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Functional Roles of Nova in RNA Metabolism

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Abstract:

Nova-1 is a neuron-specific RNA binding protein, distributed both in the nucleus and in the cytoplasm of several populations of neurons. The work presented here confirms that Nova-1 binds a sequence of RNA found in an intron of the glycine receptor $\alpha 2$ subunit pre-mRNA. Evidence that Nova-1 acts as a regulator of alternative splicing in transfected cell lines is presented. Furthermore, a direct role of Nova-1 on splicing is demonstrated by establishing an *in vitro* assay for Nova-1's regulatory role in splicing. Potential functional partners of Nova are suggested by the demonstration of physical interactions between Nova-1 and molecules whose action in the splicing machinery is well described, such as U1 70K, a protein component of U1snRNP, and U2AF65.

The sedimentation properties of Nova in neuronal cytoplasmic extracts are consistent with the engagement of Nova in heterogeneous structures, probably mRNPs, and with polysomes, suggesting a role for Nova in the regulation of cytoplasmic phenomena of RNA metabolism, such as mRNA localization and translation. These considerations prompted a gene expression screen aimed to identify differences in the pool of mRNAs associated with heavy polysomes in wild type and Nova-1-null mice. Several genes whose mRNAs have been found to undergo changes in abundance specifically in the polysome fraction in the absence of Nova have described functional roles in post-synaptic terminal structure and functions. Nova-1 was also found to be a phospho-protein, and its phosphorylation site is in an alternative cassette exon.

Chapter 1

AN INTEGRATED VIEW OF GENE EXPRESSION

The journey of RNA from transcription to translation into protein has traditionally been described as a series of discrete steps that happen in well-defined compartments of the cell. Upon the completion of transcription, the pre-mRNA, coated by hnRNPs, undergoes capping of the 5' end, splicing of the intronic sequences, cleavage and polyadenylation in the nucleus; the mature mRNA is then exported in the cytoplasm, where it is translated and eventually decays. This view of gene expression as a succession of discrete steps was suggested by the ability of in vitro assays to re-capitulate many of these events in isolation and by the non-overlapping localization in the nucleus of sites of transcription and sites containing splicing factors. While the view of a highly structurally and functionally compartmentalized cell and nucleus is still valid (Carmo-Fonseca, 2002; Spector, 2001), studies performed during the last decade are delineating a rather more integrated picture of the mechanisms of gene expression. Furthermore, it is becoming apparent that some RNA binding proteins are capable of playing more than one functional role in different compartments of the cell. The early post-transcriptional events of capping, splicing and poly-adenylation seem to all take place on RNAs which are still undergoing transcription or still engaged in the transcription machinery. I will briefly review some evidence of such integration, focusing on the splicing events.

1) Transcription and pre-mRNA splicing:

The idea that pre-mRNA splicing happens co-transcriptionally dates back to almost twenty years ago, when Fakan *et al.* suggested such relationship based on the localization of a subset of snRNPs in Perichromatin Fibrils, structures known to be enriched in newly synthesized pre-mRNAs (Fakan et al., 1984; Fakan et al., 1986). Furthermore, RNAs apparently undergoing splicing while still being transcribed were directly visualized by Beyer and Osheim by electron microscopy on chromatin spreads (Beyer and Osheim, 1988; Osheim and Beyer, 1991). In the following years, a number of lines of evidence led to the current model, according to which pre-mRNAs are spliced co-transcriptionally, while Interchromatin Granule Clusters and Cajal bodies are highly dynamic storage and/or reassembly sites for SR proteins and snRNP, respectively, (Zhang et al., 1994), reviewed in Huang and Spector, 1997).

The influence of the transcriptional promoter on the subsequent steps of RNA processing was first suggested by the observation that RNA polymerase II (RNAP II) genes expressed under the control of RNA polymerase III promoters were not processed properly (Sisodia et al., 1987). Subsequently, different RNAP II promoters, driving the transcription of the same α-globin/fibronectin reporter minigene, were shown to affect the splicing of an alternative exon (Cramer et al., 1999; Cramer et al., 1997). The ability of promoters to affect cassette exon utilization seems to correlate with their ability to influence RNAP II processivity, defined as the ability to elongate through sites where the polymerase is prone to pause or premature termination (Kadener et al., 2001; Kadener et al., 2002). Two not mutually exclusive models have been proposed to account for the observed effects of transcriptional enhancers, promoters and activators on alternative

splicing. According to one model, promoters would have differential ability to recruit different sets of splicing factors, which would then be able to differentially splice nascent RNAs. The alternative model proposes that the effect of promoters on splicing is due to their ability to provoke the commitment of more or less processive RNAP II. In the case of one alternatively included cassette exon, low RNAP II processivity and internal pause would favor the presentation of the upstream, alternative 3'splice site to the splicing apparatus before the downstream constitutive 3' splice site has been synthesized, thus favoring the exon-including product (Caceres and Kornblihtt, 2002). The kinetic model has been directly verified in recent studies, in which the processivity of RNAP II was decreased pharmacologically or by inserting pause elements downstream of the alternative exon (Nogues et al., 2002; Roberts et al., 1998).

The integration between RNA processing events described so far involve upstream events (like transcription) affecting downstream events (like splicing). A set of recent studies shows that RNA processing factors can feed back, in turn influencing transcription rate. *In vitro* studies show that TFIIH contains stochiometric amounts of U1 snRNA, and that depletion of U1 snRNA makes the TFIIH less efficient in "abortive initiation" and re-initiation assays (Kwek et al., 2002). Consistent with this finding, a promoter-proximal 5' splice site, recognized by U1 snRNA, stimulates the rate of TFIIH-dependent re-initiation *in vitro* and the overall rate of transcription *in vivo* (Furger et al., 2002; Kwek et al., 2002). Furthermore, the TAT-SF1 transcription factor was found to form a complex with U snRNPs, and to stimulate transcriptional elongation. The stimulation was more efficient when functional splice sites were present in the transcribed product (Fong and Zhou, 2001). These recent findings are beginning to

provide a mechanistic explanation for the increased efficiency of transcription of transgenes with artificial introns near the 5' end, observed more than ten years ago (Brinster et al., 1988; Palmiter et al., 1991).

2) Molecular Aspects of Transcription-RNA Processing integration:

Interestingly, RNAP II itself has been identified as a component of the pre-mRNA splicing machinery. The largest subunit of RNA polymerase II is characterized by the presence of an unusual carboxy-terminal domain (CTD), rich in potential phospho-acceptor aminoacid residues, comprised of a heptapeptide with the consensus Tyr-Ser-Pro-Thr-Ser-Pro-Ser tandemly repeated fifty two times (in human). The hypophosphorylated form of the RNApol II (RNAP II A) is associated with the pre-initiation complex, while the hyperphosphorylated form (RNAP II O) is associated with transcription elongation complexes that have cleared the promoter area (reviewed in (Howe, 2002)

RNAP IIO was found to be specifically associated with functional splicing complexes, and a dramatic inhibition of pre-mRNA splicing (and 3'processing) was observed in cells in which the only active form of RNAP II had a CTD truncation (McCracken et al., 1997b; Mortillaro et al., 1996). In a similar experimental setting, the recruitment of SC35 and U2 snRNP to the transcription sites was shown to be impaired (Misteli and Spector, 1999). These experiments suggest that the hyperphosphorylated CTD of RNAP II increases the local concentration of splicing factors, among which are SR proteins and SCAFs (SR-like CTD-associated factors), in proximity of the emerging transcript (Mortillaro et al., 1996; Misteli and Spector, 1999; (Yuryev et al., 1996). A direct role of

RNAP II in promoting splicing independent of its function in transcription was also demonstrated. In an *in vitro* splicing assay employing pre-transcribed substrates, phosphorylated RNAP II was shown to promote the assembly of an early splicing complex, whereas the unphosphorylated isoform of RNAP II was shown to have the opposite effect (Hirose et al., 1999).

The integration of transcription and splicing is only one aspect of a complex web of interactions that takes place in the nucleus during the early phases of RNA processing. The pre-RNA processing events of capping, splicing, and polyadenylation have been shown in recent studies to affect each other, and the hyperphosphorylated form of the CTD of RNAP II provides a platform for a number of biochemical interactions involved in the transcription and RNA processing events and their coupling (McCracken et al., 1997a), reviewed in (Hirose and Manley, 2000; Hirose et al., 1999; Maniatis and Reed, 2002; Proudfoot et al., 2002). Time course experiments have shown that RNA polymerases and RNA processing factors localize in large, discrete nuclear structures, the Cajal Bodies, upon their entry in the nucleus right after translation. Since no transcription or processing take place in the Cajal Bodies, these structures are currently interpreted as the assembly place of the large transcription-processing holoenzyme indicated as the transcriptosome (Gall, 2001; Gall et al., 1999). According to a recent model, from the Cajal Bodies, RNAP II and processing factors would then reach the transcription sites, where clusters of more than one polymerase would assemble into large "transcription factories", probably anchored to nucleo-skeletal structures (Cook, 1999; Iborra et al., 1996; Maniatis and Reed, 2002).

