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Control of Pre-MRNA Processing by Extracellular Signals: Emerging Molecular Mechanisms

Review Article

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Summary

Alternative splicing is an important mechanism to regulate gene expression. At least 30% of all human genes are alternatively spliced. This process can be regulated by extracellular signals that include stress and cellular activity. Splice site selection is regulated by a multiprotein complex. Its composition can be regulated by either releasing proteins from nuclear storage sites or by changing protein:protein, as well as protein:RNA interactions by serine and tyrosine phosphorylation.

I. Introduction

The expression of genetic information is controlled at several stages, such as DNA structure, transcription, pre-mRNA processing, translation and protein stability. To date, the most studied control mechanism is transcription. However, recent studies emphasized the importance of RNA metabolism in regulation of gene expression: RNA plays a crucial role in epigenetic regulation (Wolffe and Matzke, 1999), RNA editing is important for proper brain function (Sprengel et al., 1999), information stored in RNA can flow back into the genome (Cousineau et al., 2000) and almost all human genes are spliced by at least two splicing systems, and about 30% of them are alternatively spliced (Hanke et al., 1999; Mironov et al., 1999). The regulation of splicing was probably crucial for the evolution of eukaryotes (Herbert and Rich, 1999). Splice site recognition is helped by auxiliary proteins (trans factors) binding to short degenerate sequences on the RNA (exonic sequence elements). The fine tuned concentration of these trans factors governs splice site selection, both *in vivo* and *in vitro* (Black, 1995; Grabowski, 1998; Manley and Tacke, 1996). Proper splicing regulation is important for an organism, as it has been estimated that up to 15% of genetic defects caused by point mutations in humans manifest themselves as pre-mRNA splicing defects caused by changing splice site sequences (Krawczak et al., 1992; Nakai and Sakamoto, 1994) . In addition, it became apparent that point mutations in exons can cause missplicing by changing exonic sequence elements (Cooper and Mattox, 1997), for example in tauopathies (Gao et al., 2000) or spinal muscular atrophy (Lorson et al., 1999). A recent survey of disease-associated genes suggested that as much as a third of them might be alternatively spliced, suggesting that more pathologies might be associated with splicing defects (Hanke et al., 1999).

Alternative splicing pathways are not static, because an organism can dynamically change its splicing patterns, e.g. during development and/or in response to extracellular stimuli such as insulin (Smith et al., 1999), nerve growth factor (Varani and Nagai, 1998), cytokines (Eissa et al., 1996; Reddy, 1989), and neuronal activity (Daoud et al., 1999; Vezzani et al., 1995). Some changes of splicing patterns require protein synthesis and may be based on the differential transcriptional control of splicing factor expression (Shifrin and Neel, 1993). One prominent example for a protein dependent change in splice site selection are the changes in the development of cancer. Here, the processing of CD44 changes during the transition of preneoplasias to neoplasias and their metastases which is associated with *de novo* synthesis of several SR proteins

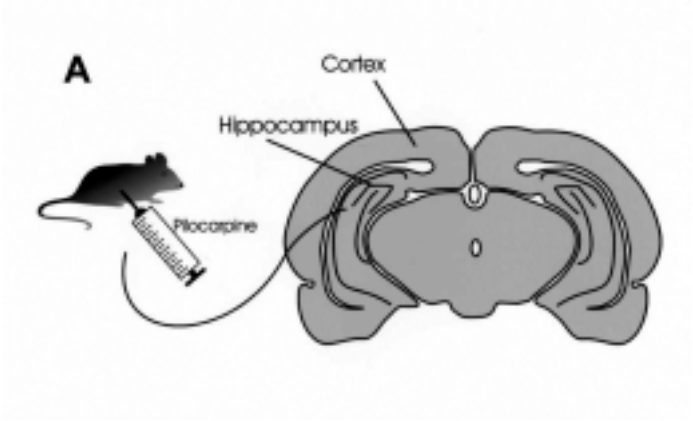

Figure 1A : The effect of pilocarpine on alternatively spliced genes
(A) Experimental paradigm. Rats are injected intraperitoneally with pilocarpine, a cholinergic muscarinic agonist that can cross the blood brain barrier. As a result the drug causes strong neuronal activity in neurons of the hippocampus resulting in seizures that resemble an epileptic episode. The diagram shows a coronal cross section of the brain.

