patients with sporadic amyotrophic lateral sclerosis (52, 77). Many instances of altered proportions of alternatively spliced products have been described for diverse genes in human tumors, although the cause and effect relationships between the splicing defects and the tumor phenotypes are not understood. Finally, a potential relationship between alternative splicing control and liver regeneration has been revealed by the finding that levels of a putative alternative splicing regulator, SRp40, become elevated after liver damage in animal models and after insulin stimulation of cultured hepatocytes (34, 36). This increase in SRp40 expression appears to cause a change in alternative splicing of fibronectin, which is postulated to aid tissue remodeling during regeneration (36).

FOUNDATIONS OF SPLICE SITE CHOICE

Pre-mRNA splicing takes place on the spliceosome, a dynamic complex of small nuclear ribonucleoprotein particles (snRNPs) and extrinsic (nonsnRNP) protein factors assembled on the juxtaposed 5′ and 3′ splice sites. Intron excision proceeds in two successive transesterification reactions whereby the upstream exon is cleaved from the intron and ligated to the downstream exon. The RNA components of snRNPs align the pre-mRNA splice sites and probably mediate catalysis, but many proteins perform auxiliary functions in the recognition and pairing of splice sites and in structural reorganizations during spliceosome assembly and catalysis (reviewed in 74, 99, 124). Alternative splicing patterns result from the use of alternative 5′ splice sites, alternative 3′ splice sites, optional exons, mutually exclusive exons, and retained introns. Except for intron retention, most alternative splicing decisions involve competition among potential splice sites; thus, splicing patterns can be controlled by any mechanism that alters the relative rates of splice site recognition, and superficially similar patterns can involve fundamentally different pathways. Tissue-specific internal exons, for example, may be excluded from the mRNA by exon skipping, whereby the splice sites for the optional exon are ignored and the upstream and downstream exons are ligated to each other directly. This is the case for neuron-specific internal exons in RNAs for the $\gamma 2$ subunit of the $GABA_A$ receptor and for c-*src* (see below). In other cases, the optional exon is excluded by removing it from partially spliced intermediates after ligation to the upstream exon, using a 5′ splice site regenerated at the exon-exon boundary. This strategy is used for neuron-specific exclusion of internal exons in the *Ultrabithorax* RNAs of *Drosophila* (AR Hatton, V Subramaniam, AJ Lopez, submitted).

Basic Splice Site Recognition

Spliceosome assembly begins with recruitment of U1 snRNP to the 5′ splice site and of U2 snRNP to the branchpoint of the 3′ splice site. The 5′ splice site

consensus sequence is complementary to the 5′ end of U1 snRNA; this contributes to recognition of the 5′ splice site via base-pairing, but extrinsic factors can also contribute to the stability of the interaction (reviewed in 2, 99). These extrinsic factors include members of the SR protein family, a group of at least eight highly conserved RNA-binding proteins characterized by one or more amino-proximal RRM-type RNA binding motifs and a distinctive carboxyl-terminal domain rich in serine-arginine dipeptides (the RS domain) that is involved in protein-protein interactions (reviewed in 44, 86). Targeting of U2 snRNP to the branch site requires the extrinsic factor U2AF (U2 snRNP auxiliary factor), which binds specifically to the intron polypyrimidine tract located between the branchpoint and the 3′ splice site (105, 123, 146). Human U2AF is a heterodimer of 65- and 35-kDa subunits (50- and 38-kDa in *Drosophila*, respectively) (69, 102, 146, 147). The large subunit contains three RRM motifs responsible for binding to the polypyrimidine tract and an amino-proximal domain rich in serine and arginine (an RS domain), which has been proposed to facilitate annealing of U2 snRNP to the branch site by shielding the negative charge of the RNA phosphodiester backbone (136, 146). In contrast, the RS domain on the small subunit is proposed to mediate interactions with SR proteins that stabilize binding of U2AF to the RNA or form a bridge to U1 snRNP at the 5′ splice site (72, 143, 149; but see 103).

Enhancement of Splice Site Recognition

Recognition of a particular splice site can be ensured by a strong match to the consensus sequence or by the assistance of *cis*-acting elements and *trans*-acting factors. Across short introns, U1 snRNP at the 5′ splice site and U2AF at the polypyrimidine tract can interact through extrinsic factors to stabilize binding and juxtapose the splice sites in a commitment complex (reviewed in 14, 99). When internal exons are flanked by longer introns, interactions can occur across the exon to stabilize the binding of U2AF and U1 snRNP (reviewed in 9, 14). These exon bridging interactions are limited to exons between 50 and 500 nt long (12, 22, 126); below this range steric interference may be a problem, whereas at the upper end the distance is too large for effective interaction. When the distances between splice sites are not propitious, or when a splice site is inherently weak, *cis*-acting enhancers can help recruit the essential splicing factors (14, 22). Splicing enhancers can be position-dependent; most are located close to the splice site that they activate, and changing their location can alter their dependence on particular *trans*-acting factors (134), determine whether they activate 5′ or 3′ splice sites (62), or even transform them into negative regulatory elements (71).