3) mRNA export is coupled with pre-mRNA splicing:

The field of mRNA export has seen striking progress in the past few years. Nucleo-cytoplasmic transport of protein and non-coding RNAs is largely mediated by transport receptors related (albeit often distantly) to Importinβ, moving their cargo (or complex of adapter and cargo) across the Nuclear Pore complex (NPC) along a gradient of RanGTP (Gorlich and Kutay, 1999). In only few instances has the export of mRNA been shown to fit this general paradigm. One such instance is the well characterized export of unspliced HIV genomic RNA. One intron in the unspliced HIV-1 RNAs contains the Rev Response Element (RRE), which is a binding site for several Rev adaptor molecules. The export of the RNA-Rev protein complex requires a nine aminoacid motif, characterized by four critically spaced Leucines, called Nuclear Export Signal (NES) (Malim et al., 1989). The NES motif has been found in several other proteins undergoing nucleo-cytoplasmic export, and has been shown to be required for the interaction with CRM1, an export receptor belonging to the importinβ family (Cullen, 2000; Fischer et al., 1995). Similarly, messages containing A rich elements (ARE) in their 3'UTR were shown to be exported through a CRM1-dependent pathway: the protein HuR binds the ARE of these mRNAs, and it interacts, in turn, with two NES-containing proteins, APRIL and pp32 (Brennan et al., 2000). Furthermore, a parallel HuR-transportin2-dependent pathway for the export of ARE-containing mRNAs was also discovered (Gallouzi and Steitz, 2001).

Unlike these few examples, though, the export of the bulk mRNA from nucleus to cytoplasm seems to follow a different route, as it is largely not affected by treatment with Leptomycin B, a specific inhibitor of CRM1 (Fornerod et al., 1997; Wolff et al., 1997).

In particular, recent studies have elucidated a strict coupling between export and pre-mRNA splicing. Experiments performed in Xenopus oocytes have shown that RNA resulting from splicing of precursors containing introns injected in the nucleus were exported with much higher efficiency than RNAs not containing introns. Despite the fact that RNAs resulting from splicing and intronless RNAs were identical in sequence, they were shown to be complexed with a different set of proteins, presumably recruited by the splicing machinery (Luo and Reed, 1999). This finding opened the hunt for the characterization of the splicing-dependent protein complex. In a series of elegant *in vitro* studies, a protein complex (called EJC: Exon Junction Complex) was shown to form twenty nucleotides upstream from the exon-exon junction following splicing, and to remain on the mRNA in the cytoplasm (Kataoka et al., 2000; Le Hir et al., 2000a; Le Hir et al., 2000b). The identification of some of the components of the complex, and genetic studies performed in yeast, led to the contemporary model of mRNA export. The splicing factor UAP56, a DEAD helicase originally discovered as a U2AF65 co-factor, was shown to recruit the protein adaptor Aly to the mRNA during the splicing reaction (Fleckner et al., 1997; Luo et al., 2001; Strasser and Hurt, 2001; Zhang and Green, 2001; Zhou et al., 2000). Aly/REF (and the yeast ortholog Yra 1) is bound, in turn, by the export receptor TAP (and the yeast ortholog Mex67), a protein that associates with the nuclear pore (Bachi et al., 2000; Segref et al., 1997). Studies in yeast indicate that TAP (Mex67) and UAP56 (Sub2, in yeast) interact with the same region of Aly (Yra1) (Strasser and Hurt, 2001). Therefore, according to the current model, the splicing machinery would recruit Aly to the mRNP through UAP56 during the splicing reaction. Aly could even be recruited co-transcriptionally, by interaction with the transcription

elongation factors of the THO complex (Strasser et al., 2002). Subsequently, TAP displaces UAP56 from its interaction with Aly and leads the mRNP to the nuclear pore for export (reviewed in (Maniatis and Reed, 2002; Reed and Hurt, 2002).

4) Possible alternative routes of mRNA export in Metazoa:

The model based on the function of EJC, Aly and TAP, which emerged mostly from studies in yeast, may not entirely account for the complexity of RNA export in metazoan cells. In a recent study, RNAi was used in Drosophila cells to ablate various components of the EJC separately and in combination. Interestingly, while TAP and UAP56 were shown to be required for export of the bulk of mRNA, even the simultaneous ablation of Aly, RNPS1, Y14 and Srm160 that is, all the known components of the EJC did not result in complete abolition of mRNA export, although a quite substantial nuclear accumulation of poly(A)+ RNA was observed (Gatfield and Izaurralde, 2002). This result raises the possibility that other essential adaptor proteins link UAP56 and TAP in higher eukaryotes, or other export receptors or parallel pathways exist. Consistent with this hypothesis, a number of RNA binding proteins have been implicated in the export of specific sets of messages. The heterogeneous nuclear ribonucleoproteins (hnRNP), a large family of around 20 proteins, are thought to bind nascent RNA during transcription, and to remain associated with nuclear mRNAs after splicing is completed (Nakielny and Dreyfuss, 1999). Certain hnRNPs, like hnRNP A1 and hnRNPK, have been shown to shuttle between nucleus and cytoplasm, while others, like hnRNPC, are found exclusively in the nucleus (Pinol-Roma and Dreyfuss, 1991; Pinol-Roma and Dreyfuss, 1992). The shuttling hnRNPs were the first group of proteins implicated in mRNA export for a

variety of reasons. They bind mRNA in both compartments (Pinol-Roma and Dreyfuss, 1992); their export is mediated by NES elements, which are not part of their RNA binding domain (Michael et al., 1995); excess of hnRNP A1, or of its M9 shuttling domain, specifically blocks export of dihydrofolate reductase (DHFR) mRNA (Gallouzi and Steitz, 2001; Izaurralde et al., 1997); an A1-like hnRNP is associated with translocating Balbiani ring transcripts from the transcription site to the cytoplasm, passing through the NPC (Daneholt, 1997); and yeast strains mutant in Np13p, a shuttling hnRNP, show nuclear mRNA accumulation (Lee et al., 1996). Therefore, it seems probable that hnRNPs play a role in the export of some mRNAs, but the details of the mechanism of this action and the features of the messages that undergo such mode of transport are not defined.

Another class of RNA binding proteins that may play a role in RNA export is the SR family of proteins. A subset of SR proteins have been shown to shuttle between the nucleus and the cytoplasm (Caceres et al., 1998). Moreover, SR proteins bind exclusively to exon sequences in pre-mRNA and remain bound to the exons after the mRNA is spliced (Graveley, 2000). Two shuttling SR proteins, SRp20 and 9G8 have been demonstrated to promote the nucleocytoplasmic export of intronless mRNA encoding histone H2A (Huang and Steitz, 2001). Furthermore, SRp20 and 9G8 can be UV-cross-linked to polyadenylated RNA in the nucleus and the cytoplasm, suggesting that they could affect some as yet unidentified step of RNA metabolism after the export from the nucleus (Huang and Steitz, 2001).

The coupling of splicing and export has been interpreted as an RNA proofreading mechanism. The mRNA, bearing protein coding information, represents a small

percentage of the total pre-mRNA transcript: the average size of an exon is 140 nucleotides while the size of introns can be in the thousands and tens of thousands of nucleotides. The splicing apparatus marks the final products of processing with a distinct set of proteins: the removed introns coated with a subset of hnRNPs, are prevented from exiting the nucleus and targeted to degradation, while the mRNA, complexed with adaptor proteins, export receptors, EJC proteins, and also some hnRNPs, is targeted for export and translation (reviewed in (Reed and Magni, 2001).