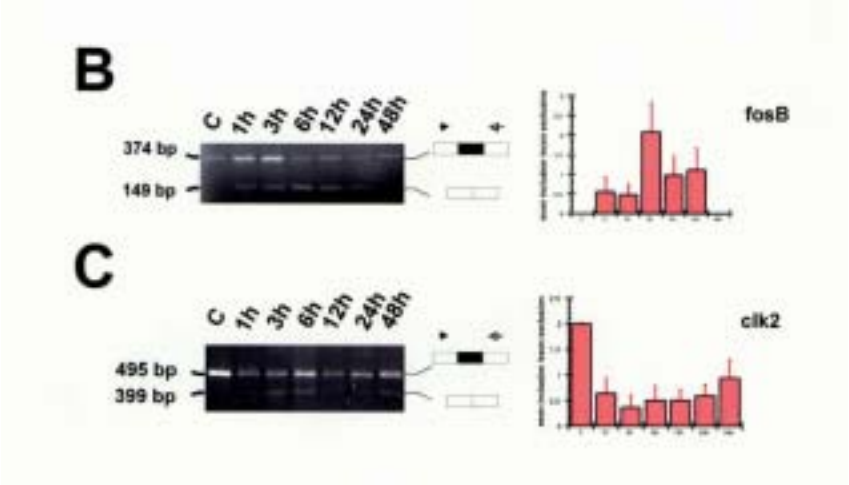

Figure 1 B-C: **The effect of pilocarpine on alternatively spliced genes** RT-PCR analysis of hippocampal RNA from pilocarpine treated rats. Pilocarpine increases neuronal activity in the brain that starts in the hippocampal formation. Removal of tissue after pilocarpine injection was at the time indicated. C: untreated control. A statistical evaluation for three experiments is given on the right. Standard deviations are indicated. Location of primers and cDNA structure are schematically indicated for each gene.
(B) RT PCR for fos B and its statistical evaluation
(C) RT PCR for clk2 and its statistical evaluation

(Stickeler et al., 1999). In addition, there is growing evidence that alternative splicing may also be regulated by transient covalent modifications of proteins implicated in mRNA splicing. For example, inclusion of CD44 exon v5 is independent of *de novo* protein synthesis and is coupled to a kinase downstream of Ras (König et al., 1998). Specific examples of changes in pre-mRNA processing after external stimuli have been compiled in this issue (Stoss et al., 2000). Here, we summarize changes of alternative splicing in response to stress and discuss the possible regulatory mechanisms.

II. Change of splice site selection evoked by cellular activity

The influence of cellular stimulation on alternative splice site selection has been mostly studied in the brain, because acute stress that is reflected in neuronal activity promotes neuroanatomic changes and increases the risk for neurodegeneration (McEwen, 1999). In several model systems, a change in alternative splice site selection after cellular stress has been observed. The gene of acetylcholinesterase generates two isoforms by alternative usage of an intron located at the end of the open reading frame (Kaufer et al., 1998). In normal brains, this "read-through" (AchE-R) variant is hardly detectable. However, when mice are subjected to acute stress by being forced to swim, this read through variant is upregulated. In contrast to the dominant "synaptic" variant (AchE-S), the AchE-R variant is soluble and monomeric. Physiologically, this switch is seen as a means to prevent neurodegenration, caused by excess activity of AchE-S (Sternfeld et al., 2000). Similar effects have been seen with potassium channels, where the effect is most likely hormone mediated (Xie and McCobb, 1998)

Another system studied is a change in neuronal activity evoked by pilocarpine (Daoud et al., 1999). Pilocarpine is a cholinergic muscarinic agonist that crosses the blood-brain barrier. This system has been used as model