Purine-rich splicing enhancers have been identified in many regulated and constitutively spliced pre-mRNAs. Most are located in exons and in general they activate splicing of the upstream intron by promoting use of a weak 3′

splice site (75, 142, 144). One class contains contiguous repeats of the motif GARGAR (R = purine). Biochemical evidence indicates that these elements function as binding sites for SR proteins (reviewed in 44, 86). The ability to bind correlates with the ability of the enhancers to activate splicing of the upstream intron (e.g. 98, 128), and in vitro splicing of an exon with weak splicing signals can be stimulated by incorporating a high-affinity SR protein binding site within the exon (129, 130). Exonic enhancers can promote the interaction between U2 snRNP and the upstream branch site (75), an effect that is probably mediated indirectly by SR protein-dependent stimulation of U2AF binding to the adjacent polypyrimidine tract (141, 149). Other enhancers are unrelated in sequence to the purine-rich exonic enhancers. Most are pyrimidine-rich and reside within the intron, close to the 5′ splice site (13, 32, 47, 106). They are required for inclusion of the adjacent upstream exon, presumably by helping to recruit U1 snRNP to the 5′ splice site. Non-SR proteins are essential for activity of an intronic enhancer in c-*src* (88, 89); although ASF/SF2 is not required, possible involvement of other SR proteins is not excluded. SR proteins play an important role with at least one intronic enhancer, the S4 element of chicken β-tropomyosin (47).

Bridging Networks

Various SR proteins can interact physically with $hU2AF^{35}$, leading to the hypothesis that SR proteins bound to exonic enhancers activate the 3′ splice site by facilitating U2AF binding (143, 149). The function of SR protein–dependent enhancers may thus be related to mechanisms for interaction between splice sites (45). SR proteins can interact simultaneously with $hU2AF^{35}$ and the 70-kDa protein of U1 snRNP (U1–70K protein, which also contains an RS domain) (143), suggesting that a network of protein-protein interactions mediated by RS domains spans the intron to pair 5′ and 3′ splice sites. SR protein–mediated interactions between $hU2AF^{35}$ and U1–70K across an exon would account for the ability of a strong downstream 5′ splice site to promote splicing at the upstream 3′ splice site (64) and to substitute for an exonic enhancer in *cis*- and *trans*-splicing reactions (21, 28, 35, 75). Recent evidence suggests another bridging mechanism across the intron involving BBP (branchpoint bridging protein), which is encoded by *MSL5* in *Saccharomyces cerevisiae* and is orthologous to mammalian splicing factor SF1 (renamed mBBP) (1, 10).

Frequently, regulated sites exhibit poor matches to the consensus and are inherently weak or their recognition is hampered by secondary structure or an exon size that is incompatible with exon bridging interactions. These features can contribute to positive regulation by rendering splice sites dependent on enhancement mediated by *trans*-acting factors whose amount or activity varies during development. Conversely, an inherently weak site may only compete

effectively with a stronger site when the latter is restrained by negative regulators. The following sections examine ways in which the basic mechanisms of splice site recognition and enhancement are modified (or disrupted) for developmental regulation.

POSITIVE REGULATION BY ENHANCERS AND SPECIFIC FACTORS

The doublesex Paradigm: Positive Regulation of a 3′ Splice Site

Activation of the female-specific 3′ splice site of *dsx*, which has a weak polypyrimidine tract, requires the regulatory factors TRA and TRA2 (Figure 1) together with sequences within the female-specific exon (the *dsx* enhancer) that include binding sites for TRA/TRA2 heterodimers (59, 66, 107, 132). TRA and TRA2 resemble SR proteins: TRA contains an RS domain, whereas TRA2 contains amino- and carboxy-terminal RS domains and an internal RRM (5, 15, 51). Splicing assays in HeLa cell extracts have shown that TRA and TRA2 can cooperate with several mammalian SR proteins to form a complex on the *dsx* enhancer (82, 83, 133, 134); formation of this complex leads to activation of the 3′ splice site by facilitating U2AF binding to the suboptimal polypyrimidine tract (133, 149). The *dsx* enhancer consists of six 13-nucleotide repeat sequences (the "*tra*/*tra2* repeat elements") and a purine-rich element (PRE). Detailed analyses of binding interactions show that in HeLa extracts TRA2 recognizes the UCAACA motif in the 3′ half of each repeat and together with TRA stabilizes binding of the SR protein 9G8, which can be crosslinked to the 5′ end of the repeat; TRA/TRA2 also binds within the PRE in a complex with the SR protein ASF/SF2 (83). Analogous interactions are seen when RNA containing the *dsx* repeat element is incubated with extracts from *Drosophila* Kc cells in the presence of exogenous TRA/TRA2, but the *Drosophila* SR protein RBP1 replaces mammalian 9G8 and SRp30 replaces ASF/SF2 (83).

Two-hybrid protein interaction assays have shown that $hU2AF^{35}$, but not $hU2AF^{65}$, can interact with ASF/SF2, TRA and TRA2 (143). $hU2AF^{35}$ is required for enhancer-dependent splicing in mammalian cell-free extracts, where protein-RNA interaction studies have also shown that $hU2AF^{35}$ mediates interactions between $hU2AF^{65}$ and proteins bound to the enhancer (149). These observations have suggested that the *dsx* enhancer complex activates the 3′ splice site by recruiting U2AF via RS domain–mediated interactions with the small subunit (83, 143, 149). The *dsx* female-specific enhancer is located unusually far from the 3′ splice site compared to other characterized exonic enhancers; when this distance is reduced enhancer function becomes independent of TRA

and TRA2 in HeLa cell splicing extracts (134). This suggests that the suboptimal polypyrimidine tract and enhancer location collaborate to render *dsx* splicing susceptible to regulation by the sex-specific factors that stabilize the interaction of SR proteins with the pre-mRNA.