5) Nonsense mediated decay (NMD):

Interestingly, Nonsense mediated decay (NMD), another level of control of the quality of the exported message, has been found to be strictly coupled to the EJC deposited in the course of the nuclear processing of RNA. NMD occurs when translation terminates more than 50-55 nucleotides upstream of the 3'-most exon-exon junction. The EJC, which lays 20-24 nucleotides upstream of the exon-exon junction, recruits Upf3/3X, a predominantly nuclear protein that remains on the mRNP upon export to the cytoplasm, where it interacts with the predominantly perinuclear protein Upf2 (Kim et al., 2001; Lykke-Andersen et al., 2000; Lykke-Andersen et al., 2001). It is thought that, in the absence of premature termination codons, the ribosomes would remove the EJC from the mRNA during the first round of translation. If a nonsense mutation occurs more than 50 nucleotides upstream from the exon-exon junction (which is considered to be the distance between the decoding site and the leading edge of the ribosome), the ribosome disassembles and the EJC remains in place. Upf 2 and Upf3/3X are then able to recruit Upf1, a group 1 helicase, and the other components of the degradation pathway (Sun et

al., 1998). The notion of a first "pioneering" round of translation, during which ribosomes scan the message for the presence of premature stop codons, was suggested by a recent study in which NMD was shown to take place in messages associated with nuclear cap binding protein CBC80, but not in actively translated mRNAs associated with the eIF4E (Ishigaki et al., 2001). Nonetheless, some important aspects remain unexplained, like the cellular compartment in which NMD takes place in the cell. While the requirement for scanning of the messages by ribosomes had suggested that NMD is a cytoplasmic phenomenon, fractionation studies have shown that most of the messages containing premature stop codons undergo NMD in the nucleus or in a functionally defined nucleus-associated compartment, which is thought to identify the set of messages in the process of exiting the nucleus but not released yet in the cytoplasm (reviewed in (Hentze and Kulozik, 1999; Maquat and Carmichael, 2001; Schell et al., 2002). Nonsense-associated altered splicing (NAS) is a putative correction response that upregulates alternatively spliced transcripts that skip premature termination codons (Cartegni et al., 2002; Maquat, 2002). This phenomenon and recent evidence suggesting translation of proteins in the nucleus (Iborra et al., 2001) suggest that scanning of the coding sequence of the message could take place in the nucleus (Wilkinson and Shyu, 2002).

6) From the nucleus to the cytoplasm: role of nuclear RNA binding proteins in translation:

In prokaryotes, which lack subcellular compartments, translation is directly coupled to transcription. In nucleated cells it is generally accepted that molecular tags, such as

components of the exon junction complex, are loaded on transcripts as a result of nuclear RNA processing events and that these ultimately affect cytoplasmic RNA metabolism. Nonetheless, in only a few instances has the same RNA binding protein been shown to affect regulation of the same RNA in the two intracellular compartments. While this apparent incongruence probably results from the fact that a genome-wide search for target sequences has been undertaken only for a handful of RNA binding proteins, it remains possible that RNA binding proteins associate with not completely overlapping sets of RNAs in the nucleus and in the cytoplasm.

In lower organisms, some RNA binding proteins have been shown to regulate expression of a single RNA both in the nucleus and in the cytoplasm (Gebauer et al., 1997). The RNA binding protein Sex Lethal (Sxl) in *Drosophila* offers one of the most striking examples of such a mode of regulation. In addition to its role in sex determination through regulation of alternative splicing (reviewed in the next section), Sxl regulates dosage compensation by inhibiting the expression of *msl-2* at the post-transcriptional level in the female fly. In the nucleus, the presence of Sxl inhibits the splicing of a small intron in the 5' UTR of *msl-2* in female flies. The retention of the intron in the 5'UTR is not in itself sufficient to inhibit the expression of *msl-2,* but provides a binding site for Sxl in the mature *msl-2* transcript. In the cytoplasm Sxl inhibits the translation of *msl-2* by binding the unspliced intron in the 5'UTR and another site in the 3'UTR (Bashaw and Baker, 1997; Gebauer et al., 1998). In a similar way, the *Saccharomyces cerevisiae* ribosomal protein L32 regulates its own expression at the level of both splicing and translation (Dabeva and Warner, 1993).

In vertebrates, there has been no description of an RNA binding protein that

regulates a single RNA in both the nucleus and the cytoplasm. Nonetheless, two RNA binding proteins structurally similar to Sxl, Polypyrimidine Tract Binding protein (PTB) and hnRNP A2 have been shown to play roles in both splicing and translation. PTB is a well-described splicing factor, and was found associated with many picornavirus IRES elements; depletion/reconstitution experiments demonstrated that PTB stimulates translation (Kaminski et al., 1995; Kaminski and Jackson, 1998; Niepmann, 1996). The mechanisms used by PTB to enhance IRES-dependent translation are presently unknown. Although a role for PTB in the translation of cellular mRNAs has only been demonstrated in two instances (Kim et al., 2000; Pickering et al., 2003), the presence of IRES and PTB-binding sites in the 5' untranslated region of a variety of cellular mRNAs suggests that the observations made in viruses may mirror a more widespread mechanism of translational control (reviewed in (Valcarcel and Gebauer, 1997). Furthermore, VgRBP60, a *Xenopus laevis* homolog of PTB, has been suggested to be involved in the localization of *Vg1* in the vegetal cytoplasm of the oocyte (Cote et al., 1999). HnRNP A2 is 68% identical at the amino-acid level to hnRNP A1, a well characterized alternative splicing regulator, and has itself a described ability to modulate alternative splicing (Bilodeau et al., 2001; Mayeda et al., 1994). In the cytoplasm of oligodendrocytes hnRNP A2 has been shown to interact with the mRNA of Myelin Basic Protein (MBP) forming large mRNPs transported along the cytoskeleton to the myelin compartment (Carson et al., 2001). Interestingly, the A2 response motif, a short purine rich sequence in the 3' UTR of MBP mRNA, has been demonstrated to act as a cap-dependent translational enhancer, the first such regulatory features described in a mammalian RNA (Kwon et al., 1999). The *Chironomus tentans* homolog of the closely related hnRNP A1, which has been mostly

characterized as a splicing regulator, has been shown to follow the Balbiani ring (BR) RNA from the nucleus to the cytoplasm, where it remains bound to the mRNA engaged in actively translating giant polysomes (Visa et al., 1996). A further example of a nuclear and cytoplasmic RNA binding protein whose functional role has been characterized in both compartments is offered by the strictly related hnRNP K, E1 and E2. HnRNP K has recently been suggested to play a role in the regulation of alternative splicing of the β-tropomyosin pre-mRNA (Expert-Bezancon et al., 2002). HnRNP K binds an intronic pyrimidine–rich element thirty-seven nucleotides downstream of the 5' splice site of a regulated exon; the presence of the intronic enhancer correlates with increased recruitment of U1 snRNP (Expert-Bezancon et al., 2002). In the cytoplasm, hnRNP K, E1 and E2 are well-characterized regulators of translation and stability of specific messages: they inhibit the initiation of translation of the 15-Lipoxygenase mRNA by binding a pyrimidine-rich repeated sequence in the 3'UTR of the message, and affecting the assembly of the two ribosomal subunits (Ostareck et al., 2001; Ostareck et al., 1997). Furthermore, hnRNP Es (also called αCPs or PCBPs) are known to stabilize α-globin mRNA during erythroid differentiation by interacting with C-rich 3'UTR determinants (Makeyev and Liebhaber, 2002). Therefore, the ultimate outcome of the interaction of hnRNP E with the 3'UTR of the 15-lipoxygenase mRNA is reduced translation, while the interaction with the α-globin mRNA results in enhanced protein synthesis.

ALTERNATIVE SPLICING AND TRANSLATIONAL CONTROL IN THE NERVOUS SYSTEM

The astonishing complexity of the architecture of the neural system and the variety of its cell subtypes with unique connectivity patterns have suggested the need for this tissue to utilize mechanisms that expand proteome complexity. Alternative splicing and RNA editing are known to play important roles in expanding the repertoire of final protein products of individual genes, while two other mechanisms responsible for the generation of the diversity of immunoglobulin and T-cell receptors, genomic re-arrangement or somatic hypermutation, have not at present been described in the nervous system.

Functional characteristics pose specific requirements on mechanisms of gene expression in the nervous system. The transmission of neuronal stimuli occurs in specialized synaptic structures that can be up to several hundred microns away from the neuronal cell body. Furthermore, studies by electrophysiology have shown that phenomena of synaptic plasticity can take place in dendritic terminations that have been severed from the cell bodies, indicating a remarkable functional autonomy of the synapse. In particular, a now substantial body of work has illustrated the role of translation of localized messages in the synapse in different aspects of synaptic plasticity. In the following section I will review some aspects of alternative splicing and translational regulation of gene expression in the nervous system.

