Functions of SR and Tra2 Proteins in Pre-mRNA Splicing Regulation (44345)

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Alternative pre-mRNA splicing is a powerful mechanism of gene expression control in eukaryotic cells that can influence cell function, cellular differentiation, and development. In some instances, as exemplified by the sex-specific regulation of *transformer* splicing by the *Sex-lethal* (*Sxl*) gene product in Drosophila, it may be used as an on/off switch of gene expression, mimicking transcriptional control (1). Other examples, like the cell-specific splicing of the calcitonin/CGRP pre-mRNA (2) or the sex-specific splicing of the Drosophila *doublesex* pre-mRNA (1), illustrate that alternative splicing provides an economical way to switch between mutually exclusive gene functions in a cell- or stage-specific manner. Its extraordinary versatility in the regulation of gene function is amply demonstrated by recent findings suggesting that electrical tuning of the hair cells in the inner ear of the chick is accomplished in part through variations in the splicing of the *cSlo* primary transcript, which has the potential of generating at least 576 different proteins (reviewed in Ref. 3). Alternative splicing can thus provide a means to store, within a single gene, finely tuned information gradients that can be distributed in a spatially regulated manner. The latter example also illustrates that in many instances there may be a need for tight control of alternative splicing patterns, which in turn may require complex mechanisms of regulation.

Cell-Specific Splicing is Governed by Combinatorial Control

How are alternative splicing patterns controlled, and what are the *trans*-acting factors involved? Although the identification of the Drosophila products of the sex-determination genes *Sex-lethal, transformer,* and *transformer 2* as splicing regulators was made possible by the unique power of Drosophila genetics, corresponding efforts to identify mammalian splicing regulators has so far had to rely on biochemical approaches. As a result of the complexity generally observed in alternative splicing patterns, it has been difficult to identify mammalian factors involved in the cell- or stage-specific control of single splicing events. A commonly employed strategy consists in first identifying *cis*-elements responsible for the cell-specific control of a specific splicing event and then trying to track down *trans*-acting factors that interact with these elements in appropriate cell-free systems, using combinations of RNA binding and *in vitro* splicing assays. To date, this approach has yielded greatest success in the analysis of the neuron-specific splicing of a single short exon in the c-*src* pre-mRNA, whose regulation involves both negative and positive control in non-neuronal and neuronal cells, respectively. An important positive regulatory sequence element located downstream of this neuron-specific exon is composed of multiple sequence elements, which in turn are targeted by a complex of multiple *trans*-acting factors. Interestingly, these include both a ubiquitous hnRNP protein, hnRNP F, and a new regulatory protein with largely neural-specific expression, KSRP (4). At least part of the function of this neural-specific complex appears to be to overcome repression by the noncell-specific PTB (polypyrimidine-tract binding protein). PTB binds specifically to polypyrimidine tracts that are part of the 3′ splice site of certain differentially spliced exons, and thereby likely antagonizes constitutive splicing factors involved in 3′-splice-site recognition, such as U2 snRNP and U2AF (U2 snRNP auxiliary factor) (reviewed in Ref. 5). Hence, the example of neural-specific c-*src* pre-mRNA splicing demonstrates that even clear-cut cell-specific splicing choices may rely on complex regulatory mechanisms, where specificity is achieved by combinatorial control. In this review, we discuss recent data suggesting that two structurally related families of splicing factors, SR and Tra2 proteins, are important parts of the cellular mechanisms devised for the regulation of alternative splicing.