Genetic and molecular studies in *Drosophila* are consistent with some of these conclusions but also paint a more complex picture. Two independent deficiencies that delete *Rbp1* can produce a dominant intersexual phenotype in a genetic background heterozygous for *tra* and *tra-2* (113); this is consistent with a biologically significant role of RBP1 in regulation of *dsx* splicing, but specific mutations in *Rbp1* alone have not been reported. Also consistent with a role for RBP1 is its ability to enhance selection of the female-specific 3′ splice site when overexpressed in cultured *Drosophila* SL2 cells (60). This activation requires the *dsx* repeat region, and a different *Drosophila* SR protein (B52) cannot substitute for RBP1. Stimulation by RBP1 occurs in the absence of TRA, indicating that at high concentrations RBP1 can interact stably with the *dsx* repeat region to form an active enhancer complex, presumably in cooperation with endogenous TRA2 and SRp30. Indeed, the RS domains of RBP1 and TRA2 interact in the yeast two-hybrid assay, and the RS domain of RBP1 is necessary for regulation of *dsx* splicing in the SL2 cell cotransfection assay (61). However, RBP1 also binds to two sites within the polypyrimidine tract that are necessary for maximum stimulation of female-specific splicing in the cotransfection assay (60). Whereas standard polypymyridine tracts exhibit no consensus, the precise sequence of this unusually purine-rich tract is highly conserved, suggesting that RBP1 plays a relevant role in regulation by binding to these sites.

How do RBP1 at the polypyrimidine tract and the *dsx* enhancer complex activate the female-specific 3′ splice site in flies? As suggested by the in vitro splicing and protein interaction assays described above, they may recruit U2AF by stabilizing or bypassing its binding to the suboptimal polypyrimidine tract (Figure 2*a*). Since hU2AF^{35} can interact via the RS domain with TRA and TRA2 (143), recruitment might occur by direct interactions between dU2AF^{38} and either of these proteins; the role of RBP1 and SRp30 would be to form a network of protein-protein and protein-RNA interactions that generate specificity and bring the key player into correct position. Since hU2AF^{35} can also interact with some mammalian SR proteins (143), it is possible that RBP1 or SRp30 interacts directly with dU2AF^{38}. It is surprising, however, that homozygotes for a hypomorphic allele that strongly reduces expression of *dU2AF38* mRNA do not exhibit defects in sex determination even when simultaneously heterozygous for both *tra* and *tra-2* mutations (102). Despite the lack of an effect on *dsx* splicing, this allele of *dU2AF38* is semilethal and causes other developmental abnormalities. Intersexuality in a doubly heterozygous *tra*, *tra2* background has otherwise been a sensitive indicator for mutations with

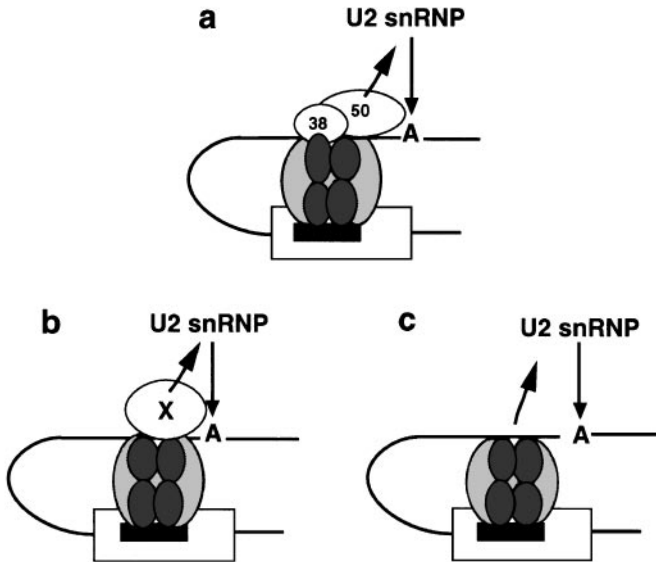

Figure 2 Alternative hypotheses for activation of the female-specific 3′ splice site in *dsx* RNA. (*a*) RBP1 at the polypyrimidine tract cooperates with the enhancer complex to stabilize binding of U2AF, which recruits U2 snRNP to the branch point. (*b*) RBP1 at the polypyrimidine tract cooperates with the enhancer complex to stabilize binding of an unknown factor (X) that recruits U2 snRNP. (*c*) RBP1 substitutes for U2AF to recruit U2 snRNP; the enhancer complex helps to stabilize RBP1 binding at the polypyrimidine tract. Diagrams do not represent precise stoichiometries or contacts. *A*: branchpoint adenosine; *dark stippled ovals*: RBP1; *light stippled ovals*: TRA and TRA2; *black rectangle*: *dsx* enhancer. "38" and "50" denote the corresponding subunits of dU2AF.

dose-dependent effects on *dsx* alternative splicing, including the haplo-insufficient effect of deficiencies that delete *Rbp1*. Genetic experiments also show that the RS domain of $dU2AF^{38}$ is not required for sex-specific splicing of *dsx* RNA in vivo (103). Nevertheless, $dU2AF^{38}$ is essential for viability, as are $dU2AF^{50}$ and the association between these two subunits (69, 103, 104).