1) Specific aspects of translational control in neurons:

Dendrites are extended and intricate structures, forming thousands of synapses that can lay hundreds of micrometers far from the cell body. Synapses are plastic structures, able to change their functional characteristics in response to activity. Since several experimental paradigms of synaptic plasticity require protein synthesis, the

distance from the cell body could pose a problem to the cell that has to provide the synapses with new proteins. The discovery that virtually all the components of the translational apparatus can be found in the post-synaptic specializations of dendrites suggested that translation of new proteins could actually happen locally, providing a mechanism for swift changes of concentration of proteins in response to stimulation (Gardiol et al., 1999; Steward and Levy, 1982). Furthermore, the local synthesis of proteins at the synapse circumvents the need of targeting proteins newly synthesized in the cell body to the activated synapse, offering a possible mechanistic explanation for the documented restriction of enduring functional modification to the stimulated synapse (reviewed in (Steward and Schuman, 2001). In the twenty years following the initial finding of ribosomes in the dendrites, several groups have pursued the identification of mRNA localized in the dendrites. There are currently about a dozen mRNAs that are universally recognized as localized in the dendrites, but it is commonly believed that many more have not been identified yet (Steward and Schuman, 2001). Notably, several identified mRNAs localized in dendrites encode for proteins for which a role in synaptic plasticity has been demonstrated: *CaMKIIα* mRNA, which encodes for the α-subunit of CaMKII, a kinase required for the establishment of both Long Term Potentiation (LTP) and Long Term Depression (LTD), *NR1,* encoding a subunit of the *N*-methyl-D-aspartate receptor (NMDAR), *Arc, BDNF,* and *TrkB* (Steward and Schuman, 2001). Dendritic translation has been described both as a consequence of synaptic plasticity and as a necessary condition for synaptic plasticity. Synaptoneurosomal preparations have been stimulated in various ways to show increase of translation of FMRP and CaMKII α-subunit by pulse labeling techniques (Bagni et al., 2000; Leski and Steward, 1996;

Scheetz et al., 2000). Furthermore, stimulation with BDNF results in the increased dendritic translation of a reporter message containing the GFP coding sequence preceded and followed by the 5' and 3' untranslated regions of α *CaMK II* mRNA in intact neurons and in dendrites severed from cell bodies (Aakalu et al., 2001). Reciprocally, in hippocampal slices, synaptic potentiation induced by BDNF is blocked by inhibitors of protein synthesis, also in severed dendrites, demonstrating the requirement of local translation for synaptic potentiation (Kang and Schuman, 1996). LTD induced through metabotropic glutamate receptor stimulation has also been demonstrated to depend on protein synthesis in isolated dendrites (Huber et al., 2000) In a culture preparation of *Aplysia* sensory and motor neurons, a branch-specific form of long-term facilitation, induced by repeated application of serotonin, has been shown to be protein synthesis-dependent (Casadio et al., 1999; Martin et al., 1997). More recently, the localization of α *CaMK II* mRNA in the dendrites was impaired by modifying the 3' UTR by homologous recombination in mice (Miller et al., 2002). The absence of localized synthesis of α CaMK II showed reduced late-phase long term potentiation, and various behavioral defects (Miller et al., 2002).

2) Proposed mechanisms of translational regulation in dendrites:

Two main modes of regulation of translation in dendrites have been described. Synaptic stimulation induces the transcription of immediate early genes, which are specifically transported to the stimulated synapse and translated. Alternatively, mRNAs are constitutively present at the synapse, and the stimulation activates their translation. The first mode of regulation has been best characterized for the gene *Arc,* which encodes

a cytoskeleton-associated synaptic protein that is required for the maintenance of LTP in the hippocampus (Guzowski et al., 2000). Patterned synaptic activation using a stimulation paradigm that is commonly used to induce LTP, both induced *Arc* transcription and caused the newly synthesized mRNA to localize selectively to activated synapses (Steward et al., 1998). An increase of Arc protein in the stimulated dendritic domains was also observed, suggesting that the events of mRNA localization and its translation are coupled in response to synaptic stimulation (Steward et al., 1998; Steward and Worley, 2001).

In other instances, the mRNA seems to be constitutively localized in the post-synaptic areas, and synaptic stimulation activates its translation. Several not exclusive mechanisms have been invoked for the coupling of synaptic stimulation and activation of translation. The inhibition by rapamycin of long term facilitation in *Aplysia* neurons, and of LTP in hippocampal neurons, suggest a role of the protein kinase target of rapamycin (TOR) in the regulation of synaptic translation (Casadio et al., 1999; Tang et al., 2002). TOR promotes translation through two pathways: it stimulates initiation of translation by phosphorylating the eI4E binding protein (4EBP), which, in the hypophosphorylated state, sequesters the initiation factor 4E. Furthermore, TOR activates p70 S6 kinase, which, in turn, phosphorylates the ribosomal protein S6, and facilitates the translation of mRNAs containing a 5' terminal oligopyrimidine tract (5' TOP), a group of RNAs that encode several factors involved in translation (reviewed in (Raught et al., 2001).

Besides general activation of cap-dependent translation through activation of TOR, other mechanisms have been proposed for activation of specific mRNAs in response to synaptic stimulation. NMDAR stimulation has been shown to rapidly increase α CaM II

synthesis, while reducing overall translation in synaptoneurosomes from rat superior colliculus (Scheetz et al., 2000). The increase of α CaM II synthesis is accompanied by phosphorylation of eukaryotic elongation factor 2 (eEF2), a modification known to reduce the elongation rate. A similar stimulation of α CaM II synthesis is obtained by treatment with cyclohexamide, a drug that inhibits the elongation step of translation. It has been speculated that, when the elongation step of translation becomes rate-limiting, the translation of messages that are abundant, but usually inefficiently initiated, like *α CaM II,* are up-regulated (Scheetz et al., 2000).

Cytoplasmic polyadenylation has also been proposed as a possible mechanism for the regulation of dendritic translation. This mechanism was first characterized in *Xenopus* oocytes: a number of maternally transcribed mRNAs contain a very short poly(A) tail and are kept untranslated until fertilization. Upon fertilization, the inhibition of polyadenylation and translation exerted by the cytoplasmic polyadenylation element binding protein (CPEB) is removed by activation of the Aurora protein kinase, a longer poly(A) tail is synthesized, which is bound by Poly(A) Binding Protein (PABP), which, in turn, recruits eI4F complex, promoting initiation of translation (reviewed in (Mendez and Richter, 2001). It has been shown that the *α CaM II* mRNA contain two CPE in its 3'UTR and that these two elements are functional in repressing translation when *α CaM II* is micro-injected in oocytes (Wu et al., 1998). Furthermore, other components of the cytoplasmic polyadenylation apparatus are present in dendrites of hippocampal neurons and increased length of the poly-A tail of *α CaM II* was observed in response to visual experience (Wu et al., 1998). These findings were further explored in cultivated neurons, where stimulation of NMDAR receptors was shown to induce translation of a reporter

under the control of *α CaM II* CPE (Huang et al., 2002; Wells et al., 2001). A recent study shows that CPEBP also facilitates the transport of *α CaM II* mRNA in the dendrites, presumably in a dormant state, since maskin, the protein involved in translation repression of CPE-containing messages, is also part of the mRNP complex. Neurons lacking CPEB or expressing a mutated form of the protein show slower and decreased transport of the substrate mRNAs (Huang et al., 2003).

The local synthesis of proteins requires the translocation of the mRNAs encoding them from the cell body to the synapses, either in response to stimulation, as in the case of *Arc,* or constitutively, as in the case of *α CaM KII* (Ouyang et al., 1999). The trafficking of mRNA to sites of translation involves the recognition of cis-acting sequences, frequently in the 3'UTR, by trans-acting proteins. These messenger ribonucleoprotein (mRNP) complexes bind to cytoskeletal elements, which drive their transport and localization. The physical nature of the mRNPs is debated. The existence of extremely large RNA "granules" has been proposed based on studies in cultured primary neurons (Krichevsky and Kosik, 2001). These structures sediment faster than the largest polyribosomes on linear density gradients and seem to contain messenger RNAs, ribosomes and RNA binding proteins. Depolarization of neurons provokes redistribution of some mRNAs from the granules to the actively translating polysomes (Krichevsky and Kosik, 2001; Krichevsky and Kosik, 2002). Other studies have proposed that the mRNP structures devoted to transport and storage of mRNAs are rather heterogeneous in size, but smaller than the polyribosomes (Feng et al., 1997; Khandjian et al., 1996).

It has been postulated that through the recognition of shared cis-acting sequences in discrete sub-populations of RNAs, RNA-binding proteins can post-transcriptionally

regulate the expression of functionally related genes (Keene and Tenenbaum, 2002). It is conceivable that, in the neurons, specific sets of RNA binding proteins may target mRNPs to specific synapses and confer translational regulation in response to stimulation.

3) Alternative splicing as a mean to generate proteome complexity:

The contemporary view of genome complexity is remarkably different than what was anticipated ten years ago, when numbers of genes higher than 10^5 were commonly estimated. With a number of genes probably not much higher than thirty thousand, only around twice the number of genes of the Drosophila genome (according to December 2002 estimations (Waterston et al., 2002), the mammalian genome has to account for the startling complexity of higher vertebrates. One probably important source of complexity of the vertebrate proteome is the presence of vertebrate-specific protein domains and motifs (7% of the total), and the arrangement of pre-existing domains in a more variegate repertoire of architectures (Lander et al., 2001). The richer set of protein domains suggests a higher potential for regulation at the level of interactions among proteins and pathways. Other mechanisms often invoked to explain the complexity of vertebrates are pre-mRNA editing and post-translational protein modifications (Banks et al., 2000; Keegan et al., 2001).