The SR Protein Family: Essential Splicing Factors that Influence Alternative Splicing

ASF/SF2, the first mammalian alternative splicing factor to be identified, was initially characterized as an activity that could switch 5′-splice-site usage in HeLa nuclear extracts in a concentration-dependent manner (6). ASF/SF2 is now known to be one of at least eight members of the so-called SR protein family of essential splicing factors, all

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of which can also influence alternative splicing (reviewed in Ref. 7). This group of RNA binding proteins is believed to play important roles at several steps in spliceosome assembly by promoting splice site recognition and providing a network of essential protein-protein interactions through their RS domains (RS domains are characterized by a high content of arginine and serine residues). The definition of SR proteins as essential splicing factors was originally based on their ability to individually activate splicing of certain model substrates in splicing-deficient cytoplasmic S100 extracts (8). (S100 extracts contain all factors necessary for splicing except SR proteins.) Although these early experiments suggested a high degree of functional redundancy for SR proteins, it is now clear that individual members of the family possess distinct RNA binding specificities and influence alternative splicing in quantitatively and qualitatively distinct ways (7). In addition, genetic depletion experiments showed that at least some SR proteins must have unique functional properties that are essential for cell viability and/or differentiation (9). It is probable that some of these properties are related to a role of SR proteins in splicing control. For instance, apoptosis induced in chicken DT40 cells by genetic depletion of ASF/SF2 is correlated with a change in 5′-splice-site selection in the pre-mRNA of the apoptotic regulator *Bcl*-X. As a result, the ratio between the apoptotic activator *Bcl*-X_L and the apoptotic inhibitor *Bcl*-X_S is increased (Wang and Manley, unpublished data). This observation is in agreement with the initial prediction that variations in the concentration or activity of ASF/SF2 may play important roles in the regulation of alternative splicing (6).

How does ASF/SF2 influence alternative splicing? *In vitro* splicing experiments with splicing substrates containing alternative splice sites showed that increasing concentrations of ASF/SF2 gradually shifted 5′-splice-site selection from a distal 3′-splice-site to proximal splice sites. Since proximal splice site selection is still preferred when the sequences of and surrounding the alternative sites are exchanged (10), it seems that sequence-specific RNA binding is not required, which is consistent with the finding that many different alternatively spliced pre-mRNAs behave identically (7). A current hypothesis is based on findings suggesting that ASF/SF2 initiates and stabilizes interaction of the U1 snRNP with 5′ splice sites *via* direct interaction with the U1 snRNP 70-kDa protein (11, 12). High concentrations of ASF/SF2 would then result in saturated occupancy of all 5′ splice sites leading stochastically to preferential use of the site closest to the 3′ splice site (7). However, the effects on splice-site selection observed *in vivo* and *in vitro* are not always consistent (7, 13), suggesting that multiple mechanisms may apply, including perhaps interference of ASF/SF2 with the activity of other essential splicing factors or splicing regulators.

In addition to their effect on splice-site usage, ASF/SF2 and other SR proteins participate in splicing control through their ability to activate exonic splicing enhancers (reviewed in Ref. 7). These are mostly, but not exclusively, purine-rich sequences, which are frequently encountered downstream of introns with weak 5′ or 3′ splice sites, and whose deletion causes intron retention or exon skipping. SR proteins can be sufficient for activation of such enhancers *in vitro* (14), but this is frequently not the case. For example, artificial splicing enhancers can be composed of high-affinity RNA binding sites for individual SR proteins and are activated specifically by their cognate binding proteins, but additional nuclear factors are required (15, 16).

Much of our current understanding of how SR proteins regulate enhancer-dependent splicing has come from the biochemical analysis of the female-specific control of *double-sex* pre-mRNA splicing in Drosophila. The female-specific *transformer* gene product (Tra) and the nonsex-specific *transformer*-2 gene product (Tra2) act in concert to promote inclusion of the female-specific exon 4 into the *doublesex* (*dsx*) mRNA. In male flies, exon 4 is skipped because of the weakness of its associated 3′ splice site. Female-specific splicing relies on a positive regulatory sequence within exon 4, termed the *dsx* enhancer, which consists of six 13-nucleotide repeats and a purine-rich element. Tra and Tra2 bind cooperatively and recruit members of the SR protein family to the enhancer (17–19). As a result, a splicing enhancer complex is formed that activates use of the weak 3′ splice site by stabilizing interaction of the splice site with components of the splicing machinery. An essential role in this stabilization is played by SR proteins, which provide bridging interactions with Tra2 and the 35-kDa subunit of U2AF, a factor that aids in the recognition of the branch site by U2 snRNP (20).

