Histol Histopathol (2000)15: 239-249

http://www.ehu.es/histol-histopathol

Histology and Histopathology

Cellular and Molecular Biology

Invited Review

Splicing and the single cell

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Summary. The selection of alternative splice sites is an important component of cell-specific gene regulation in eukaryotic cells. Use of splice sites can be positively and negatively regulated, and often physiologically appropriate splice site choice is achieved by a balance of the two. RNA elements controlling splice site choice are found in both exons and introns, and these determine management by the cellular splicing machinery. However, the molecular basis of how the splicing machinery responds to these signals in different cells is somewhat of a paradox. Thus far the identified proteins which bind to tissue/cell-specific regulatory elements in mammals are expressed in many different tissues, and not just in the regulating tissue. Potential tissue-specific splicing regulators have been identified by non-biochemical means. However, alternative splicing choices are likely to be affected by subtle differences in the splicing machinery in different cells. In this review I suggest that one important factor is the ratio of proteins in different nuclear compartments, which might be established in a cell type specific fashion.

Key words: Pre-mRNA, spliceosome, nucleus

Introduction

The individual cells in a complex multicellular organism are often dramatically different to each other, despite in most cases containing essentially the same genetic information. These phenotypic differences between cells result from differences in how their genes are expressed. A major contributor to differential gene expression between different cell types and during development is transcriptional control, with specific arrays of genes being switched on or off in different tissues by tissue-specific transcriptional regulators (for example see (Ericson et al., 1997). However, it is becoming increasingly apparent that pre-mRNA splicing (reviewed in Zaphiropoulos, 1998) exerts another important level of control. For example alternative splicing has been shown to be particularly important regulating gene expression in the nervous system. Recent reports show that the electrical tuning of avian cochlea to different sound frequencies is at least partially achieved through the elaborate alternative splicing of a pre-mRNA which encodes a calcium activated potassium channel, affecting the sensitivity of the resulting protein to calcium (Navaratnam et al., 1997; Rosenblatt et al., 1997). At least 576 proteins are potentially encoded by possible splice variants of this gene with the splicing pattern directly dependent on the position of the cell in the ear-fewest exons are included transcripts by cells at the base of the basillar papilla. Alternative exon usage is also critical to other tissues and developmental decisions. For example, sex-specific exon usage regulates the activity of DNA methyl-transferase in mammalian gametogenesis (Mertineit et al., 1998).

The spliceosome itself is composed of a set of snRNPs (small nuclear RNPs, made up of snRNAs and associated proteins), along with additional non-snRNP proteins. Five snRNAs are usually found in the spliceosome (U1, U2, U4, U5 and U6). Splice site complementarity plays a critical role in splice site recognition. Base-pairing takes place between U1 and the 5' splice site, between U2 and the branchpoint and between U5 and sequences in the exons to be spliced together (Moore et al., 1993; Madhani and Guthrie, 1994). Intriguingly an alternative spliceosome with a different set of snRNAs is involved in the splicing of some pre-mRNAs with variant splice sites (Tarn and Steitz, 1996, 1997). The protein content of spliceosomes has been estimated at over 50 by the 2-dimensional gel analysis of in *vitro* assembled splicing complexes and from the number of genes genetically identified as essential for splicing in yeast (Reed and Palandjian, 1997; Neubauer et al., 1998). A large family of non-snRNP proteins called SR proteins (since they contain many serine/arginine dipeptides clustered in RS domains) are critical for both splicing and splice site selection (Fu, 1995; Chabot, 1996; Valcarcel and Green, 1996; Caceres and Krainer, 1997). SR proteins are likely to be involved in recognition of 5' splice sites (Eperon et al., 1993; Kohtz et al., 1994), and in fact some 5' splice sites can be recognised by SR proteins in the absence of

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U1 snRNP (Crispino et al., 1994). At the 3' splice site, another protein with an RS domain (although not usually considered a member of the SR family) U2AF binds to the branchpoint sequence before U2 snRNP. U2AF is one of the earliest components of the assembling spliceosome and so is likely to be a critical component for committing pre-mRNA to the splicing pathway (Valcarcel et al., 1996). A second set of non-snRNP proteins which also impact both on splicing and splice site selection (but are not usually considered to be components of the spliceosome) are the hnRNPs. These are among the earliest proteins to assemble on pre-mRNA, probably prior to the spliceosome components (Reed and Palandjian, 1997).

Understanding splice site selection is the key to how alternative splicing operates. Both the basic biochemistry of splicing and the minimal sequence elements of the pre-mRNA *required* for intron removal have been identified *in vitro* by examining the splicing of short synthetic pre-mRNAs (which can be experimentally manipulated) in cell extracts (Reed and Palandjian, 1997). Splicing occurs in biochemically separable steps which contain different proteins and snRNAs in a molecular machine called the spliceosome. In these experiments, the critical pre-mRNA sequence elements required for splicing are restricted to nucleotides around the 5' splice site and the 3' splice site, and follow a regular consensus (though subject to some variability) (Fig. 1). The 3' splice site is tri-partite, being composed of the branchpoint nucleotide (to which the 5' splice site attaches covalently after it is cleaved to give a lariat intermediate), a polypyrimidine tract, and the 3' splice site itself.