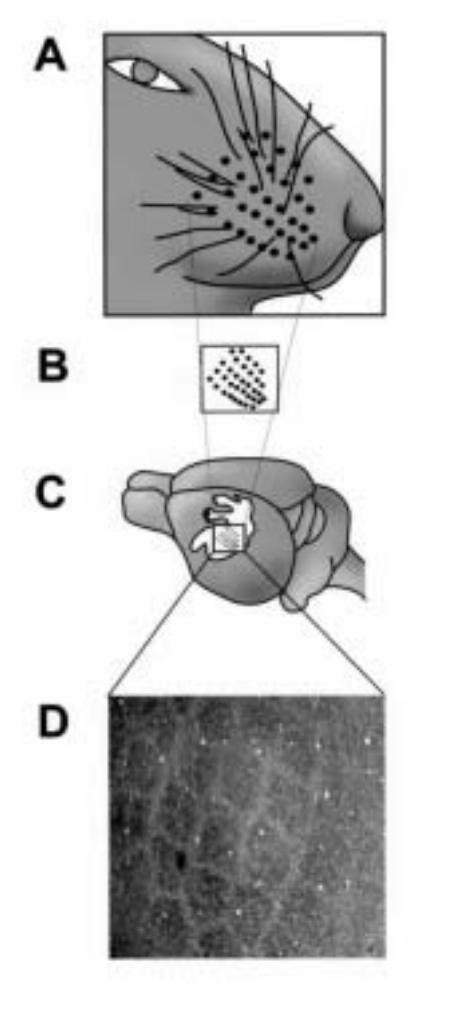

Figure 2 A-D: The effect of whisker removal on alternative splice site selection in the rat barrel cortex.
The experimental system. (A) shows the rat's face and its whisker pad. Some whiskers are already removed. (B) is a diagram of the whisker pad. (C) is a drawing of a rat brain, in which the somatosensory representation of the rat's body surface is indicated. The representation of the whisker pad is indicated. (D) is a cytochrome oxidase staining of the region in the somatosensory cortex S1. Each of the barrel shaped structures represents a single whisker. Removal of a whisker decreases the neuronal activity in this region ("barrel").

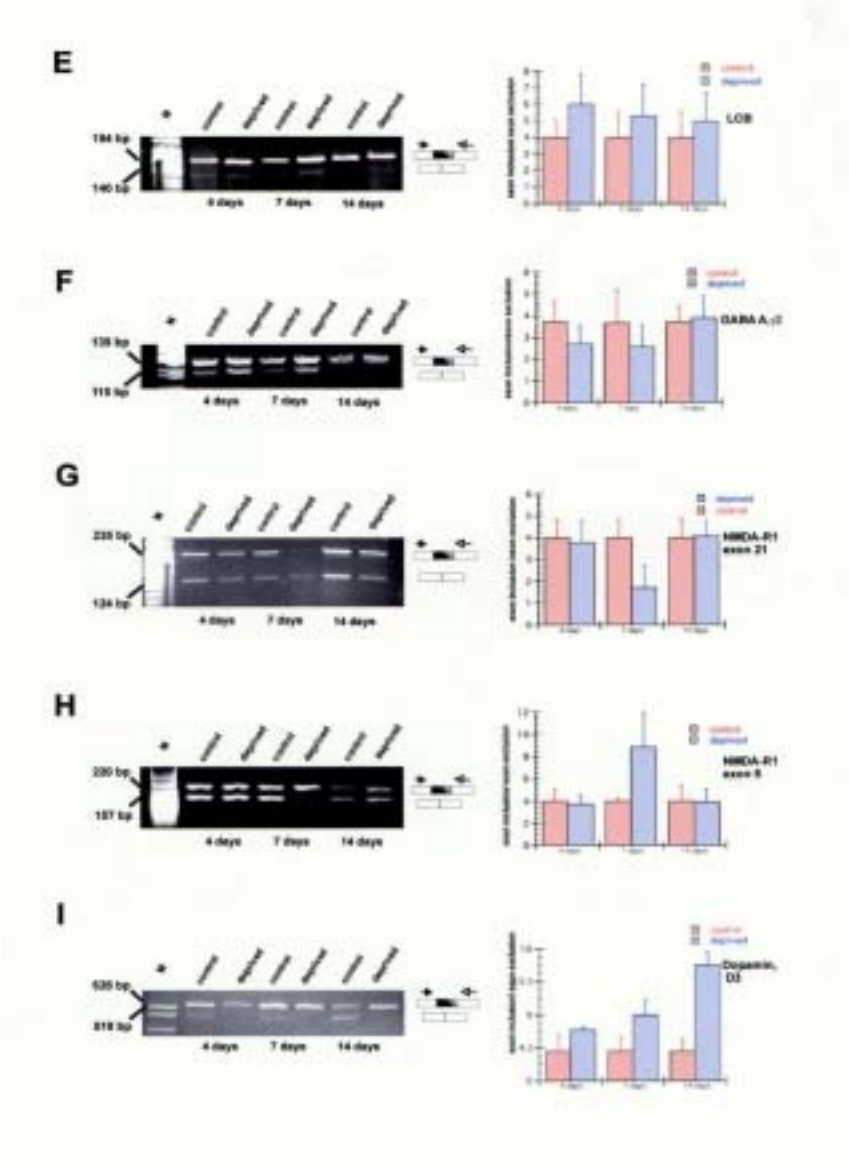

Figure 2: E-I: Effect of whisker removal on alternative splicing patterns in the barrel cortex.
RNA was isolated after the whiskers were repeatedly removed (deprived) for the time indicated (4, 7, 14 days). As a control, the colateral side was used. Whiskers corresponding to this side were not removed. A statistical evaluation for three experiments is given on the right. Standard deviations are indicated. Location of primers and cDNA structure are schematically indicated for each gene. The alternatively splice genes were:
(E) Clathrin light chain B, exon EN (Stamm et al., 1992)
(F)GABA A receptor, gamma2 subunit, alternative exon (Wang and Grabowski, 1996)
(G) NMDA receptor1, exon 21, (Hollmann et al., 1993; Zimmer et al., 1995)
(H) NMDA receptor1, exon 5, (Hollmann et al., 1993; Zimmer et al., 1995)
(I) Dopamin D3 receptor, (Giros et al., 1991)