Tra2 Has Evolutionarily Conserved Functions in Splicing Control

Until recently, two long-standing questions had been whether aspects of the mechanisms employed in the regulation of Drosophila sex-determination were conserved in mammals, and by extension, whether mammalian homologs of the splicing regulators Sxl, Tra, and Tra2, exist. The answers to these questions seemed to be especially uncertain in view of the fact that sex-determination strategies as a whole are not conserved between mammals and flies. Given in addition that Sxl and Tra are exclusively female-specific, it is perhaps not surprising that mammalian homologs of these two factors have so far not been identified. However, several reasons suggest that Tra2 might be a good candidate for an evolutionarily conserved splicing regulator. First, unlike Sxl and Tra, Tra2 is not a sex-specific factor. Apart from its Tra-dependent role in females (21), Tra2 is also essential for spermatogenesis in the male germ line. In this tissue, Tra2 regulates alternative splicing of its own and at least one other transcript, the *exuperantia* pre-mRNA. The mechanism of regulation is not understood in either case, but Tra2 has apparently opposite effects on intron retention in the two different transcripts, promoting it in the former and preventing it in the latter (22). The observation

that autoregulation of *transformer 2* splicing occurs in the germ line but not in somatic tissue indicates that it may involve additional, tissue-specific factors. Secondly, Tra2, like SR proteins, is ubiquitously expressed, and is also structurally related to these proteins. SR proteins and Tra2 are composed of the same domains, but differ in their overall domain organization: SR proteins contain one or two RNA binding domains and a C-terminal RS region; Tra2 contains a central RNA binding domain flanked by two RS regions (Fig. 1; reviewed in Ref. 7). Since SR proteins are highly conserved during evolution, the prediction that this might also be true of Tra2 seemed justified.

The prediction that Tra2 is evolutionarily conserved was borne out when Dauwalder *et al.* (23) identified two expressed sequence tags with strong similarity to a 19-amino acid region immediately downstream of the Tra2 RNA binding domain. This region was chosen because of its high evolutionary conservation between *Drosophila melanogaster* and *Drosophila virilis*. Subsequent screening of a HeLa cell library resulted in the identification of Tra2α, one of two identified mammalian Tra2 homologs. The second mammalian homolog, Tra2β, was identified independently by three different groups using three distinct approaches, including differential display of mRNA from rat astrocytes exposed to hypoxia and hypoxia/reoxygenation (24), screening of a subtractive cDNA library of mouse macrophages exposed to mineral particles (25), and a two-hybrid screen of a HeLa cell library with the SR protein SC35 as a bait (26). Tra2α and Tra2β are highly homologous to each other and to the Drosophila protein, particularly in the RNA binding domain (Fig. 1).

Are the regulatory splicing functions of Tra2 evolutionarily conserved? Using genetic complementation of flies homozygous for a loss-of-function mutation in the *transformer 2* gene, Dauwalder *et al.* (23) provided evidence that a transgene expressing human Tra2α can substitute for the Tra-dependent but not for the Tra-independent functions of the endogenous gene. The transgene induced female sexual differentiation and promoted female-specific *dsx* pre-mRNA splicing in chromosomally female Tra2 knockout flies. Since both effects were not obtained with chromosomally male flies, they were likely dependent on Tra expression. Consistent with this, flies that carry a deletion in the *dsx* gene that removes the *dsx* enhancer, did not show female-specific *dsx* splicing in presence of the transgene. In contrast to its effect in females, the human transgene was unable to prevent sterility in Tra2 knockout males. In agreement with the idea that autoregulation of splicing of its own pre-mRNA is an important part of Tra2 functions in the male germ line, Drosophila but not human Tra2α promoted intron retention in a corresponding *transformer 2* reporter gene.