An alternative hypothesis is that RBP1 and the enhancer complex help to stabilize the interaction of an unknown factor with the branchpoint region, and this factor helps to recruit U2 snRNP (Figure 2*b*). This hypothesis is intriguing, given that the $U2AF^{65}$-like protein Mud2p contributes to U2 snRNP recruitment in *S. cerevisiae* but is not essential for this process, which instead hinges critically on binding of BBP to the branchpoint (1, 10). As noted above, BBP is orthologous to mammalian SF1/mBBP, which also interacts with the branchpoint and is required for recruitment of U2 snRNP into the prespliceosomal complex (10).

A more radical model proposes that an RBP1 dimer bound within the polypyrimidine tract substitutes for U2AF to recruit U2 snRNP to the female-specific branch site (60) (Figure 2*c*). This possibility is supported by the demonstration that mammalian SR protein SC35 can substitute for $hU2AF^{65}$ to splice some pre-mRNA substrates (85). Other in vitro experiments have shown that the positively charged RS domain of $hU2AF^{65}$ interacts with the pre-mRNA branch site and facilitates base-pairing with U2 snRNA by shielding the negative charge of the phosphodiester backbone (136). The RS domains of SR proteins might be able to perform similar functions if targeted appropriately; indeed, although different in size, distribution of RS dipeptides, and content of other amino acids, the RS domain of ASF/SF2 can substitute for that of $hU2AF^{65}$ to promote annealing of U2 snRNA to the branchpoint (136).

fruitless: *Dual Potency of a Regulated Enhancer*

TRA and TRA2 switch the *fru* splicing pattern (Figure 1) by activating the female-specific 5′ splice site through an exonic splicing enhancer located 38 nt upstream (62). The *fru* enhancer consists of three copies of a repeat element like that in the *dsx* enhancer; when placed at a position similar to that of the *dsx* enhancer, the *fru* enhancer can promote activation by TRA/TRA2 of the female-specific *dsx* 3′ splice site (62). This dual potency implies that similar factors and molecular mechanisms can positively regulate both 5′ and 3′ splice sites, the target being specified by the location of the enhancer; it is also consistent with the conclusion that the specific regulator TRA/TRA2 functions by stabilizing a complex with general factors, such as SR proteins, that can interact with both the 5′ and 3′ splice site to promote spliceosome assembly. In agreement with this, RBP1 can also stimulate female-specific splicing of *fru* RNA in SL2 cell cotransfection assays (62).

Mammalian Homologues of TRA2 Implicated in Enhancer-Dependent Splicing

Several mammalian homologues of TRA2 have been identified. At least one of the human proteins can substitute partially for *Drosophila* TRA2 to regulate female sexual differentiation and alternative splicing of *dsx* when expressed in flies (31). Two human TRA2 proteins bind preferentially to RNA sequences containing GAA repeats like those in purine-rich enhancers, and although neither appears to stimulate constitutive splicing in mammalian extracts, both can activate enhancer-dependent splicing in a sequence-specific manner (131). Therefore, TRA2 homologues may function like their *Drosophila* counterpart to help regulate certain types of alternative splicing decisions in mammalian cells.

NEGATIVE REGULATION

In principle, negative regulation could be achieved simply by providing a binding site for a factor that blocks access to a 5′ or 3′ splice site, but most cases seem more complicated. The regulated splice sites must be used in at least some cell types or stages, and this may constrain the degree to which the sequence and organization of splice sites can vary without requiring compensatory regulatory mechanisms to ensure activation when appropriate. Binding to nontarget splice sites must be avoided as well as binding to authentic targets in the wrong developmental context. This may require cooperative or antagonistic interactions, particularly if the repressors are not highly sequence-specific or developmentally restricted. Many examples of inhibitory *cis*-acting elements have been described, showing that splice sites can be blocked by secondary structure, by specific or general factors binding to regulatory elements within introns or exons, or by the splicing machinery itself binding at regulatory elements near the repressed sites (reviewed in 2, 6, 23, 26, 139).

Blocking Access to the Polypyrimidine Tract: SXL and PTB

SXL controls the use of alternative 3′ splice sites in *tra* pre-mRNA (Figure 1). Regulation has been explained by SXL binding to the polypyrimidine tract of the upstream site in females, thus blocking access by U2AF and favoring the use of a weaker downstream 3′ splice site. In the first place, analyses of *cis*-acting elements point to the upstream 3′ splice site as mediator of the SXL effect (67, 122). SXL protein binds to a poly(U) run in the polypyrimidine tract of the regulated 3′ splice site (67, 111, 121, 135), and in vitro experiments with recombinant proteins show that binding of SXL precludes binding by U2AF (135). If the RS domain of $U2AF^{65}$ is attached to SXL, however, SXL is transformed into an activator of the upstream 3′ splice site and substitutes functionally for U2AF in HeLa extracts (135). Fusing the RS domain of $U2AF^{65}$ to SXL also relieves the inhibitory effect of SXL in transgenic flies (55). These results argue that SXL acts as a repressor because it displaces U2AF and lacks the RS effector domain.

Molecular and biochemical experiments have implicated another pyrimidine tract binding protein (PTB/hnRNP I) in negative regulation of 3′ splice site choice in vertebrates (reviewed in 2, 6, 26). The effect in several cases has also been attributed to reduced access of U2AF to the polypyrimidine tract because binding competition can be demonstrated in vitro and preincubation with recombinant U2AF counteracts the PTB-dependent inhibition (26, 76, 121). However, it is not clear whether PTB functions in vivo as an alternative splicing factor or as a constitutive component of repressor complexes whose assembly or activity is dictated by more specific regulators.