Spliceosome assembly in the nucleus is likely to be much more complex than *in vitro*, although probably involves the same principles. One important difference is

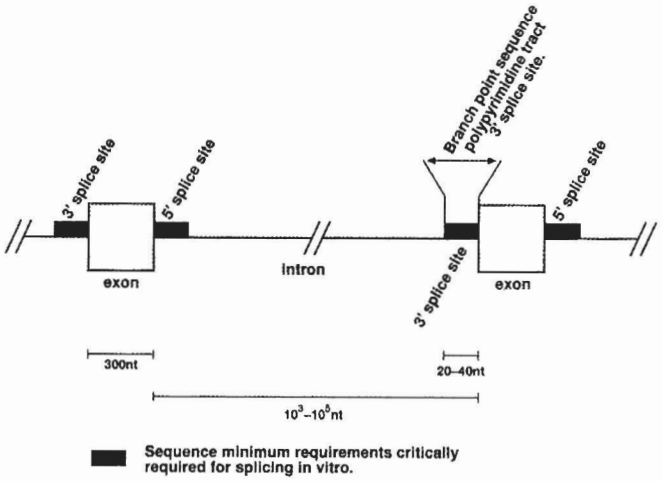

Fig. 1. Schematic organisation of a constitutively spliced pre-mRNA. Exons are shown as white boxes, and introns as lines. The sequence elements identified as critical for splicing in *in vitro* experiments of artificially short pre-mRNAs are shown as black boxes, and are confined to the intron, close to the exonic boundaries.

the architecture of the substrate: naturally occurring introns are often very large (typically 1Kb-100Kb) while exons are much smaller (around 300b), in direct contrast to the artificially short introns which have been used for *in vitro* splicing studies. Hence the splicing machinery has to identify fairly short and somewhat variable splice sites within the context of a potentially enormous target RNA. Exon definition has been suggested as a solution for this 'needle in the haystack' problem (Berget, 1995; Black, 1995). This proposes that to facilitate splice site selection, the earliest steps in spliceosome assembly actually occur across the exon immediately after the intron to be removed. Exons are recognised because they are flanked by correctly oriented 5' and 3' splice sites (in different introns). Because exons are usually quite short, this occurs over comparatively short distances. After exon recognition, the spliceosome then assembles across the adjacent intron and splicing proceeds.

A second critical factor regarding the substrate is that splicing in the nucleus almost certainly occurs on nascent RNA, i.e. while transcription is still going on (Wuarin and Schibler, 1994; Zhang et al., 1994). An important mediator between transcription and splicing is the carboxy-terminal domain or CTD of the large subunit of RNA polymerase II, which interacts with a sub-family of SR splicing proteins (reviewed by Corden and Patturajan, 1997).

The organisation of genes into distinct modules (exons) imposes an element of choice. In practice individual splice sites can compete with each other to ensure that their exon is spliced into the final mRNA, perhaps meaning that other exons are ignored. Here I review what is known about how these decisions are made both at the level of the individual cell and indeed the individual transcript.

Positive regulation of exons

Two important parameters which influence splice site choice are the strength of splice sites, and the length of exons to be spliced. Splice sites which differ from the consensus sequence are often recognised less well by the splicing machinery and exons of less than 50 nucleotides are usually spliced less efficiently. For example the alternatively spliced exons of the agrin gene (a neural extracellular matrix protein) range from 33 nucleotides to a mere 2 nucleotides (Wei et al., 1997). Such exons require help to be recognised by the splicing machinery. This provides the basis for positive regulation of splice site selection, and results in positive selection of exons.

A detailed analysis of the sequence elements influencing alternative splicing has recently been possible because of the development of primary cell lines derived from tissues which regulate alternative splicing: critically these reproduce patterns of alternative splicing correctly, they are transfectable and cell extracts can be made. A good example of this is the pre-mRNA encoding chicken cardiac Troponin T (cTNT). This has an additional regulated exon which is included in

embryonic skeletal muscle but absent in adult cardiac muscle (Cooper, 1992; Xu et al., 1993). Transfection experiments in primary cultures of embryonic muscle have shown that sequence elements in both introns and exons which have quite different properties are important for inclusion of this exon (Fig. 2). An exonic splicing enhancer (ESE) located in the alternatively spliced exon is likely to act as a general splicing enhancer required for the splicing machinery to identify the alternatively spliced exon (Xu et al., 1993). This ESE is not cell type-specific since it can function as an enhancer in different pre-mRNAs and in other cell types. In contrast three synergistic intronic elements downstream of the regulated exon, and one upstream are involved in embryonic-specific cTNT splicing. Proof that the important controlling elements are within introns came from the demonstration that these intron sequences can direct muscle cell-specific splicing of a synthetic exon 5 lacking an exonic splicing enhancer (Ryan and Cooper, 1996; Cooper, 1998).