for human temporal lobe epilepsy (Turski, 1983; Turski et al., 1984). After neurons were stimulated with this drug, the pre-mRNA processing of the splicing factor transformer2-beta (tra2-beta) is changed (Daoud et al., 1999). The tra2-beta gene generates at least five isoforms that can encode three open reading frames (Nayler et al., 1998a) from which two, htra2-beta1 and beta2, are translated into protein (Daoud et al., 1999). Neuronal stimulation causes a shift from the beta1 to the beta3 isoform. Since both transformer proteins have different abilities to regulate splice site selection when tested in cotransfection experiments (Stamm et al., 1999), it is likely that this switch changes the alternative splicing patterns of several still unknown target genes. In agreement with this hypothesis, the splicing patterns of clathrin light chain B, exon EN, NMDA receptor 1, exon 22 and the neuron specific exon of c-src are changed after pilocarpine treatment (Daoud et al., 1999). In addition, the splicing patterns of the fosB gene and the SR protein kinase clk2 change after pilocarpine induced neuronal activity as well (**Figure 1**). A change of the splicing pattern of the NMDA receptor1 has also been observed in a kindling model, in which neuronal activity is evoked by repeated electrical stimulation (Vezzani et al., 1995). Finally, induction of long term potentiation was shown to regulate syntaxin 3 isoforms (Rodger et al., 1998).

Together, these data indicate that after a strong burst of neuronal activity, different isoforms of splicing regulatory proteins are generated, which changes the processing of a number of genes. In both of these systems a change in splice site selection was observed after an increase of neuronal activity. We used the rat barrel cortex as a third model, in which neuronal activity is decreased. In rodents, the facial whiskers indirectly project to a region in the primary somatosensory cortex S1 (**Figure 2A**) (Van der Loos and Woolsey, 1973; Woolsey and Van der Loos, 1970). Each whisker is represented by an arrangement of cortical neurons that resembles a barrel. Stimulation of a whisker increases the activity of the neurons corresponding to this whisker, which can be detected histologically by cytochrome oxidase staining (Wong-Riley and Welt, 1980). When whiskers are completely removed on one side of the animal's snout, alternative splice site selection in several genes of the corresponding barrel cortex are changed (**Figure 2B, C**). This indicates that a change in cellular activity, not just an increase, results in differences of pre-mRNA processing.

Cellular activity is not the only stress condition that alters splice site selection, as a change of alternative splicing patterns is also seen in response to temperature shock. Alternative splicing of the human neurofibromatosis type 1 (NF1) gene (Ars et al., 2000) and the potato invertase gene (Bournay et al., 1996) are induced by cold shock, whereas a rise in temperature changes the splicing pattern of heat shock protein 47 (Takechi et al., 1994) and the collagen A1 gene of a Danlos syndrome patient (Weil et al., 1989). Finally, osmotic stress changes the pre-mRNA processing of an adenovirus reporter gene (van Oordt et al., 2000).

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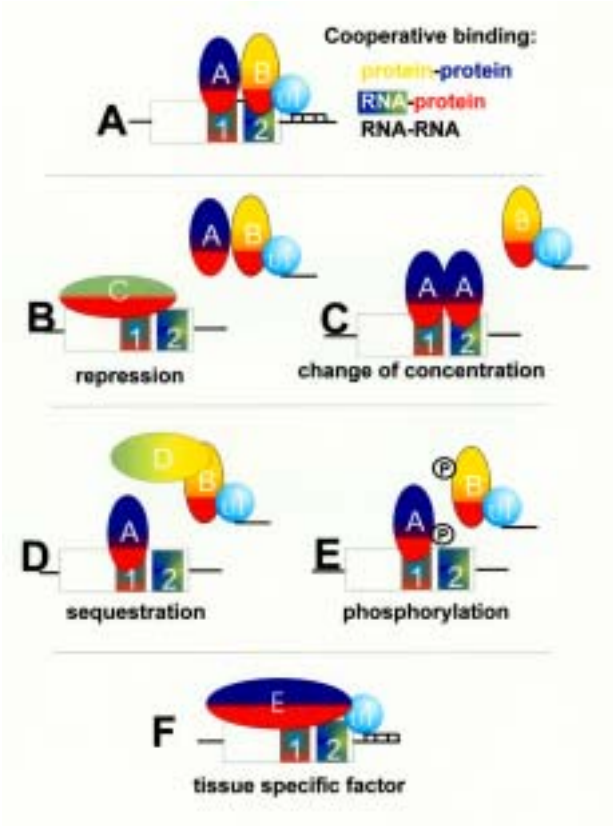