SOURCES OF SPECIFICITY Although SXL, PTB, and U2AF bind pyrimidine-rich sequences, they have distinct sequence preferences. $U2AF^{65}$ exhibits a broad specificity for degenerate U-rich sequences, which is consistent with the need to recognize diverse polypyrimidine tracts in many 3′ splice sites (121). SXL and PTB prefer more restricted and distinct sets of pyrimidine-rich sequences (95, 121). However, these preferences seem insufficient to account for discrimination between regulated and unregulated 3′ splice sites. PTB is an abundant hnRNP protein, and SXL has been observed to bind with equal affinity to regulated and unregulated polypyrimidine tracts in vitro (70). Another problem is the ubiquitous expression of PTB, given that it represses sites used for splicing in some cell types and not others.

The target selectivity of the pyrimidine-binding regulators may be enhanced by cooperative binding mechanisms (138) and by competition with other factors and signals that promote spliceosome assembly. In vitro repression of the 3′ splice site for the neural-specific exon of the $GABA_A$ $\gamma 2$ receptor subunit involves recognition by PTB of a repressor array consisting of three polypyrimidine blocks both upstream and downstream of the branch site (6) and not just competition for binding to the canonical polypyrimidine tract. These negative regulatory elements are balanced against positively acting elements located within the exon and between the canonical polypyrimidine tract and the intron/exon junction (6, 148). In α-tropomyosin, PTB also represses 3′ splice site use by binding to multiple widely spaced pyrimidine-rich elements (including the polypyrimidine tract); in this case, a low constitutive level of repression seems to be enhanced specifically in smooth muscle cells by the action of unknown factors without obvious changes in the level of PTB (50, 76, 95). A detail whose possible significance is not understood is the existence of multiple isoforms of PTB, including a possible brain-specific version (6).

Regulation of *tra* also requires more than just SXL competing with U2AF for binding to the polypyrimidine tract. The products of at least two other genes—*virilizer* (*vir*) and *female-lethal-2-d* (*fl(2)d*)—are required for female-specific splicing of *tra* pre-mRNAs (54, 63). These genes are essential for viability in both sexes, indicating that they do not function exclusively in sex determination, but particular roles in assisting SXL function are revealed by hypomorphic alleles that exhibit female-specific lethality or sterility and by genetic interactions with *Sxl* mutations (53, 63). Genetic mosaics show that XX somatic cells lacking *vir* function differentiate with male characteristics. In addition, the vir^{2f} allele is lethal for XX but not for XY animals. This lethality is due to inappropriate activation of male dosage compensation in females and reflects an additional role of *vir* in regulation of *Sxl* splicing (see below); the mutant XX flies can be rescued by constitutive expression of SXL or by mutations in the male-specific lethal (*msl*) genes, which are required

for hyperactivation of transcription from the single X in males. The rescued XX animals develop as strongly masculinized intersexes or pseudomales and express *tra* mRNA that is spliced in the male-specific pattern, showing that constitutively expressed SXL is not sufficient to switch the splicing pattern of *tra*. *Sxl* mRNA itself is spliced in the male-specific pattern in XX mutant flies rescued by *msl* mutations. Similar results are obtained in rescue experiments for *fl(2)d*. The precise functions of the *vir* and *fl(2)d* products are not known. Their products may act as cofactors to assist binding of SXL to the target polypyrimidine tract. Alternatively, they might help to activate the weak female-specific 3′ splice site so that it can compete effectively with the upstream site repressed by SXL. *vir* seems to play a dose-dependent role in at least one alternative splicing decision that is not controlled by SXL, since the inclusion of a tissue-specific exon of the homeotic gene *Ultrabithorax* is reduced in *vir* heterozygotes of both sexes (A Hatton, J Burnette & AJ Lopez, unpublished results).

Coordinated Repression of 5′ and 3′ Splice Sites

SXL and PTB can also exert negative effects on 5′ splice sites, and coordinated repression of both splice sites by pyrimidine-binding proteins is emerging as a common theme in several cases of exon skipping. Coordination may be required to ensure skipping and to prevent unproductive splicing of either the upstream or downstream intron. Several mechanisms are suggested by data discussed below (Figure 3).

AUTOREGULATION BY SXL SXL maintains its own expression in female flies by excluding from its message an exon that aborts translation (Figure 1). Although the polypyrimidine tract for this exon contains a long poly(U) run similar to that which mediates repression of 3′ splice site use by SXL in *tra*, this sequence is dispensable for regulation of *Sxl* splicing in vivo. Instead, SXL-induced exon skipping requires multiple SXL binding elements both upstream of the 3′ splice site and downstream of the 5′ splice site (65, 108). The downstream elements appear to play a more important role, hinting that regulation hinges critically on suppressing the activity of the 5′ splice site (65). The upstream and downstream elements are located more than 200 nt from the splice sites, suggesting that the SXL protein does not function in autoregulation by directly competing with or blocking the binding of U2AF or U1 snRNP.