Further data from patients with muscular diseases underline the importance of the cTNT intronic sequences in regulating alternative splicing of the cTNT transcript. There are aberrant, embryo-like cTNT splicing patterns in the muscle of adult patients with Duchenne muscular dystrophy (Philips et al., 1998), a disease caused by the expansion of a CTG trinucleotide in the DM protein kinase gene. Two of the downstream intronic elements

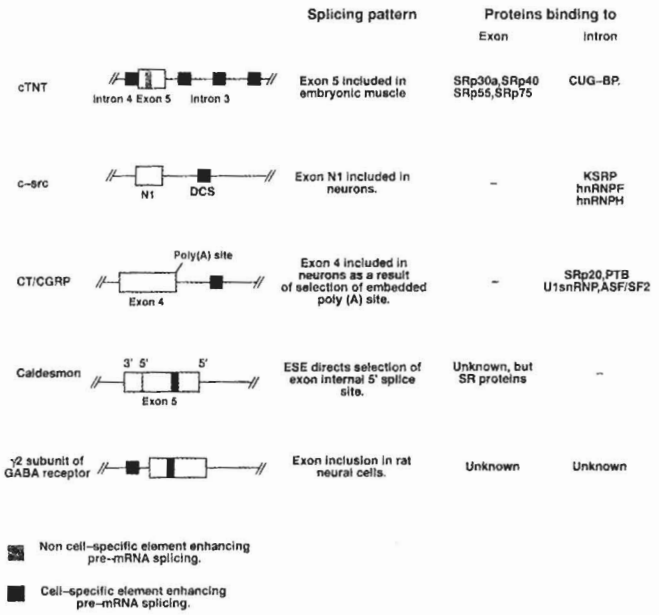

Fig. 2. Cartoon showing the location of sequence elements positively regulating the alternative splicing exons of pre-mRNAs of a number of genes. Introns and exons are represented as in Fig. 1. Sequences identified as critical for cell-specific exon inclusion are shown as black boxes, while sequence elements which promote exon inclusion independent of cell type are shaded. Identified proteins binding to these elements are indicated. In the case of the γ2 subunit of the GABAA receptor the protein binding during positive regulation is unknown, but the protein binding during negative regulation is PTB (see Fig. 4).

regulating cTNT splicing in the human cTNT gene (and conserved in the chicken) contain CTG trinucleotide repeats. These CTG trinucleotides are bound by an hnRNP-like protein called CUG-binding protein. It is thought CUG-BP is likely to be an important trans-acting factor regulating cTNT alternative splicing, and that the CTG expansion in the DM transcript results in an increased nuclear concentration of this protein and aberrant cTNT splicing. However, CUG-BP is not a muscle-specific protein and so is unlikely to be responsible by itself for establishing the muscle tissue-specific splicing pattern.

Intronic tissue-specific splicing regulatory elements have been located adjacent to a number alternatively spliced exons (Fig. 2). In many cases the proteins binding to these are unknown, but a complex of proteins binding to the downstream control sequence (DCS in Fig. 2) controlling neural-specific exon inclusion of the c-src N1 exon have been identified (Min et al., 1995, 1997) (Chou et al., 1999). Perhaps the most surprising thing about the proteins in this complex is that they are not tissue-specific, being present in both neuronal and non-neuronal cells. Three of the proteins are known to be ubiquitously expressed RNA-binding proteins: hnRNPs F and H (members of the hnRNP family of proteins) and KSRP (a novel protein with a KH-RNA binding domain).

On the other hand, ESEs are typically located within the regulated exon and are quite variable in sequence. A striking illustration of this came from experiments in which randomised sequences were used to replace the endogenous IgM splicing enhancer (Liu et al., 1998). As many as 10-20% of random 20mers had ESE function. It is likely that degeneracy of ESEs may be necessary to allow them to fit into coding sequences. Despite a high sequence complexity, ESEs are frequently purine rich (Cooper and Mattox, 1997). ESEs are likely to work by acting as a nucleation site for SR proteins (Ramchatesingh et al., 1995; Coulter et al., 1997; Tacke et al., 1997; Liu et al., 1998), which either stabilise interactions of splicing components with splice sites via protein-protein interactions, thereby 'anchoring' them to the splice site, or act as a nucleation site for splice site assembly. A key interaction is likely to be with protein U2AF which binds to the polypyrimidine tract in the downstream portion of the intron (Fig. 1) and might be critical for committing pre-mRNAs to particular pathways of splicing (see introduction) (Wang et al., 1995). ESEs are probably particularly important for the stabilisation of weak 3' splice sites with poor ability to base pair with U2 snRNA (Buvoli et al., 1997).

A generalisation from the experimental results from primary cell lines (which presumably most accurately recapitulate molecular events in tissues) is that ESEs are required as general splicing enhancers needed for the splicing machinery to recognise weak splice sites, but they do not provide the critical switches. Support for this concept has also come from the observation that ESEs are important for constitutive splice site choices in the ß

globin pre-mRNA (Schaal and Maniatis, 1999). However exceptions to this generalisation are found for neural splicing of the γ2 subunit of the $GABA_A$ receptor, in which the critical sequences are both within the regulated exon and the adjacent intron (Zhang et al., 1996) and in the slightly atypical ESE of the caldesmon gene, which directs the choice between two alternative 5' splice sites within an exon (Fig. 2). In this latter case the ESE is itself important for splice site choice, since substitution with an unrelated ESE changes 5' splice site choice (Elrick et al., 1998). Despite their frequent role as non-specific splicing activators, ESEs and SR proteins are almost certainly critical for cell-type specific splicing. ESEs are required for efficient splicing of regulated introns (Xu et al., 1993) and SR proteins are essential for the viability of both cells and organisms (Ring and Lis, 1994; Wang et al., 1996). On the other hand, SR proteins are more or less ubiquitously expressed, so a prediction is that if ESEs are important in regulating splicing, additional components must bind to them which are tissue/cell specific (see also below).