Figure 3: Model for alternative splice site regulation

(A) An exon (box) is recognized by binding of multiple proteins (A, B) on splicing enhancers located on the pre-mRNA (1, 2). Splicing of this exon is initiated by contact with components of the constitutive splicing machinery, shown here as the U1 snRNP (U1). The formation of this complex is cooperative and involves protein: protein interactions, RNA:protein interaction and RNA: RNA interactions that are indicated by different colors.

This complex can be regulated by several ways:

(B) A protein C with higher affinity to the enhancer sequences can repress the exon usage by competing with proteins A and B for binding to the exon.

(C) The recognition is concentration dependent, as an increase of the concentration of protein A could compete for binding with protein B.

(D) Likewise protein B could be sequestered by a different protein D

(E) Phosphorylation can influence the binding of individual factors assembling around exon enhancers

(F) Tissue specific factors can recognize a protein in a cell type or tissue specific way.

Figure 4

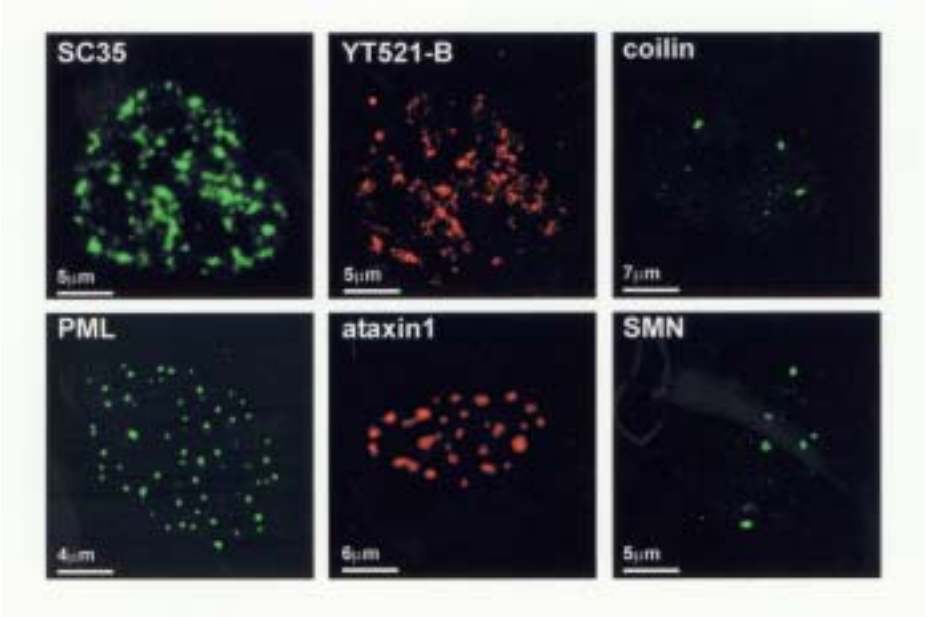

Figure 4: Subnuclear compartments visualized with endogenous antibodies of as GFP-fusion proteins (ataxin-1)
Subnuclear compartments include SC35-speckles (Fu and Maniatis, 1992) , YT-bodies containing YT521-B (Nayler et al., 2000) , coiled bodies (Carmo-Fonseca et al., 1992) containing coilin, PML bodies (Grande et al., 1996) ,, nuclear inclusion, such as the ones formed by ataxin-1 (Skinner et al., 1997) , and gems (Liu and Dreyfuss, 1996) formed by SMN (survival of motoneuron).