A clue to the mechanism of SXL autoregulation comes from analysis of the gene *sans-fille* (*snf*), named for the synergistic female-lethal phenotype caused by reduction of *snf* function in the mother combined with partial reduction of SXL expression in the zygote (4, 92, 125). These female-lethal interactions are due to failure of SXL autoregulation (4). Similar interactions involving

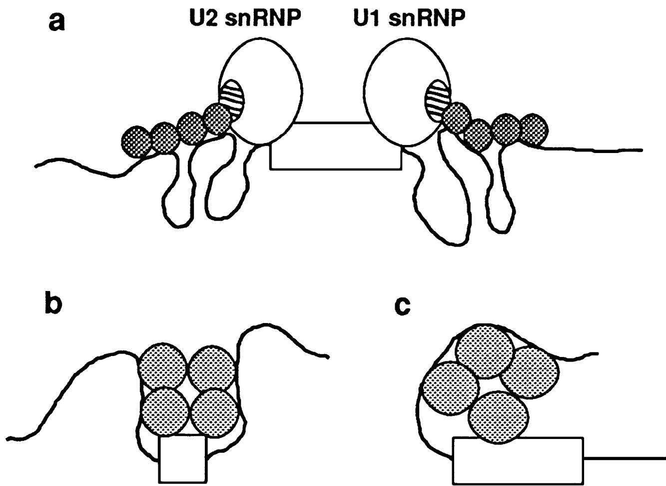

Figure 3 Mechanisms to coordinate repression of 3′ and 5′ splice sites for exon skipping. (*a*) Independent repression of the 3′ and 5′ splice sites by the same developmentally specific factor binding cooperatively to elements near each splice site. The diagram represents a specific model for exclusion of the male exon in *Sxl* RNA (adapted from Reference 33). SXL (*filled circles*) binds cooperatively and interacts with SNF (*striped ovals*) on U2 and U1 snRNP, thereby inactivating the snRNPs or blocking interactions required for spliceosome assembly. (*b*) The repressor(s) bind cooperatively to upstream and downstream sites and also interact across the exon; this interaction may stabilize binding of the repressor(s) or may itself hinder access to the splice sites through formation of a large complex. Repression of exon N1 in c-*src* may involve a mechanism like this mediated by PTB. (*c*) The repressor(s) bind cooperatively to sites in or around the polypyrimidine tract, blocking access by U2AF, and also interact with repressor(s) bound at the exon to form a large complex that hinders access of U1 snRNP to the 5′ splice site. Exclusion of a neural exon in the $GABA_A$ $\gamma 2$ RNA may involve a mechanism like this mediated by PTB. In all cases, factors other than SXL or PTB may also form part of the repressor complexes.

strong viable *snf* alleles also demonstrate a zygotic requirement for *snf* function in this process (92, 109, 125). Furthermore, certain *snf* alleles can suppress the male-specific lethality of the Sxl^{M1} allele, which causes female-specific splicing of *Sxl* in males (109, 125). The first allele, snf^{1621}, was isolated as a female-sterile mutation; subsequent characterization revealed inappropriate exclusion of the male-specific exon of *Sxl* in mutant female germ cells (19, 93). Accordingly, this sterility can be rescued by *Sxl* mutations that cause constitutive exclusion of the male exon from *Sxl* RNA (11, 109, 125). These phenomena were established with viable alleles that might cause effects unrelated to normal *snf* function, but three results argue for involvement of the wild-type product in SXL autoregulation (110). First, a null allele is a nonsex-specific lethal but can be shown to interfere with female sex determination in genetic mosaics where small groups of cells are homozygous. Second, *snf* is dosage sensitive

in its maternal-effect female-lethal interaction with *Sxl*; the null allele itself exhibits a weak dominant effect in this assay, and of the extant viable alleles, one behaves as a hypomorph and the other two behave as antimorphs that antagonize the function of the wild-type product. Third, both the antimorphic and hypomorphic alleles can suppress the male-specific lethality caused by initiating the SXL positive feedback loop with low-level expression of a female *Sxl* cDNA transgene.

snf encodes an RNA-binding protein that is homologous and functionally equivalent to the vertebrate proteins U1A and U2B″ (42, 58, 97). Like U1A, SNF binds U1 snRNA and is incorporated into U1 snRNP; like U2B″, SNF binds with U2A′ to U2 snRNA and is incorporated into U2 snRNP. The antimorphic *snf* alleles are missense mutations that affect residues predicted to be important for RNA binding based on homology to vertebrate U1A (42, 110). The hypomorphic allele encodes a truncated protein that lacks the second of two RNA-binding motifs (RRM-2). Consistent with the prediction that these mutant proteins have reduced RNA-binding activity and are not incorporated efficiently into snRNPs, high levels of the antimorphic proteins are found in the cytoplasm as well as in the nucleus, whereas the wild-type protein is found predominantly in the nucleus (110). The antimorphic behavior could be explained if SNF normally mediates an interaction between SXL and the U1 and U2 snRNPs that is required to repress splicing of the male exon; the free mutant proteins would interfere with regulation by competing with snRNP-associated SNF for binding to SXL (110). This hypothesis is supported by the demonstration that bacterially expressed SXL and SNF bind to each other in vitro (33). In *Drosophila*, SXL and SNF associate in an RNA-dependent fashion as judged by coimmunoprecipitation; when these complexes are stabilized by protein crosslinking before immunoprecipitation, they are also found to contain U1 and U2 snRNAs and other protein components of U1 and U2 snRNP, but not hnRNP proteins 40 and 48 (33). The protein encoded by the snf^{1621} allele also coimmunoprecipitates with SXL, as required by the hypothesis to explain the antimorphic behavior of this allele.