The prototypic ESE was identified not in mammals, but in the doublesex gene of *Drosophila melanogaster*, where it is involved in the cell-type regulation of splicing events controlling sexual differentiation (reviewed by Wang et al., 1998). In this case the key regulatory protein responsible for mediating the sex-specific splicing switch is the female-specific Tra protein, which forms a complex with the more widely expressed Tra-2 protein and a number of SR proteins, together resulting in a female-specific splice site choice. However, experiments suggest that the situation is likely to be more complex in many mammalian pre-mRNAs (and perhaps other *Drosophila* pre-mRNAs), with both ESEs and intronic sequences providing important signals to the splicing machinery.

Does a similar situation pertain in mammals, with some ESEs binding cell-specific complexes of proteins? Although mammalian sexual differentiation does not seem to be controlled at the level of differential splicing, mammals do contain two homologues of Tra-2 (Tra-2α and Tra-2ß) and these may be important positive regulators of splicing. In a recent twist to this story it was reported that in nuclear extracts the major protein binding to an optimal RNA sequence derived by SELEX for ASF/SF2 was in fact Tra-2ß (Tacke et al., 1998). Hence although SR proteins *can* activate ESE dependent splicing, whether they always are the key proteins *in vivo* is unclear. Tra-2ß is similar in domain organisation to SR proteins, with an RNP-type RNA Recognition Motif and an RS region but important functional differences suggest it is distinct. Most importantly, tra-2ß can rescue splicing of an enhancer-dependent intron in a nuclear extract which contains SR proteins diluted to the point that they are unable to support splicing by themselves, suggesting it is a very potent positive regulator of splice site selection, but it is unable to replace SR proteins *in vitro* in a general splicing reaction. A prediction is that Tra-2ß-dependent ESEs might be targets for tissue-specific splicing, but since it is widely expressed in mammalian tissues Tra-2ß is likely to complex with tissue-specific splicing regulators (the mammalian equivalents of Tra).

As well as positive splice site selection, exons can also be positively regulated as a result of 3' end selection immediately downstream of the regulated exon. This means the removal of downstream competing splice sites, which otherwise might lead to exon removal by splicing. Calcitonin mRNA is normally made in the thyroid in this way, with 3' end formation immediately downstream of an internal exon which contains its own polyadenylation site (Fig. 2). In neurons transcription continues past this poly(A) site. As a result of downstream splice sites being synthesised the regulated internal exon is skipped and is replaced by downstream exons to generate CGRP (calcitonin gene-related peptide) (Amara et al., 1982). In humans, use of the calcitonin-specific polyadenylation site is promoted by an intronic element 150 nucleotides downstream which binds a number of generally-expressed splicing factors including U1 snRNP, PTB and SRp20 (Lou et al., 1995, 1996, 1998). Importantly, these proteins are believed to work by interacting with components of the poly-adenylation machinery, perhaps mediated by RS domains (which are present both in SRp20 and the cleavage/polyadenylation factor CFI).

Negative regulation of exons

While exons can be positively selected in a cell-type specific fashion, the converse can also be true. In a number of cases exons are not selected in specific cells or tissues as a direct consequence of negative regulation. For example, the neural-specific exons of c-src and $GABA_A$ receptor γ2 subunit are selected in neurons (see above) but excluded in HeLa cells. In other cases such as the alternative splicing of α-tropomyosin, the regulated exon is only included in smooth muscle: as a result of competition between splice sites the strong non-muscle splice site is used in all other tissues (apart from smooth muscle where it is negatively regulated). While positive regulation of splice site selection is thought to occur as a result of protein-protein interactions strengthening the recognition of weak splice sites in certain cell types, negative regulation of splice site choice often results from occlusion of splice sites to prevent them being recognised. However, in some cases sequences binding within an exon can also negatively regulate splicing. These sequences are called Exonic Splicing Silencers (ESSs) by analogy with ESE. In at least two cases these ESSs bind hnRNPA1 (DelGattoKonczak et al., 1999).

A key molecule implicated in the negative regulation of a number of splice sites is the polypyrimidine tract binding protein (PTB). Based on SELEX experiments which showed that the RNA targets of PTB overlap with those of U2AF, it was proposed that PTB functions as a competitive inhibitor, masking U2AF binding by binding to the 3' end of the intron (Singh et al., 1995; Valcarcel

and Gebauer, 1997). Some evidence in support of this model has come from the splicing of α and ß tropomyosins, in which PTB binding does seem to block U2AF access (Lin and Patton, 1995) (Fig. 3). However, in many cases repression by PTB is likely to be somewhat more complex than this simple model predicts (Fig. 3). In the case of α-tropomyosin additional *in vitro* and *in vivo* experiments have shown that PTB binding both upstream and downstream of the negatively regulated exon is important in the negative regulation (Perez et al., 1997; Gooding et al., 1998). Additionally, the PTB-mediated negative regulation of c-src neural-specific exon N1 in HeLa cells occurs at a distance. In this case PTB is thought to bind to sequences in the intron upstream (intron 3) of that which contains the splice sites it negatively regulates (intron 4) (Fig. 3) (Chan and Black, 1997). Negative regulation of the γ2 subunit of the $GABA_A$ receptor pre-mRNA involves PTB-binding sequences in both the exon (corresponding to the site which is positively regulated in neural cells: see above) and the intron (Ashiya and Grabowski, 1997). Additionally PTB is not likely to bind by itself, but as part of a stable complex. In HeLa cells monoclonal antibodies to PTB immunoprecipitate a complex of proteins assembled on the negatively-regulated introns of the rat ß-tropomyosin pre-mRNA including PTB and SAM 68 (Grossman et al., 1998).