Alternative splicing offers an obvious mechanism through which an organism can expand the proteome from a limited number of genes, by combining exons in multiple different ways. While some general principles governing alternative splicing and examples of remarkably complex patterns of alternative usage of exons have been known

for more than a decade, only in the past two years has a systematic appreciation of the quantitative significance of this mode of gene expression begun to emerge. The completion of the human and murine genome drafts and the progress of the expressed sequence tag (EST) project and the RIKEN Mouse Gene Encyclopedia Project allow for the first time to compare cDNA sequences to the entire genome (Gregory et al., 2002; McPherson et al., 2001; Okazaki et al., 2002; Venter et al., 2001). Several groups undertook comparisons between ESTs derived from the same gene, followed by assessment of the candidate splice sites by alignment of the ESTs to the genomic sequence of the gene (reviewed in (Modrek and Lee, 2002). They estimated an alternative splicing frequency ranging between 35 and 59% of the human genes. These estimations were confirmed by a large international effort to compare 60,770 full-length cDNA sequences to the newly released draft of the mouse genome (Okazaki et al., 2002). This study estimated a 41% frequency of alternative splicing, in good agreement with the most recent EST-based studies (Modrek et al., 2001). These studies also agree on a high ratio (more than 70%) of alternative splicing events affecting the coding sequence (Modrek et al., 2001). While the quantitative impact of alternative splicing on gene expression appears indisputable, its role in expanding the proteome in vertebrates relative to other organisms is still debated. A survey of the frequency of alternative splicing across different organisms shows no appreciable difference between vertebrate and invertebrate (Brett et al., 2002). However, due to the incomplete coverage of the EST database in some of the organisms analyzed, the authors of this study were forced to compare a limited set of cDNAs, leaving out cDNAs with more complex patterns of alternative splicing. It is possible that once the comparison of all the cDNAs becomes possible,

differences across species may emerge (discussed in (Maniatis and Tasic, 2002).

4) Alternative splicing and the Nervous System:

A recent analysis of the public databases shows that 10-30% of human alternatively spliced genes show a tissue-specific pattern of exon utilization (Xu et al., 2002). The tissue with the largest total number of tissue-specific splice form was brain, which represented 18% of all the observed tissue-specific alternative splicing events (Xu et al., 2002). This is consistent with results of previous studies that had shown that nervous and immune system account for the highest frequency of alternative splicing (Modrek et al., 2001; Stamm et al., 2000). These studies provide the first quantitative support to the concept that the Central Nervous System (CNS) utilizes alternative splicing as a mean to increase the complexity of its proteome, and, therefore, its ability to give rise to many different cell subtypes and innumerable synaptic connections, an idea that has been quite popular for a number of years (Darnell, 1998; Grabowski, 1998).

The concept that the CNS needs a very large repertoire of different molecules derives intuitively from the large number of neurons (estimated to be roughly 10^{10}), the vast variety of neuronal subtypes and the high number of specific synaptic connections among them (up to 10^5 in a single Purkinje neuron). Sixty years ago, Roger Sperry's seminal experiments provided the first evidence that axon guidance and establishment of synaptic connections are governed by *chemospecificity,* or by specific sets of molecules guiding axons to their targets. It is currently widely believed that the diversity of neuron subtypes and the complexity of their synaptic connections reflect differential gene expression that confers each neuron's morphology, position, connectivity and

physiological characteristics. For example, motor neurons of the spinal cord have been shown to be grouped in multiple pools, each characterized by the expression of a unique combination of transcription factors and signaling molecules, with different muscle targets and afferent connections (reviewed in (Jessell, 2000). Another striking example of neuronal molecular diversity is the olfactory system, where each olfactory neuron expresses only one of over 1000 odorant receptor genes, and the receptor defines the neuron's connection in the brain (Mombaerts et al., 1996).

Consistent with a suggested role of alternative splicing in expanding the ability of the CNS to establish specific intercellular contacts and communications, Modrek et al. show that 29% of the messages undergoing tissue-specific alternative splicing encode membrane-anchored receptors, integral membrane proteins and proteins involved in cell surface adhesion (Modrek et al., 2001). It is indeed among genes involved in adhesion and axonal guidance that the most complex patterns of alternative splicing have been described. *Dscam*, a *Drosophila* homologue of human Down Syndrome cell adhesion molecule expressed in the axon processes, can potentially give rise to as many as 38,016 isoforms, by alternative splicing of four clusters of tandemly arranged exons (Schmucker et al., 2000). Nearly 3,000 neurexins, a group of neuronal cell surface receptors proposed to have a key role in synaptic formation, are predicted to arise by alternative splicing from 6 primary transcripts, deriving from three genes with two alternative transcription initiation sites each (Missler and Sudhof, 1998). It has been proposed that the diversity of neurexins might be a component of a code that specifies neural connectivity (Missler et al., 1998). Interestingly, the alternative splicing pattern of neurexin is much simpler in *Drosophila* than in vertebrate (Tabuchi and Sudhof, 2002), suggesting that the much

higher number of neurexin isoforms may have evolved to match the more complex architecture of the vertebrate neural system. The family of the α, β, γ protocadherins (Pcdh) is another source of neuronal protein diversity. The organization of the *Pcdh* genes is reminiscent of Immunoglobulin and T cell receptor loci, with clusters of 14 to 22 tandemly arranged alternative exons encoding the variable extracellular domain, and 3 constitutively spliced exons encoding the intracellular constant part. A separate promoter precedes each variable exon. Cis-splicing and, to a minor extent, trans-splicing, has been shown to take place between the variable and the constant exons, and the differential promoter choice is proposed to determine the usage of the 5'-most variable exon (Tasic et al., 2002; Wu and Maniatis, 2000; Wu et al., 2001).

5) Mechanisms of Alternative Splicing:

The fundamental step in alternative splicing, as in constitutive splicing, is discrimination of the part of the pre-mRNA that will be utilized in the message (exon), from the part that will be excised and eventually degraded in the nucleus (intron). The definition of exon-intron boundaries is dictated by the interaction of *cis*-elements and *trans*-acting factors. The information content of the primary sequence is rather limited: the splice sites consensus sequences are short and degenerate in metazoans. The splicing reaction occurs in the spliceosome, a complex molecular machinery constituted by the five small nuclear Ribonucleoprotein Particles (snRNP U1, U2, U4, U5, U6), and more than 60 non-snRNP proteins (Will and Luhrmann, 2001). The early steps of splicing involve the recognition of the 5' splice site by U1 snRNP, the binding of SF1 to the branch point, and of U2AF65 and 35 to the polypyrimidine tract and the AG at the 3'

splice site, respectively. Subsequently, U2 snRNP interacts with the branch point, and U4,U5,U6 enter the complex giving rise to the catalytically active spliceosome (reviewed in Burge, C.B., et al., 1998). Vertebrate exons are usually short (140 nucleotides on average) while introns can be tens of thousands of nucleotides long. The recognition of the splice sites is enhanced by interactions across the exon between splicing factors recognizing the 3' splice site and splicing factors bound to the 5' splice site, in a process called exon definition (Berget, 1995). A number of non-snRNP proteins take part in both the constitutive assembly of the spliceosome and the regulation of alternative splicing. Splicing regulators, most of which are RNA binding proteins, can modulate splicing by binding constitutive consensus sequences at the splice sites, branch point and polypirymidine tract, or specialized *cis* regulatory elements known as exonic enhancers and silencers (ESE and ESS) or intronic enhancers and silencers (ISE and ISS).

Three not mutually exclusive mechanisms have been shown to be responsible for alternative splicing in different cell types or in cells in different physiological conditions.

Transcription promoters can affect the pattern of splicing by recruiting splicing factors to the promoter area through interactions with transcriptional activators bound to transcription enhancers or with the Carboxy-Terminal-Domain (CTD) of the RNAP II, or by affecting the processivity of RNAP II (see the section on coupling of transcription and splicing).