Figure 5

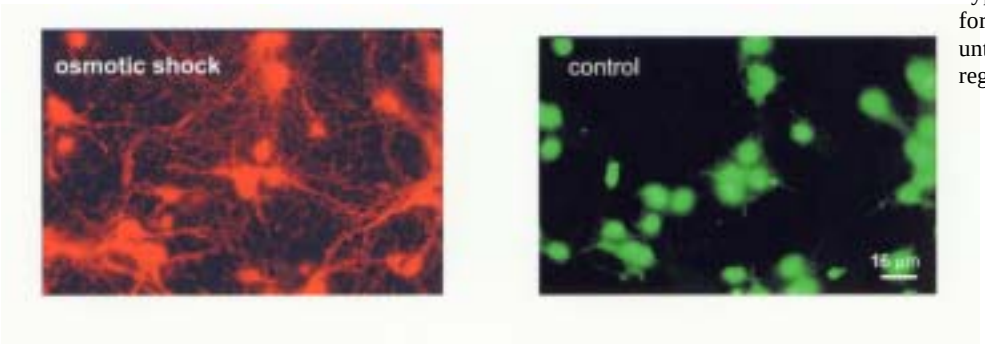

Figure 5: Stress evoked by osmotic shock changes the intracellular localization of the splicing regulatory proteins htra2-beta1
Cells were subject to 3 hours of hyperosmotic medium and then stained for htra2-beta1 (left). When compared to untreated cells, a translocation of splicing regulatory proteins is apparent.

Together, these results show that a change in pre-mRNA splicing patterns is a common cellular adaptation to stress and cellular activity. This raises the question how the signal is transduced to the spliceosome

III. Mechanisms of alternative splice site selection

Although there has been tremendous progress in elucidating the mechanisms regulating constitutive splicing, the rules governing alternative splice site selection still remain elusive. Since the general mechanism has been reviewed in this volume (Stoss et al., 2000), we concentrate on the question how the recognition of an alternative exon can be modulated. All the elements on the RNA that govern splice site selection are only weakly conserved (Berget, 1995; Breitbart et al., 1987; Stamm et al., 2000) . The high fidelity observed in splicing is therefore achieved by the formation of a protein-RNA complex, that involved cooperative binding of several molecules (**Figure 3**). On the pre-mRNA, sequences known as exonic enhancers or silencers have been identified and were shown to bind to splicing regulatory proteins, such as SR proteins and hnRNPs (reviewed in: (Cooper and Mattox, 1997; Hertel et al., 1997; Manley and Tacke, 1996; Stamm et al., 2000; Stoss et al., 2000)). SR proteins multimerize and can bind to components of the spliceosome, e.g. to the U1 snRNP particle (Wu and Maniatis, 1993). The 5' end of the U1 snRNA present in this particle hybridizes to the 5' site, which initiates the recognition of an exon (Zhuang and Weiner, 1986). As a result the initial recognition of an exon is regulated by protein:protein interaction (e.g. between different SR proteins), by protein:RNA interactions (e.g. between an SR protein and a splicing enhancer) and by RNA:RNA interactions (e.g. between U1snRNA and the 5'

splice site) (**Figure 3A**). *In vitro* models suggest that the formation of this multi-protein:RNA complex involves cooperative binding of the individual components (Hertel and Maniatis, 1998) . As a result, splice site recognition is dependent on the relative concentration of regulatory proteins and can be influenced change in concentration of a constitutively expressed factor can alter the composition of the protein complex forming around an enhancer, which can either decrease (**Figure 3C**) or increase exon usage (Cáceres et al., 1994; Caputi et al., 1999; Coulter et al., 1997; Hanamura et al., 1998; Mayeda and Krainer, 1992; Wang and Manley, 1995) (iii) factors necessary for recognition can be sequestered by binding to a different protein (**Figure 3D**) (Hartmann et al., 1999; Nayler et al., 1998c) (iv) the interaction of proteins can be regulated by phosphorylation (**Figure 3E**) (Colwill et al., 1996; Duncan et al., 1997; Fu, 1995; Prasad et al., 1999) (v) tissue specific factors can recognize exons in a cell type specific way (**Figure 3F**) (Jensen et al., 2000; Polydorides et al., 2000).