Surprisingly SR proteins have been implicated in the negative regulation of pre-mRNA splicing as well as its positive regulation. SR proteins bind to negative regulatory elements in the introns of both adenovirus and Rous sarcoma virus, blocking U2 snRNP access to the 3' splice site (Kanopka et al., 1996; McNally and Mcnally, 1998), and SR proteins have been implicated in the negative regulation of both SV40 and HIV pre-mRNA splicing (Wang et al., 1996, 1998). Hence the precise location of binding sites for proteins on the pre-mRNA

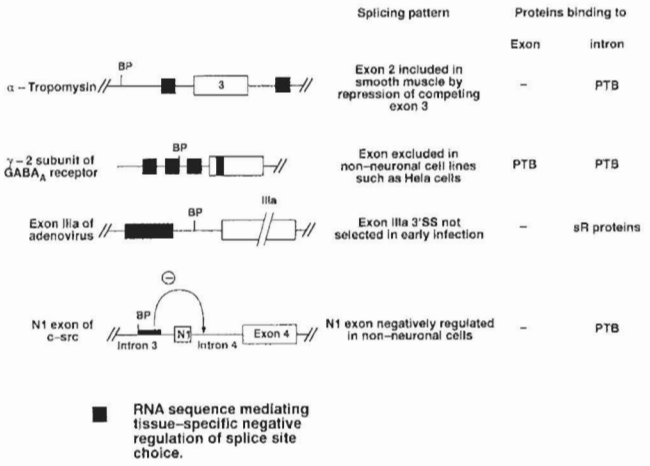

Fig. 3. Cartoon showing the location of sequence elements in pre-mRNA which negatively regulate exon inclusion (black boxes). Proteins identified as binding to the sequence elements are indicated. The branchpoint is indicated as BP.

is an important determinant as to whether they will act as positive or negative regulators of splicing. Any complex of proteins which occludes a splice site, even if it contains SR proteins, is likely to act as a negative regulator. Splice site occlusion can also be dynamically regulated. Such a strategy has been adopted by the influenza virus to control production of M2 ion channel protein by alternative splicing. A strong 5' splice site is blocked by a complex of viral polymerase proteins synthesised during infection, which causes a switch to a weaker 5' splice site (activated by ASF/SF2) (Shih and Krug, 1996).

Competing positive and negative signals often determine splice site choice

Often exons which are positively regulated in one cell type are negatively regulated in other cell types, suggesting that a balance of controls are important for establishing appropriate cell type-specific splicing patterns (Grabowski, 1998). A number of alternatively spliced exons contain both positive and negative splicing signals (Caputi et al., 1994; Delgatto et al., 1997; Staffa et al., 1997). Perhaps the most interesting example of this occurs in alternatively spliced exons of the cell surface molecule CD44, which is a key molecule in determining the behaviour of cells. At least one alternatively spliced exon from this gene contains both ESEs and ESSs, and these composite elements are respond to extracellular stimuli mediated by signal transduction (Konig et al., 1998).

What are the important cell-specific molecular switches controlling alternative splicing?

A major paradox in the alternative splicing field has been that the proteins thus far identified in *in vitro* mammalian systems are neither cell-type nor tissue-specific. Do these proteins interact with tissue-specific factors? Molecules like Tra with regulatory roles in enhancers have not yet been described in mammals. Hence tissue-specific families of splicing factors either do not exist, or a more likely possibility is that they have yet to be found.

A possible explanation for the dearth of cell-specific splicing regulators is they might not have been identified to date, either because they do not bind tightly to their target sequences/complexes, or do not cross-link to RNA. Consistent with this, strong *candidates* for cell type-specific splicing regulators have been identified by genetic as opposed to biochemical means. One of these is the product of the Wilms Tumor candidate gene WT1, which is restricted to cells of the mesoderm. In an embryonic kidney cell line WT1 protein is enriched in regions of the nucleus which contain high concentrations of splicing factors (Charlieu et al., 1995; Larsson et al., 1995). WT1 protein is associated with spliceosomes in *in vitro* extracts from kidney cells and directly interacts with the splicing protein U2AF65, suggesting that it

might operate by stabilising (or perhaps de-stabilising) the interaction of this protein with the branchpoint sequence of a regulated pre-mRNA (Davies et al., 1998). Since WT1 has an RNA-recognition motif it might also directly interact with target RNAs (Caricasole et al., 1996; Kennedy et al., 1996)

Another candidate is the germ cell-specific RNA binding protein RBM. In sections of human testis RBM co-localises with pre-mRNA splicing factors in nuclei at certain stages of germ cell development (Elliott et al., 1998). No RBM protein is detected in neighbouring somatic cells in the testis, suggesting that RBM plays a specific role in germ cell RNA metabolism. Both WT1 and RBM were genetically identified by positional cloning of disease loci. Similarly the ELAV protein in *Drosophila*, originally identified genetically by a mutation which affects neuron development, is a neural-specific splicing regulator (Koushika et al., 1996). It also should not be forgotten that two of the key components in the alternative splicing pathway regulating sexual differentiation in *Drosophila*, Tra and Tra-2, were also originally identified genetically (Bell et al., 1988).