Varying the concentration of general splicing factors in *in vitro* splicing assays or by transfection in cell culture has been shown to affect the pattern of alternative splicing. ASF/SF2 and hnRNP A1 have been shown to have competing effects on the splicing of a reporter minigene that could produce two splicing products differing in the usage of two

alternative 5' splice sites. Excess ASF/SF2 has been shown to promote the utilization of the more proximal 5' splice site. The addition of hnRNP A1 competes the ASF/SF2 effect, promoting the usage of the more distal 5' splice site (Caceres et al., 1994; Ge and Manley, 1990; Krainer et al., 1990; Mayeda and Krainer, 1992). HnRNP A1 can promote the usage of the most distal 5' splice site by reducing the occupancy of both 5' splice sites by U1 snRNP, by cooperative binding that seems to be independent of the presence of high affinity binding sites (Eperon et al., 2000). Furthermore, a number of studies show that opposite actions of SR proteins and hnRNPs A and B regulate the inclusion of cassette exons. SR proteins usually activate the inclusion of cassette exons by binding to ESE sequences contained in the alternative exon and enhancing exon recognition, or by antagonizing the repressing actions of hnRNP A or B bound to nearby ESS elements (Zhu et al., 2001). HnRNP A1 mediates silencing by recognizing initially a high-affinity binding site in the exon, which promotes the eventual interaction of hnRNP A1 with the upstream region of the exon, and possibly with intronic sequences (Zhu et al., 2001). The opposing effects of SR proteins and hnRNPs have been shown to regulate many RNAs in a variety of systems (Bilodeau et al., 2001; Caceres and Kornblihtt, 2002; Caputi et al., 1999; Cartegni and Krainer, 2002; Dirksen et al., 2000; Hou et al., 2002; Marchand et al., 2002; Pollard et al., 2002; Smith et al., 2002; van der Houven van Oordt et al., 2000; Zhu et al., 2002). The abundance of both hnRNP A1 and ASF/SF2 was found to vary widely across tissues, with variations in the molar ratio between the two proteins greater than 100 times among certain tissues (Hanamura et al., 1998). It is conceivable that other general splicing factors belonging to the SR and hnRNPs families vary in a similar way across different tissues. Therefore, changes in the ratio between SR proteins and hnRNP

A/B may be crucial for the regulation of tissue-specific alternative splicing of a variety of messages.

Another mechanism of alternative splicing regulation involves tissue- or developmental stage-specific factors. At present, there are only few examples of such factors, and they have been mostly discovered in systems amenable to genetic screening. This mode of regulation has been mostly investigated in the sex-determination cascade in *Drosophila* by a combination of genetic and biochemical means. The female-specific factor Sex-Lethal (Sxl) inhibits the utilization of male-specific 3'splice sites in *Transformer* (*Tra*) and on its own pre-mRNA. By competing with U2AF65 for the *Tra* polypyrimidine tract, Sxl promotes the utilization of a downstream female-specific 3' splice site in exon 2, and the skipping of a stop codon that produces an inactive Tra protein in male flies (Valcarcel et al., 1993). The female-specific Tra isoform, in turn, promotes the cooperative binding of two proteins containing RS domains, RBP1 and Tra2, to six ESEs in exon 4 of *Doublesex* (*Dsx*) pre-mRNA, which contains a stop codon (Hertel and Maniatis, 1998; Lynch and Maniatis, 1996). Exon 4 is included in the *Dsx* mRNA exclusively in female flies, resulting in an isoform of dsx that transcriptionally represses several male-specific genes.

The *Drosophila* Half pint (HFP), which is expressed exclusively in the ovary in the adult fly, specifically regulates the alternative splicing pattern of *ovarian tumor, eIF4E,* and the constitutive splicing of *gurken* (Van Buskirk and Schupbach, 2002). The mammalian homolog of Half Pint, PUF 60, does not seem to have conserved the tissue- and target-specificity of HFP, and appears to have a rather general splicing activation action (Maniatis and Tasic, 2002).

The *Drosophila* neural-specific RNA binding protein ELAV (embryonic lethal abnormal visual system) has been shown to be necessary and sufficient to produce the neural specific splicing pattern of *neuroglian* (Koushika et al., 1996), *erect wing* (ewg) and *armadillo* (Koushika et al., 2000). The effect on *neuroglian* splicing is dependent on the interaction of ELAV with uridine-rich intronic sequences (Lisbin et al., 2001). However, a neural-specific vertebrate member of the ELAV/Hu family, HuD, when expressed in *Drosophila* CNS under the control of an ELAV promoter, did not reproduce the ELAV effect on the *neuroglian* pre-mRNA splicing (Toba et al., 2002).

Drosophila P-element somatic inhibitor (PSI), which is expressed exclusively in the somatic cells, prevents the production of a functional P-element transposase by inhibiting the excision of intron 3 (reviewed in Wang et al., 1997).

NOVA, A NEURON-SPECIFIC RNA BINDING PROTEIN

1) Paraneoplastic Neuro-degenerations:

The paraneoplastic neurologic degenerations (PNDs) are a group of very rare neuronal degenerations described in patients suffering a neoplasia outside the nervous system. It is a rather heterogeneous group of diseases, associated with various cancers and affecting different areas of the nervous system (reviewed in (Darnell and Posner, 2003; Musunuru and Darnell, 2001). The immunologic nature of these diseases was first suggested by the discovery that the patients have high-titer autoantibodies reacting with antigens in both tumor and central nervous system (Posner, 1992). The sera of patients

were used to screen cDNA expression libraries, leading to the identification of autoantigens (reviewed in (Darnell, 1996). The targets of the autoantibodies were found to be proteins normally expressed exclusively in the neural system. It has been suggested that the expression of neural specific proteins in tumors growing outside the CNS elicits an immune response against those proteins, which are normally considered to be non-self because of their exclusive location in the "immuno-privileged" environment of the CNS (reviewed in (Darnell, 1996).

Nonetheless, the observation that transfer of antibodies does not reproduce the disease in animals suggests that the presence of autoantibodies is not sufficient to cause the neurological degeneration (Darnell, 1996). Furthermore, most of the identified onconeural antigens are intracellular. A cell-mediated mechanism in paraneoplastic cerebellar degeneration has been unveiled based on the presence of expanded populations of onconeural antigen-specific cytotoxic T lymphocytes in patients (Albert et al., 1998). The cytotoxic activity of the T lymphocytes was shown to be much enhanced when the onconeural antigen, deriving from cells undergoing apoptosis, was cross-presented to the T-lymphocytes by dendritic cells (Albert et al., 1998). While the presence of a protein normally in immuno-privileged tissues in apoptotic cancer cells could lead to the observed tumor immunity, the mechanism responsible for the neurologic degeneration is still unexplained. It has been proposed that the auto-antibodies could enter neurons, and lead to neuronal death by interfering with the physiological functions of the onconeural antigens (Musunuru and Darnell, 2001). Even a limited number of apoptotic neurons could provide the antigenic material for cross-priming of cytotoxic lymphocytes, which would in turn trigger massive neural degeneration through a cell-mediated mechanism

(Musunuru and Darnell, 2001).

The PND sera have proved a valuable reagent to identify a number of onconeural antigens. Based on the biological characteristics, these exquisitely neuron-specific proteins have been classified in four groups: neuromuscular junction proteins, vesicle-associated nerve terminal proteins, neural signaling proteins, neuron-specific RNA-binding proteins (Darnell, 1996). The two groups of RNA binding proteins identified as onconeural antigens, Hu and Nova, are of interest for their potential role in tissue-specific regulation of different steps of gene expression.

Hu proteins are targets of autoantibodies in a set of PNDs called paraneoplastic Encephalomyelitis and Sensory Neuropathy (PEM/SN), a condition most commonly associated with small-cell lung cancer (SCLC), and some types of prostate cancer (Ball and King, 1997; Dalmau et al., 1992a; Dalmau et al., 1992b; Manley et al., 1995). There are four known *Hu* genes in mammals, termed *HuA* (or *R*) through *D*. While Hu B, C and D are neural specific and autoantibody targets in PND, HuA is ubiquitous. Hu proteins have been reported to be involved in mRNA export (Gallouzi and Steitz, 2001), stabilization of mRNAs containing AU-rich elements (ARE) in their 3'UTR (Fan and Steitz, 1998) and translational control (Antic et al., 1999). Interestingly, Hu exerts opposite effects on translation depending on the location of its binding site in the messages. Hu enhances translation of transcripts that interact with the protein in their 3'UTR (Antic et al., 1999), but exerts an inhibitory effect on the translation of *p27*, by binding in the 5'UTR in a region of the transcript that functions as an internal ribosome entry site (Kullmann et al., 2002). Overexpression of Hu in P19 and P12 cells and ectopic expression in mouse embryos promotes a neural phenotype (Akamatsu et al., 1999; Antic

et al., 1999; Gao and Keene, 1996). In mouse embryonic cortex a hierarchy of different *Hu* messengers in neurons at different stages of migration was described (Okano and Darnell, 1997).