A number of studies have revealed that transcription and splice site selection (Cramer et al., 1999) are occurring concomitantly in a large complex that was termed 'RNA factory' (McCracken et al., 1997) or transcriptosomal complex (Corden and Patturajan, 1997), which is probably associated with components the nuclear matrix (Bode et al., 2000; Nayler et al., 1998c). Various components of this complex are stored in subnuclear compartments (**Figure 4**) and can be released into the nucleoplasma by regulatory mechanisms, such a phosphorylation. One of the best studied example of this domains are nuclear speckles in which splicing factors are stored until they are released e.g. by phosphorylation (Huang and Spector, 1996; Misteli et al., 1998; Misteli et al., 1997; Spector, 1993) . Nuclear factories and storage compartments are dynamically linked to RNA polymerase activity. Speckles change their morphology under the influence of transcriptional inhibitors (Carmo-Fonseca et al., 1992; Misteli et al., 1998; Misteli and Spector, 1999; Nayler et al., 1998b). This suggests the existence of a dynamically regulated nuclear architecture supporting the compartmentalization of the nucleus (Jackson et al., 1993; Ma et al., 1998; Nakayasu and Berezney, 1989) . The importance of proper regulation is apparent under cellular stress conditions. Activation of the MKK(3/6)-p38 pathway changes the subcelluar localization of several factors involved in pre-mRNA processing and ultimately results in a change in splice site selection (van Oordt et al., 2000) . Similar results are observed when primary neuronal cultures are subjected to stress evoked by osmotic shock. In this system, the splicing factor htra2-beta1 (Beil et al., 1997) is translocated into the neurites and the alternative splicing patterns of several genes change (**Figure 5**).

IV How are signals transduced to the spliceosome?

Despite the accumulating evidence that cellular activity influences splice site selection, the molecular mechanism that underline this phenomenon remain to be determined. It is clear however, that phosphorylation plays a fundamental role in regulation. The majority of proteins regulating alternative splicing are phosphorylated and it was shown that pre-mRNA processing is influenced by the tyrosine kinase activity of src (Gondranand Dautry, 1999; Neel et al., 1995). Since src is anchored to the cell membrane, the question how the phosphorylation signal reaches the nucleus needs to be addressed. Only a limited number of tyrosine kinases have been identified in the nucleus, among them Abl, Rak, Fes, Fer, Wee1 and Sik/Brk (reviewed in (Pendergast, 1996; Wang, 1994)). Most of these kinases shuttle between nucleus and cytosol and it is possible that they phosphorylate proteins participating in pre-mRNA processing. For example, Sik/BRK was shown to phosphorylate Sam68, a process which regulates the RNA binding activity of Sam68 (Derry et al., 2000) and most likely the composition of the protein complex forming around Sam68 (**Figure 3E**) (Chen et al., 1997; Hartmann et al., 1999). The composition of this complex will most likely influence splice site selection by controlling the recognition of exon enhancers (**Figure 3**).

Furthermore, serine phosphorylation of SF1 (Berglund et al., 1998; Rain et al., 1998), a factor that recognizes the branch point and is therefore important for the formation of the spliceosomal A complex, was shown to be regulated by PKG-I (Wang et al., 1999). This kinase is activated by cGMP. The cGMP level itself can be regulated by a membrane bound guanyl cyclase receptor that is activated by natriuretic peptides or by a cytoplasmic guanyl cyclase which is activated by nitric oxide (NO). Phosphorylation of SF1 on Ser20 inhibits the SF1-U2AF65 interaction, leading to a block of pre-spliceosome assembly. One of the best studied class of splicing regulatory proteins are SR proteins that are regulated by serine phosphorylation. Several SR protein kinases have been identified (reviewed by (Stoss et al., 2000), this volume) and the identification of their upstream regulatory kinases will help to identify the signal transduction pathways regulating pre-mRNA processing.