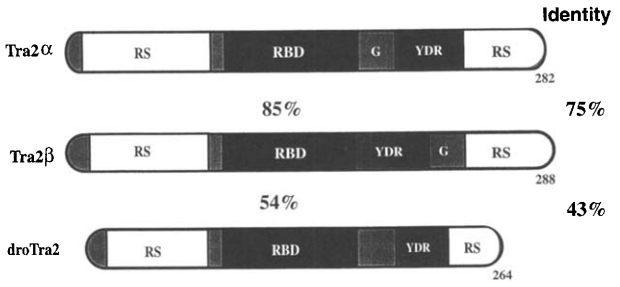

Figure 1. Tra2α and Tra2β are human homologues of the Drosophila splicing regulator Tra2. The human proteins are 75% identical and both share 43% sequence identity with Drosophila Tra2 (droTra2). Sequence identity among all three proteins is highest in and immediately downstream of the RNA binding domains. In this region the human domains are 85% identical, and both share 54% identity with the corresponding region of Drosophila Tra2. This may explain the findings that the RNA binding specificities of the human proteins are indistinguishable and likely similar to that of the Drosophila protein (see text). The human proteins differ mainly in the location of a polyglycine stretch in the C-terminal RS domain, and in the first 49 amino acids of the N-terminal RS domain. The C-terminal domains of all three proteins contains several copies of a characteristic repeat of three to four amino acids containing a tyrosine residue (YDR). The lowest degree of sequence homology between Drosophila and human Tra2 is found in the N-terminal RS domain.

What is the molecular basis for the functional differences of human and Drosophila Tra2? Indirect evidence suggests that the poor evolutionary conservation of the N-terminal RS domain might be the decisive factor. Drosophila Tra2 exists in three different isoforms generated through alternative splicing, including $Tra2^{179}$, which essentially lacks the N-terminal RS domain (22). Genetic experiments addressing the functional differences of these isoforms indicated that all three can support female-specific functions. However, $Tra2^{179}$ was unable to carry out male germ-line functions such as autoregulation of *tra2* splicing (22). This strongly indicates that the presence and possibly the nature of this RS domain is crucial for some functional properties of Tra2, but not for female-specific *dsx* splicing. Consistent with this, the N-terminal RS domain is the part of the molecule that is the least conserved between Drosophila and mammalian Tra2. In addition, the first 49 amino acids of the mammalian domains are highly divergent. As these structural differences contrast with the high degree of sequence identity in the corresponding RNA binding domains, they may account for the observed functional differences of Drosophila Tra2 and human Tra2α, and for possible functional differences between the two human proteins.

How do the mammalian Tra2 proteins function in splicing? A recent biochemical study suggests that Tra2 proteins may play important roles in the control of splicing enhancers not only in Drosophila but also in vertebrates. Using an artificial splicing enhancer with homology to enhancers located in alternatively spliced exons of the human calcitonin/CGRP (2) and the chicken cardiac troponin T (14) transcripts, Tacke *et al.* (27) demonstrated specific binding of authentic Tra2 proteins present in HeLa nuclear extracts. Gel mobility–shift assays with purified recombinant proteins further indicated that binding to the enhancer was direct and not mediated by other nuclear factors. These experiments also showed that Tra2α and Tra2β have indistinguishable RNA binding specificities, binding preferentially to GAA repeats. As Drosophila Tra2 can be replaced by