2) POMA and the discovery of Nova:

The Paraneoplastic-opsoclonus-myoclonus-ataxia (POMA) is a neural degeneration characterized by deficit of inhibitory control of the eye, limbs and trunk movements (Anderson et al., 1988; Budde-Steffen et al., 1988). It is a rather rare complication of breast and gynecological tumors and small cell lung cancers. In western blot analysis, sera from patients affected by POMA recognize two sets of four to five bands in neural extracts, migrating slightly above 50 kDa and around 70 kDa (Buckanovich et al., 1993). By screening expression libraries with POMA patient sera, two genes were cloned, termed *Nova-1* and *Nova-2* (Buckanovich et al., 1993; Yang et al., 1998). *Nova-1* mRNA is expressed in neurons, mostly in the motor neurons of the ventral horns of the spinal cord, in the motor neurons of the hindbrain and in ventral midbrain (Buckanovich et al., 1993). *Nova-2* mRNA is also exclusively neuronal, and its distribution in the CNS is roughly complementary to that of *Nova-1*. *Nova-2* is most highly expressed in the cortex, olfactory bulb, thalamus, dorsal midbrain and inferior olive, with weaker levels of expression observed in the hindbrain and spinal cord (Yang et al., 1998). Both Nova proteins are most abundant in the nucleus, but they are also present in the cytoplasm and in the dendrites (Triller, A and Darnell, R.B., unpublished observations).

3) Nova is a sequence specific RNA binding protein:

Nova-1 and Nova-2 contain three repeats of the KH domain (hnRNPK Homology domain), the second most common RNA binding motif, originally discovered in the hnRNPK protein (Siomi et al., 1993). Since the characterization of the original KH motif, a superfamily of more than 100 KH proteins have been identified in eukaryotes, eubacteria and archea (Lewis et al., 1999). KH motifs span about seventy residues, with a characteristic α/β fold consisting of a three-stranded, antiparallel β-sheet backed by three α-helices, a Gly-X-X-Gly loop invariant among all the members of the superfamily, and a variable loop (Lewis et al., 1999). KH-containing proteins play a functional role in splicing, as PSI in *Drosophila*, MER-1 in yeast, SF1 and KSRP in mammals, and in translation, as hnRNPK, E1 and E2, and FMRP (Berglund et al., 1998; Brown et al., 2001; Labourier et al., 2002; Min et al., 1997; Ostareck-Lederer et al., 1998; Spingola and Ares, 2000) . Each Nova protein contains three KH motifs, the first two in the N-terminal half of the protein being separated by a short connecting region, with the third close to the C-terminus. A similar arrangement is observed in other KH domain-containing proteins, like hnRNPK, hnRNPE1 and E2. Nova-1 and Nova-2 are very similar in their amino acid sequence: Nova-2 is 75% identical to Nova-1. The highest level of sequence identity is observed in the KH motifs (87-93% amino acid identity).

Extensive *in vitro* Selection-Amplification studies have been performed to identify specific RNA sequences that Nova binds with high affinity (Buckanovich and Darnell, 1997; Jensen et al., 2000b; Yang et al., 1998). Selection-Amplification performed with the entire Nova protein resulted in identification of a $[UCAU(N)_{0-2}]_3$ motif, in the context of a stem-loop structure (Buckanovich and Darnell, 1997). The same study showed that the third KH domain was necessary and sufficient to bind the target

sequence. Additional RNA selection experiments performed with isolated KH3 domain resulted in the selection of a single UCAY motif in the context of a stem-loop structure, confirming the major role exerted by KH3 in the determination of the target sequences of the entire molecule (Jensen et al., 2000b).

This target RNA was co-crystalized with the isolated KH3 domain and the structure was solved at 2.4 Å resolution (Lewis et al., 2000). The single-stranded RNA sequence is pinioned between the invariant and the variant loops, and it stacks on a hydrophobic surface made of aliphatic chains of amino acids belonging to α helices and β strands. The central bases of the consensus sequence, C and A are specifically recognized thanks to the spatial arrangement of several polar and charged residues surrounding the hydrophobic surface, which mimic Watson-Crick-like C-G, A-U contact (Lewis et al., 2000). The bordering residues on the two sides of the CA core of the consensus sequence are also tightly specified, although less stringently, such that only a pyrimidine can fit. Furthermore, the crystallized RNA/KH3 complexes arrange themselves in dimers by interactions through their third α helix. This mode of dimerization, which has been confirmed in solution by Nuclear Magnetic Resonance (Ramos et al., 2002), would give the KH3 dimers the ability to bind two UCAY motifs, in agreement with the target consensus sequences selected by the entire protein, and with the few validated *in vivo* target sequences (see in the next sections), which all contain multiple repeats of the UCAY motif. The role of the first and second KH domains is not clear at present; while differences in critical amino acids make it improbable that they recognize the UCAY motif, they could recognize other RNA sequences, further specifying the *in vivo* targets, or participate in inter or intra-molecular dimer formation,

or engage in interactions with other proteins (Lewis et al., 2000; Ramos et al., 2002).

Chapter 2

MATERIALS AND METHODS:

DNA preparation and restriction digest analysis

Unless otherwise stated, all plasmid DNA was prepared using Spin mini-prep kits (Qiagen) and analyzed by restriction digestion using enzymes, buffers and protocols supplied by New England Biolabs. DNA was separated by electrophoresis in standard 1x TBE/1-1.5% agarose gels and photographed under UV light. Plasmid DNA for transfections into cell lines was prepared by a modified cesium chloride method (Molecular Cloning, by Sambrook, Fritch and Maniatis, Cold Spring Harbor Laboratory Press, 1989), and dialyzed against 1xTBE.

Plasmid Constructs:

The GlyRα2 minigene (cloned in pCDNA3, Invitrogen) corresponds to the following GlyRagr2 E2 mouse genomic sequences: bases 81–276 (GenBank accession number X75842), 63–583 (GenBank accession number X75843), and 10–300 (GenBank accession number 75844). The mutated GlyRα2 minigene, in which the Nova-1 binding site 5'-UCAUCAUCAUUUUCAUUUUGUUU-3' was mutated to 5'-UAA UAAUAAUUUUAAUUAAGGUU-3', was generated by PCR. A cDNA encoding T7-tagged Nova-1 was cloned into pcXHook (Invitrogen) and into pCI-neo (Promega).

The β-globin minigene was generated by PCR using primers glo-KpnF and glo-Rc using plasmid sp64-HβΔ6 (a kind gift from A. Krainer) as a template, and was cloned into pCDNA3 (Invitrogen). Exon 2 was shortened by PCR. Heterologous minigene

constructs were generated by PCR.

Mutations of both minigene and Nova-1 expression constructs were made with the QuikChange Site-directed Mutagenesis Kit (Stratagene) and specifically designed primers (Operon).

All DNA constructs generated by PCR were sequenced in their entirety.

Cell transfection

N2A and 293T cells were grown to 60% confluence in 6-well plates or chamber slides in 10% FBS/DMEM with penicillin/streptomycin. For in vivo splicing assays, 2.25 μg of total DNA, comprised of 0.25 μg of the appropriate minigene, and variable amounts of pNova-1 and empty pcIneo vector, were incubated with 95 μl of DMEM and 5 μl of Fugene6 (Roche) for 15 min at room temperature. The mixture was then added to the cells in a total volume of 2 ml 10% FBS-DMEM. After 40 hrs the cells were washed with 1xPBS, collected by scraping and halved for RNA extraction and protein extraction. Transfection in chamber slides was performed by adding a mix of 0.25 μg of plasmid, 48 μl of DMEM and 2 μl of Fugene6 to cells in a total volume of 1 ml 10% FBS-DMEM. 293T cells (ATCC) are a primary human embryonic kidney cell line transformed with the SV40 T antigen. N2A cells (ATCC) are a mouse neuroblastoma cell line.

RNA preparation

Total RNA was extracted from mouse tissues using a modified guanidine-acid phenol protocol (Chomczynski et al., 1997). RNA from tissue culture cells was purified using the RNeasy Mini kit system (Qiagen), including on the column DNase1 digestion.

RT-PCR

Purified RNA was reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen). cDNA products were amplified using PfuTurbo (Stratagene) with 40 pmol each primer, and 0.5 pmol of one γ-^{32}P-ATP-labeled primer. PCR products were separated by denaturing PAGE (6% acrylamide/7 M urea) and confirmed by sequencing. Quantification was performed using a Molecular Dynamics Storm Phosphorimager and ImageQuant software. PCR was linear with respect to input cDNA and cycle number (data not shown).