The available information can be incorporated into the following model (33) (Figure 3*a*). SXL binds cooperatively (111, 138) to the poly(U) tracts upstream and downstream of the male exon at the same time that U1 and U2 snRNPs are recruited to the 5′ and 3′ splice sites. SXL proteins bound at the poly(U) tracts interact via SNF with U1 and U2 snRNP bound at the splice sites of the male exon and prevent them from participating in spliceosome assembly across the downstream and upstream introns, respectively. In consequence, a spliceosome would assemble instead between the upstream and downstream exons, causing the male exon to be skipped. SXL might prevent spliceosome assembly by physical interference or by induction of a conformational change that inactivates the snRNPs; the latter is suggested by the finding that snRNPs

coimmunoprecipitated by SXL antibody are destabilized (33). Regulation of *Sxl* splicing also requires the function of *vir* and *fl(2)d* (53, 63; see above), but their precise role is not known.

The mechanism of *Sxl* autoregulation appears more complex than that for regulation of *tra*. Unlike TRA, accidental production of a small amount of SXL in males can have lethal consequences by initiating the positive autoregulatory loop and disrupting dosage compensation. Cooperative binding to multiple sites and formation of active inhibitory complexes at both ends of the exon may steepen the response of the system, making splicing of *Sxl* insensitive to slight fluctuations in the amount of SXL protein (138). A tighter mechanism may also be required for highly consistent skipping of the *Sxl* male exon, which resides within a 4-kb intron, whereas *tra* regulation involves suppression of a 3′ splice site that is not far from its competitor in a small intron.

PTB-MEDIATED EXON SKIPPING Simultaneous action of PTB at the 5′ and 3′ splice sites may be at play in vertebrate α-tropomyosin, where PTB-binding sites that function as repressor elements are located within the polypyrimidine tract and downstream of the 5′ splice site of exon 3 (50, 95). Communication across the exon is suggested by results with rat c-*src* (25, 26). Inclusion of internal exon N1 is restricted to neuronal cells by a combination of positive and negative regulatory mechanisms. Repression affects both splice sites and is mediated in vitro by CUCUCU elements within the polypyrimidine tract and downstream of the 5′ splice site. PTB binds to these elements and is required for repression. In vitro experiments using 2-exon splicing substrates reveal that the 5′ splice site can be derepressed by mutations in the upstream regulatory elements and the 3′ splice site can be derepressed by mutations in the downstream elements. This suggests that exon skipping requires regulatory factors bound at both ends that perhaps interact by forming a bridge across the exon (25). Whether repression occurs by blocking access of splicing factors or by engaging them in inhibitory interactions is not known. Yet another variation is seen in $GABA_A$ $\gamma 2$, where PTB-binding elements upstream of a neural-specific exon repress the 3′ splice and also the 5′ splice site for the downstream intron (6). The effect on the 5′ splice site also involves a PTB-binding element within the exon. PTB may help to form a large complex involving all elements that represses the 3′ and 5′ splice sites simultaneously. The regulatory complex has been proposed to interfere indirectly with activation of the 5′ splice site by hindering access of U1 snRNP (6).

Diversion or Cooption of Essential Splicing Factors

Transposition of the P-element in *Drosophila* is restricted to the germline because the third intron (IVS3) of the transcript is not spliced in somatic cells; consequently, a repressor of transposition is made instead of transposase, which

is produced in germ cells. Genetic and biochemical experiments have demonstrated that IVS3 splicing is inhibited in the soma and they have identified a *cis*-acting regulatory sequence located within the upstream exon (reviewed in 2, 3). In *Drosophila* somatic cell extracts, a multiprotein complex forms on the exonic repressor element and prevents U1 snRNP from binding to the 5′ splice site of IVS3; instead, U1 snRNP binds to the more upstream (F1) of two pseudo-5′ splice sites located within the repressor element (118, 119). Two of at least four components in the complex have been identified. One of these accounts for the somatic specificity of repression, since it is expressed at high levels in the soma and not in germ cells. This factor, designated PSI (P-element somatic inhibitor), contains 3 KH RNA-binding domains and binds specifically to the regulatory element. Antibodies against PSI derepress IVS3 splicing in somatic extracts and an anti-PSI ribozyme derepresses IVS3 splicing in somatic cells of *Drosophila*, while ectopic expression of PSI in the germline represses IVS3 splicing (3, 119, 120). The other identified factor is HRP48, a ubiquitously expressed protein related to hnRNP A1 that makes contacts in the F2 pseudo-5′ splice site (119). Analysis of *hrp48* mutants with partial loss of function confirms that HRP48 is required for effective repression of IVS3 splicing (56).

What, if any, is the role of U1 snRNP in the repressor complex? It may help to stabilize the complex, whose principal function may be to block access to the authentic 5′ splice site. U1 snRNP may itself hinder access to this splice site. Either case would represent cooption of U1 snRNP to serve a regulatory role. Recruitment of U1 snRNP to regulatory elements also occurs in other systems. U1 snRNP associates with some vertebrate exonic splicing enhancers (123, 142) and with a 5′ splice site consensus sequence within an intronic element that stimulates a regulated cleavage/polyadenylation site in the pre-mRNA for calcitonin/calcitonin gene-related peptide (CT/CGRP) (81). Although the functional significance is not known in the former cases, enhancement of in vitro polyadenylation in the CT/CGRP RNA requires U1 RNA. The involvement of PSI in recruiting U1 snRNP to the F1 pseudo-splice site may have a parallel in positive regulation of c-*src* exon N1 inclusion: KH domain splicing regulatory protein (KSRP), which plays a key role in activation of N1 splicing through a downstream element, has significant homology to PSI in the KH domains and in a proline-rich carboxy-terminal region and may function to recruit U1 snRNP to the 5′ splice site (89). SF1/mBBP and yeast BBP, which are required for prespliceosomal A complex formation as discussed above, also contain a KH domain and a proline-rich carboxy-terminal domain (1, 10). In the one case of developmentally regulated splicing known in yeast, MER1 (also a KH domain protein) activates splicing of *MER2* RNA by stimulating the use of a weak 5′ splice site (38, 90). Thus, PSI, KSRP, SF1, and MER1 may represent a class of proteins with shared capacity to recruit U1 snRNP to suboptimal splice sites through interactions with specific RNA sequences and other proteins. The

consequence of recruitment is not necessarily activation of splicing, however, and may be determined by the location of the site and additional factors in the complex.