Tra2α in the activation of the *dsx* enhancer and has also been reported to bind preferentially to the purine-rich element within this enhancer (19), its RNA binding specificity is likely similar to that of the mammalian proteins. Significantly, using *in vitro* splicing assays with distinct enhancer-containing substrates, Tra2 proteins were shown to activate enhancer-dependent splicing in a highly sequence-specific manner. A similar degree of specificity was observed previously with some SR proteins (15, 16). However, UV cross-linking assays in nuclear extracts also suggested that, perhaps as a result of overlapping RNA binding specificities, at least some purine-rich splicing enhancers may bind multiple proteins, including SR and likely Tra2 proteins (14, 15). In this context it is noteworthy that the enhancer used in the study by Tacke *et al.* (27) was composed of three copies of a sequence element initially identified as a high-affinity ASF/SF2 binding site (15), to which Tra2 was unexpectedly found to bind with high affinity. Accordingly, both Tra2 and ASF/SF2, but not SC35, were capable of activating enhancer-dependent splicing when added to nuclear extract. It is intriguing that ASF/SF2 and Tra2 proteins display such similar RNA binding specificities, but the functional significance of this is unknown. An important functional difference between Tra2 and ASF/SF2, however, was that, unlike ASF/SF2, neither Tra2 protein could activate splicing in S100 (27). This finding indicates that Tra2 proteins, unlike SR proteins, are not essential splicing factors, and likely participate only in splicing regulatory functions.

An as yet unresolved question is whether mammalian Tra2 proteins play important roles in cell- and stage-specific splicing control. Support for this idea comes from the fact that Tra2β was initially identified as a factor rapidly induced during re-oxygenation of astrocytes after hypoxia (24). A dramatic induction of Tra2β transcripts was detected in hypoxic cells within 15 min of re-oxygenation. This effect was specific, as it was not observed with general splicing factors such as U2AF and the U1 snRNP 70-kDa protein. These data suggest that an important part of the pathways leading to the metabolic changes induced by re-oxygenation may involve regulation at the level of pre-mRNA splicing. In addition, mRNA expression levels of Tra2β were shown by semi-quantitative reverse transcription/PCR to differ substantially in various mouse tissues (25). For instance, expression levels were found to be at least 10 times higher in brain than in liver. Although these findings are suggestive of a role for Tra2 proteins in tissue-specific splicing, more definitive evidence will require the development of appropriate *in vitro* systems in which alternative splicing of potential target genes can be analyzed. In this context it is important to note that the significance of purine-rich splicing enhancers for truly tissue-specific splicing control is still unclear (28). Purine-rich sequences resembling Tra2 binding sites are not only found in alternative exons but also in many constitutive exons, where their function is likely to prevent skipping by default. In view of the increasing evidence for combinatorial control in tissue-specific splicing, the ability of Tra2 proteins to activate such enhancers may be only one aspect of their potential properties as splicing regulators. An attractive hypothesis is based on the observation that the regulation of *dsx* splicing in Drosophila depends on the ability of Tra2 to engage in cooperative RNA binding activities with the sex-specific factor Tra and/or SR proteins such that its RNA binding properties are changed (19, 29). One might envision an analogous scenario in the cell-specific splicing control in mammals, whereby cell-specific factors alter the RNA binding properties of Tra2 proteins as a means to exploit a large number of different *cis*-regulatory elements. It is thus conceivable that mammalian Tra2 proteins might act as stage-specific regulators of pre-mRNA splicing not only by themselves but also as part of stage-specific, multicomponent complexes that bind RNA cooperatively. In this context it is worth noting that mammalian Tra2 proteins, like Drosophila Tra2, can interact with SR proteins (26; Shin C, Tacke R, Manley JL, unpublished data), but the significance of these interactions for RNA binding and function has not been determined.

For the present, the precise mechanisms by which mammalian Tra2 proteins participate in splicing control are largely a matter of speculation, as are the identities of the genes and pathways they may control. However, given the rapidly growing number of splicing events known to undergo developmental and tissue-specific regulation, the creation of genetically manipulated mice may be the fastest road to a general understanding of the significance of mammalian Tra2 proteins in these processes.

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