A second possible explanation for the apparent absence of tissue-specific splicing regulators would be if alternative splicing was often controlled by differences in the levels of expression of constitutive splicing factors. A number of potentially different isoforms of PTB are generated by alternative splicing. Low levels of PTB are expressed in the brain, but there is evidence for a protein immunologically related to PTB which might function as a tissue-specific replacement (Patton et al., 1991; Ashiya and Grabowski, 1997). In addition there are alternatively spliced forms of U2AF (Ding et al., 1996).

A further possibility for regulating the splicing machinery comes from splicing factors being expressed in different ratios. Changes in the ratio of the SR protein ASF/SF2 to hnRNPA1 can affect splice site selection (Caceres et al., 1994) and different mouse tissues have different ratios of these proteins (Hanamura et al., 1998). Individual SR proteins are able to commit different pre-mRNAs to the splicing pathway with different efficiencies *in vitro* (Zahler et al., 1993). Members of the SR protein family are known to be expressed at different levels in different tissues (Zahler et al., 1993; Neugebauer et al., 1995), as are members of the hnRNP family of proteins (Kamma et al., 1995). These are likely to be underestimates of cellular variation, since tissues represent an average of their component cells which might include both high and low levels of expression. This means that differences in ratios might not be apparent in whole tissues, even if there are in fact differences between cells. This happens in the testis, where hnRNPA1 is expressed only in spermatogonia and Sertoli cells, and not in the later stages of germ cell development, while hnRNPU is more widely expressed (Fig. 4). SRp20 was initially isolated in a differential screen as a gene which was transcribed on B cell activation and its level is differentially regulated over the cell cycle (Jumaa et al., 1997). Tra-2ß expression is upregulated by hypoxia (Matsuo et al., 1995) and is differentially expressed in mouse tissues (Segade et al., 1996). A number of SR proteins are induced in response to T-cell activation (Screaton et al., 1995). In addition, SRp20 and other SR proteins including SF2/ASF negatively regulate their own splicing (Wang et al., 1996; Jumaa and Nielsen, 1997) as do some of their associated kinases (Colwill et al., 1996b).

Do changes in nuclear organisation of splicing factors contribute to cell-specific splicing - a hypothesis

Splicing factors are not uniformly distributed in the cell nucleus. In tissue culture cells splicing factors are found both in punctate structures ('speckles') and

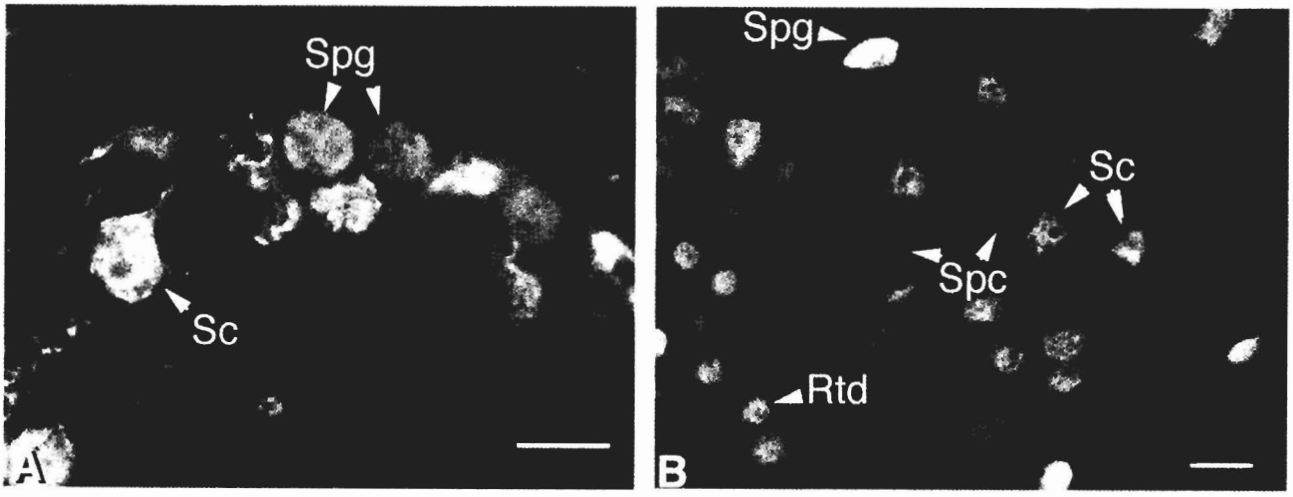

Fig. 4. Expression pattern of **(A)** hnRNPA1 and **(B)** hnRNPU in the human testis. In either case the hnRNP was visualised by probing a section of adult human testis with a primary monoclonal antibody specific to the hnRNP and indirect immunofluorescence. Sc: Sertoli cell; Spg: spermatogonium; Spc: spermatocyte; Rtd: round spermatid. Bars: 10μm.