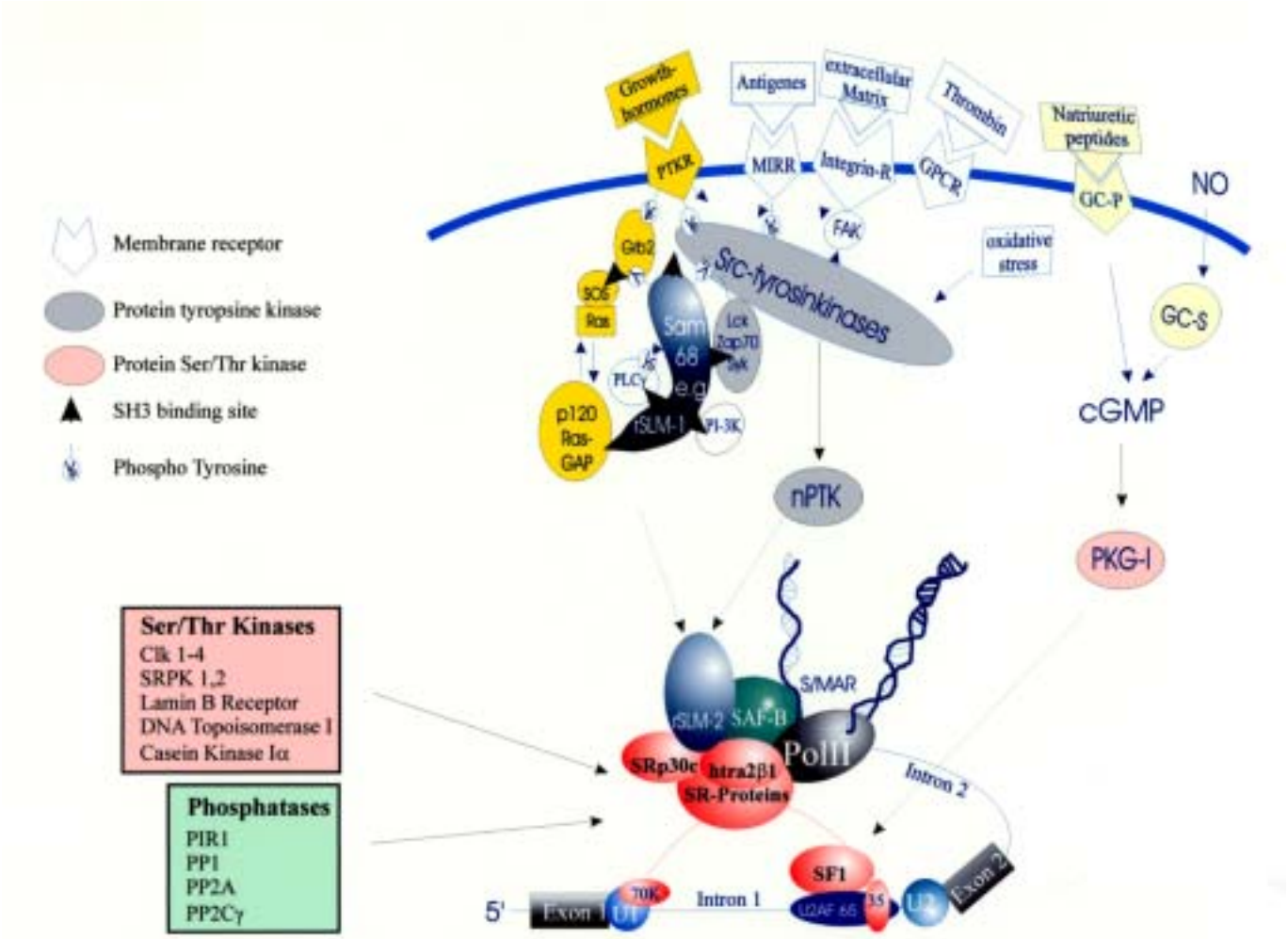

Figure 6. Signal transduction and splicing: A model

An overview of possible signal transduction pathways and their links to the spliceosome is shown. Extracellular stimuli and factors are indicated on the top and are boxed. Some of the receptors are shown below. Their phosphorylation (YP) leads to activation of src tyrosine kinases, which likely stimulate nuclear tyrosine kinases (nPTK), such as abl, Rak, Fes, Fer, Wee1 and Sik/Brk (Pendergast, 1996) . These proteins phosphorylate nuclear proteins, which affects splice site selection by changing the composition of splice site enhancer complexes.

Similar, a change in serine phosphorylation can change the composition of splice site enhancer complexes. Several nuclear kinases and phosphatases have been identified, but the upstream kinases and signals remain to be determined (Stojdl and Bell, 1999) . Nitric oxide (NO) and natriuretic peptides activate guanylyl cyclase either in the plasma membrane (GC-P) or in the cytosol (GC-S), which leads to the activation of phospho-kinase G-I (PKG-I) that phosphorylates splicing factor 1 (SF1) (Wang et al., 1999)

PTKR: phosoph-tyrosine receptor; MIRR: multichain immune recognition receptor; FAK: focal adhesion kinase; GPCR: G-protein coupled receptor; GC-P: GTP cyclase, plasma membrane bound; GC-S GTP cyclase, soluble; PKG-I: Phospho kinase G-I; sos: son of sevenless; clk: cdc2 like kinase; SRPK: SR protein kinase; PP: protein phosphatase; SAF-B: scaffold attachment factor B; rSLM-2: rat SAM68 like molecule; polII: RNA polymerase II; SR: protein: serine-arginine-rich protein; U1, U2: U1, U2: U1-U4 snRNP; U2AF: U2 auxiliary factor

IV. Conclusions

The regulation of transcription by signal transduction pathways is well documented. Since at least 30% of all human genes are subject to alternative splicing, regulation of splice site selection after an extracellular signal seems to be another important mechanism to regulate gene expression. Although it is clear that phosphorylation events are involved in mediating this signal, the identification of the molecular players remains the challenge of the future.

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