DOSE-DEPENDENT REGULATION BY GENERAL FACTORS

Highly specific alternative splicing factors have not been identified in vertebrate cells, and few have been identified in invertebrates. Although their existence cannot be ruled out, numerous observations point instead to the involvement of broadly distributed factors, with developmental and target specificity arising from variations in the relative concentration or activity of different competing and cooperating factors, on the one hand, and the strength and arrangement of binding sites for regulators and constitutive splicing factors, on the other. Substantial experimental and correlational evidence has implicated SR proteins as dose-dependent positive regulators that influence alternative splicing decisions through stabilization of U1 snRNP binding or enhancer activation (reviewed in 23, 44, 86, 139). Another example may be the KH domain protein KSRP. Inclusion of the c-*src* N1 exon requires the cooperative assembly of an intronic activator complex that depends critically on KSRP; KSRP is enriched in neuronal cells, where N1 is normally included, but it is not restricted to this cell type (89). Components of the N1 activator complex may also regulate splicing of other RNAs that share sequence features with c-*src* (see 89).

In many cases, the key determinant of splice site choice may not be the level of an individual protein but its concentration relative to factors with opposing effects. This became evident from studies of 5′ splice site competition where the activity of SR proteins in promoting the use of proximal 5′ splice sites in vitro and in transfected cells was antagonized by hnRNP A1 and related proteins (reviewed in 23, 86, 139). As for SR proteins, variations in the levels of hnRNP A/B proteins or mRNAs among different cell types have been reported (41, 57, 68). The molar ratio of hnRNP A1 to SF2/ASF varies over a range of at least 100-fold among different rat tissues (57), well beyond what suffices to induce a complete switch between some alternative 5′ splice sites in vitro. Hence, it seems reasonable to predict that the variations observed in vivo have important effects on alternative splicing of particular transcripts.

SR proteins may also antagonize one another. The β-tropomyosin gene of chicken contains a pair of mutually exclusive internal exons: exon 6A is included in fibroblasts and smooth muscle cells, while exon 6B is specific to skeletal muscle. A pyrimidine-rich element (S4) in the downstream intron is essential for recognition of the 5′ splice site for exon 6A. In vitro, the SR protein ASF/SF2 binds to the S4 element and stimulates splicing of exon 6A (47). In contrast, the SR protein SC35 antagonizes the S4-mediated stimulatory effect

of ASF/SF2, while a different SR protein, 9G8, has no effect. Thus, variations in the ratio of ASF/SF2 to SC35 might influence the splicing efficiency of exon 6A in vivo; this is supported by the observation that the ratio of SC35 to ASF/SF2 is at least twofold higher in SR protein preparations from muscle cells than in those from HeLa cells (47). Accordingly, SR protein preparations from skeletal muscle cells support in vitro splicing of exon 6A poorly compared with preparations from HeLa cells (47).

CONCLUSIONS

Proteins that play roles in alternative splicing exhibit remarkable functional versatility. SXL acts in very different ways to regulate splicing of *tra* and *Sxl* RNAs and is also a translational repressor of *msl2* and perhaps other RNAs (see 49). The regulation of *dsx* and *fru* by TRA exhibits few similarities with the regulation of its other known targets (see 62). In addition to its roles in splicing regulation, PTB also appears to be required for some internal translation initiation events. SR proteins play numerous roles in spliceosome assembly and perhaps also in mRNA transport, and various hnRNP proteins have similarly been implicated in multiple aspects of RNA processing. Thus, splicing regulation exploits the RNA-binding and protein interaction properties of diverse factors that are not dedicated to one type of regulatory function nor even to splicing itself.

Collaboration between general and more-or-less specific factors is common in both positive and negative regulation. Even highly restricted regulators such as SXL and TRA function in cooperation with ubiquitous or broadly distributed factors. Antagonistic interactions between *trans*-acting factors and between positive and negative signals are also known. The combinatorial effects that arise from cooperative and antagonistic interactions probably play an important role in enhancing the specificity of the regulatory systems; they may also generate flexibility for finely tuned quantitative regulation of alternative splicing in different cell types.

Even for the best-characterized model systems many molecular details remain to be resolved, including how enhancers stimulate splice site recognition in vivo. In vivo splicing takes place on nascent transcripts, and SR proteins and other components of the splicing machinery can associate physically with RNA polymerase II. What consequences does this have for regulation? What limitations or regulatory opportunities arise from the time required to transcribe long introns? Does phosphorylation play a role in alternative splicing regulation (discussed in 44, 139), and if so, do alternative splicing factors serve as targets for signal transduction pathways? It may be expected that in coming years a continuing combination of genetic, molecular, and biochemical aproaches will bring even greater improvements in our understanding of alternative splicing regulation.

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