throughout the general nucleoplasm (reviewed by Huang and Spector, 1997). The punctate structures correspond to nuclear bodies identifiable by electron microscopy called interchromatin granules, perichromatin fibrils and coiled bodies, and are probably the sites of different biochemical processes. Coiled bodies do not contain detectable levels of SR proteins. Interchromatin granules correspond with the majority of the punctate structures, and are thought to be storage sites for splicing factors not currently involved in splicing. In contrast, the nucleoplasmic pool of splicing factors are likely to be involved in splicing reactions taking place on nascent transcripts. Splicing factors are mobile between these populations, and are thought to be recruited to sites of active transcription from interchromatin granules by cycles of phosphorylation and de-phosphorylation (Misteli and Spector, 1997). It has been suggested that these dynamic re-organisations are driven by transcription, with splicing factors being recruited by active transcription complexes (GamaCarvalho et al., 1997; Misteli et al., 1997; Zeng et al., 1997), perhaps mediated by the CTD of RNA polymerase II (Corden and Patturajan, 1997; Huang and Spector, 1997).

Recently it was shown that ubiquitously expressed SR proteins have dramatically different spatial organisations in the different cell types of the human testis (Elliott et al., 1998) (Fig. 5). In spermatogonia, SR proteins are distributed between a punctate (speckled in appearance) population and a more general nucleoplasmic population. Differentiation into spermatocytes is associated with a change in this nuclear organisation. The level of nucleoplasmic protein falls, while there is an increase in the punctate population. The nuclear organisation subsequently changes again in round spermatids, with an increase in nucleoplasmic SR proteins and usually a single punctate site containing high levels of SR proteins. Moreover, individual SR proteins have distinct, while overlapping nuclear distributions. Figure 5a shows the spatial organisation of SR proteins recognised by monoclonal antibody 16H3 (a subset of SR proteins which contain runs of arginine alternating with glutamate and aspartate), while Figure 5b shows the spatial organisation of SF2/ASF. It has been argued from studies of tissue culture cells that splicing factors are recruited by the transcriptional machinery to sites of local demand (Huang and Spector, 1997). However, differences in the distribution of splicing factors in germ cell development are not driven by changes in transcriptional activity alone, since transcription is maximal in the pachytene stage in which the punctate structures are more pronounced, and lower in the preceding spermatogonial stage (Monesi, 1964).

A number of transcripts are known to be differentially spliced over the course of germ cell development including DNA methyltransferase (Mertineit et al., 1998), nuclear lamins (Furukawa and Hotta, 1993), and sox proteins (Kanai et al., 1996). Hence differences in alternative splice site choice are likely to take place on a background of differences in nuclear organisation.

These observations raise an intriguing possibility. As discussed above the ratio of SR proteins can profoundly affect splice site choice, and so it is possible that differential splicing patterns might be established in part by changes in the intranuclear ratios of splicing proteins within individual nuclear compartments. In this case it will be the intranuclear ratio which is of paramount importance, established between the SR proteins directly involved in splicing, and those which are in storage compartments. Moreover, these intracellular ratios might be established and modulated by signal transduction pathways independently of the transcriptional activity of the cells. Importantly, differences in intracellular ratios would not be detectable by comparing total levels of

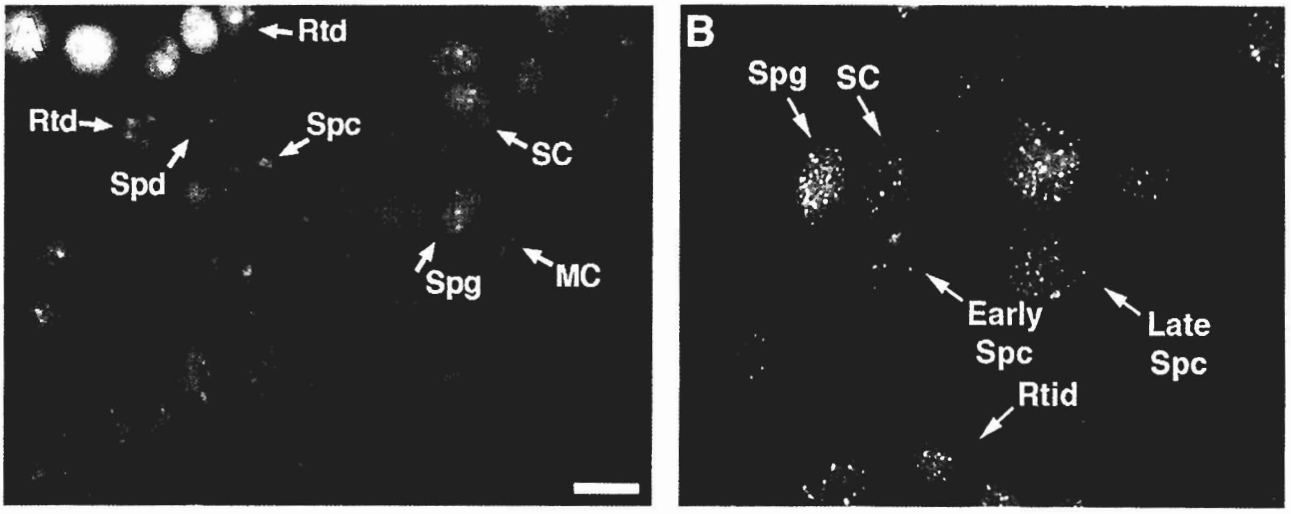

Fig. 5. Expression pattern of **(A)** SR proteins recognised by monoclonal antibody 16H3 and **(B)** ASF/SF2 visualised by indirect immunofluorescence in sections of adult human testis. Cell types are labelled as in Fig. 4, with the addition of MC (myoid cells). Bar: 10μm.

protein between tissues on Western blots, only by direct examination of sections of tissue (Elliott et al., 1998). A conceptually similar model has been proposed to account for regional differences in gene transcription during early insect development in which the local ratio of transcription factors is an important component regulating promoter response (reviewed in Gerhart and Kirschner, 1997). Similarities in the modular nature of the splicing machinery assembling on ESEs, and proteins binding to tissue-specific transcriptional enhancers for RNA polymerase II have been described (Hertel et al., 1997). Hence both RNA and DNA sequence elements which are responsive to different concentrations of interacting proteins may be a central paradigm of differential gene expression.

What other evidence is there with which to judge this model? In tissue culture cells the splicing factor SRp20 is found only in a subset of regions of the nucleus containing other splicing factors (Neugebauer and Roth, 1997), suggesting functional heterogeneity between different nuclear regions containing splicing factors even in homogeneous populations of cells. However, the model proposed here applies to tissue as opposed to tissue culture, and so will have to be examined in this context. The spatial organisation of splicing machinery has only been examined in very few intact tissues containing different cell types, but in these cases it has been found to be more complex than that which might be anticipated from cultured cells. Tra-2 protein in *Drosophila melanogaster* spermatocytes is found in large punctate structures (Dauwalder and Mattox, 1998), and splicing factors in *Drosphila* neural tissues also do not have speckled localisation typical of cells grown in culture (Yannoni and White, 1997). Mutations in the *Drosophila* LAMMER kinase Darkener of Apricot (Doa) affect both alternative splicing choices, and the subnuclear distribution of splicing factors (Du et al., 1998). While not directly tested in this study it is a prediction of our model that changes in splice site selection will occur in the same cell types as those in which subnuclear location of splicing factors changes. In mammals there are even fewer examples of the distribution of splicing factors in intact tissues, but it is known that coiled bodies are re-organised during neuronal differentiation in the rat hippocampus (Santama et al., 1996). In a tissue culture model of human mammary gland differentiation, a pre-mRNA splicing factor became re-localised from a number of heterogeneous speckles into around 7 large speckles on differentiation (Lelievre et al., 1998). Other tissue culture models have also shown splicing factor re-distributions on differentiation (Antoniou et al., 1993).

How are these cell-type specific nuclear organisations of pre-mRNA splicing factors established in tissue? A strong possibility is by the differential modification of ubiquitously expressed splicing factors in different cell types. Good candidates for this kind of regulation are the SR family of proteins, which are modified by phosphorylation of their RS domains. A number of SR-specific kinases which carry out this kind of phosphorylation have been identified. These fall into two families which likely have different targets (Colwill et al., 1996a). There are two SR protein-specific kinases, SRPK1 and SRPK2, and these show different (although overlapping) levels of expression in different mammalian tissues with SRPK1 expressed at high levels in the pancreas and SRPK2 at high levels in the brain (Wang et al., 1998). The second family are the cdc-like kinases (clk) 1-4. In either case the ratio of these kinases within the cell types making up the tissues is unknown. It is possible that tissues with similar overall levels of kinases/phosphatases might represent the average of cell types expressing both very high and low levels of the respective enzymes. It has been shown that expression of both SRPKs and the clk family of protein kinases in tissue culture cells causes the re-organisation of SR proteins from a speckled distribution to a more general nucleoplasmic location (Colwill et al, 1996; Wang et al., 1998). Expression of clk1 protein kinase affects the splicing pathway both of its own pre-mRNA, and an adenovirus pre-mRNA, presumably mediated by changes in the phosphorylation status of SR proteins (Duncan et al., 1997). Phosphatases are also likely to be important for organising the nuclear location of SR proteins (Misteli and Spector, 1997).

Conclusions

Cell type-specific pre-mRNA splicing patterns are frequently observed. In a few cases the sequence elements controlling alternative splicing have been identified. These elements either positively regulate splice site choice by stabilising the interaction of the splicing machinery with weak splice sites, or negatively regulate splice site choice, usually by occluding splice sites which would otherwise be used. Physiologically correct splice site choice frequently is achieved by a balance of positive and negative regulation. Surprisingly, the proteins which have been implicated in splice site choice are expressed in cell types making different splice site choices. This might mean that the critical cell-specific proteins have yet to be identified; or alternatively that more subtle mechanisms are in operation, perhaps including differential modification of splicing factors in different cell types, or differences in their cellular ratios. Based on recent observations of the nuclear distributions of splicing factors in different cell types within tissues, it is possible that an important factor might be ratios of ubiquitously expressed splicing factors in different nuclear compartments.

Acknowledgements. This work was supported by the MRC and the Caledonian Research Foundation. DJE is a Caledonian Research Fellow. We thank Drs. Neugebauer, Dreyfuss, Kamma and Caceres for monoclonal antibodies, and Professor Howard Cooke, Dr. Javier Caceres and Julian Venables for comments on the manuscript.

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Accepted July 14, 1999