Western blot analysis

Tissue culture cells were lysed in PXL lysis buffer (1xPBS, 0.1% SDS, 0.5% DOC, 0.5% NP-40). Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Equal loading of total protein was determined by Bradford assay. Membranes were blocked for one hour at room temperature in 5% non-fat milk in R buffer (0.15 M NaCl, 10 mM Tris-Cl, 1 mM EDTA, 0.1% Triton X-100), incubated with HRP-conjugated anti-T7 tag monoclonal antibody (Novagen) in 5% milk in R buffer at 4°C overnight and washed four times with alternating high salt (1 M NaCl, 20 mM Tris-Cl, 0.1% Triton X-100) and high detergent (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-Cl, 1% Triton X-100, 0.05% SDS) buffers. After a final rinse with R buffer, signal was detected by chemiluminescence (NEN). The membranes were wrapped (Saran Wrap) and exposed on Biomax MR film (Kodak).

Nitrocellulose Filter Binding Assays

Labeled RNA (100–200 nmol preheated to 75°C and bench-cooled 5 min) was incubated with the indicated concentrations of protein. Protein-RNA mixes were incubated in a total volume of 50 μL SBB (200 mM KOAc, 10 mM TrisOAc [pH 7.7], and 5 mM MgOAc), SBB 500 (500 mM KOAc, 10 mM TrisOAc [pH 7.7], and 5 mM MgOAc) or SBBhep (SBB + 1mg/ml heparin) for 10 min at room temperature. Binding solutions were passed through MF-membrane filters (0.45 HA, Millipore) and washed with 5 ml SBB (or SBB500). Data were plotted as percentage of total RNA bound versus log of the protein concentration, and Kds were determined with Kaleidograph software (Synergy Software). GABA was transcribed using T7 RNA polymerase and gel-purified PCR templates generated using primers T7-GABA-9CF and xR' (wildtype, 159nt RNA) or i9AtoCR2 (mutant, 156nt RNA) and $GABA_AR\gamma2$ minigenes as PCR templates. Glo RNA was transcribed using T7 RNA polymerase directly from minigene glo2A digested with BbsI, to yield a 175nt RNA.

PCR Primers:

The primers used were

E2F: 5'-AGCTTTCTGCAAAGACCATGAC-3';

E4R: 5'-GAAGATCTCCAAATCCAAGGAATCATCTGGG-3';

agr2GlyRE3A: 5'-CATGGTGGTTTCTGTGACTGATC-3';

agr2GlyRE3B: 5'-CATTGTAGTTTCTGCTATTGACCCAAAG-3';

agr2GlyRE4: 5'-TCCAAATCCAAGGAATCATCTGGG-3';

actinF: 5'-GTGGGCCGCTCTAGGCACCA-3'

actinR: 5'-CCCCCCTGAACCCTAAGGCCAACCG-3';

mGluR1F: 5'-CCTGGGGTGCATGTTTACTCC-3';

mGluR1R: 5'-AGGCCGTCTCGTTGGTCTTCA-3';

ICH-1F: 5'-GTCTCATCTTCATCAACTCC-3';

ICH-1R: 5'-ATGCTAACTGTCCAAGTCTA-3';

ClaBF: 5'-ACCGAACAGGAGTGGCGGGAG-3';

ClaBR: 5'-GGGGTCTCCTCCTTGGATTCT-3';

ggr2GABAF: 5'-GTATGGCACCCTGCATTATTTTGTC-3';

ggr2GABAR: 5'-TTGAATGGTTGCTGATCTGGGACG-3';

ChATF: 5'-ATGCCTATCCTGGAAAAGGTCCC-3';

ChATR: 5'-AGTGCTCCGAGCAAAGATCACAG-3';

SrcF: 5'-CCAAGCTCTTCGGAGGCTTCAACTC-3';

SrcR: 5'-CACATAGTTGCTGGGGATGTAACCG-3';

AgrinF: 5'-GGGATAGTTGAGAAGTCAGTGGGGG-3';

agrin R: 5'-CGAAGCCAGCGGTTGGTGTTG-3'.

T7-GABA-9CF: 5'- GAAATTAATACGACTCACTATAGGGAGCAGCTGCA CTGCTTAAGCGC-3'

xR': 5'- TTTTGCTAGCCACAATGTCATCAATGAGATGATG-3'

i9C-A R2: 5'- GTACTTCCACAATGTCATCAATTAGATTATTAATCTGAAAA TTAGTATTCAGAAAATTGCTT-3'

RT-PCR

Purified RNA (Chomcynski and Sacchi, 1987) was reverse transcribed using random

hexamers, and cDNA products were amplified using PfuTurbo (Stratagene) with 40 pmol of each primer and 0.5 pmol of one ggr-32P-ATP-labeled primer. PCR products were confirmed by sequencing. PCR was linear with respect to input cDNA and cycle number (data not shown).

In vitro Transcription and UV Cross-Linking

RNAs for crosslinking were generated by PCR using the wild-type or mutant GlyRagr2 minigene, followed by in vitro transcription. The primers used were T7primer1 (sense) (5'-AGTAATACGACTCACTATAGGGATCATGCAGTTCTGGTTTAAT) and primer2 (antisense) (5'-AGCTCCATCAACATCTGTGG), which amplified a 90 nt fragment surrounding the Nova-1 binding site. Gel-purified PCR products were used as templates for in vitro transcription. Thirty microliters of total cell extracts from mouse brainstem (50 μl/μg) were irradiated with UV light for 15 min. The samples were treated with 80 U of RNAse A (Worthington) for 30 min at 37°C. UV cross-linking reactions were immunoprecipitated with a 5 μl polyclonal rabbit anti-Nova-1 antisera in a total volume of 100 μl of lysis buffer. The immune complexes were precipitated with protein A–Sepharose beads (Sigma), run on 10% SDS–PAGE gels, transferred to nitrocellulose, and exposed to film.

Purification of Nova-1 fusion protein:

Full length Nova-1 was cloned into pET21a (Novagen) and transformed into BL21 competent cells, followed by standard IPTG-induction and purification by nickel-chelation chromatography. The fractions containing recombinant Nova were further purified by affinity chromatography using an anti-T7 monoclonal antibody covalently

coupled to cross-linked agarose beads (Novagen). The bound protein was eluted with 0.1 M citric acid, pH 2.2 directly in tubes containing 2 M Tris Base. Fractions containing protein were pooled and affinity purified with a poly(G) resin (Sigma) and eluted in 0.25, 0.5 and 1 M NaCl. The fractions were pooled, dyalized against buffer D and concentrated in Slyde-A-Lyzer concentrating kit (Pierce). Nova fusion protein preparation were assessed for purity by electrophoresis on 5-20% polyacrylamide-SDS gels and Coomasie blue (BioRad) staining.

***In vitro* splicing assay:**

Substrates GABA/Globin A, B and mutant were transcribed in the presence of α-^{32}P-UTP. Transcription conditions were as follows: 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine 0.5 mM ATP, CTP, GTP, 0.05 mM UTP, 1.65 μM α-^{32}P-UTP (ratio cold UTP/labeled UTP= 30.3), 0.2 mM m7dG(5')ppp(5')G, 1 mM DTT, 3 μg template DNA, 50U T7 RNA polymerase. The transcription mix was incubated 2 hrs at 37°C. After treatment with Dnase I (final concentration 30U/ml) 20 min at 37°C, the product was gel-purified using a 6% polyacrylamide 7M Urea gel. *In vitro* splicing assays were performed as described in Mayeda and Krainer, 1999. Briefly, the reaction was carried out in 25 µl total volume, with approximately 20 fmol of ^{32}P-labelled, 7CH_3-GpppG-capped substrate, in the presence of 0.5 mM ATP, 2 mM creatine phosphate, 3.2 mM $MgCl_2$, 2.6% (w/v) polyvinyl alcohol (PVA). The reaction contained 7.5 µl of HeLa nuclear extract in buffer D (20 mM Hepes-KOH, pH 8.0, 0.2 mM EDTA, 20% (v/v) glycerol, 100 mM KCl, 1 mM DTT, 0.5 mM PMSF), to which 1 to 3 pmol of NOVA-1 recombinant protein in buffer D were added. Following incubation for 4 hrs at 30°C, the

RNA was extracted with Tryzol LS (Invitrogen), precipitated and analyzed on a 6% Acrylamide-Bisacrylamide (19:1), 7M Urea, 1X TBE gel. The gel was dried and exposed. The intensity of the bands on the gel was quantified using a phospho imager.

Debranching Assay:

Bands were cut out of polyacrylamide dried gel, crushed in extraction buffer (0.5 M NH_4Ac, 10 mM $MgAc_2$, 1 mM EDTA, 0.1% SDS), eluted at 4°C for 6 to 12 hrs, and the gel debris eliminated by filtering the slurry through a glass pre-filter. The recovered RNA was precipitated. The de-branching assay was performed essentially as described in (Ruskin and Green, 1990). Briefly, the RNA was incubated in the presence of 15 µl of HeLa cytoplasmic S100 extract, 8 mM EDTA, in a final volume of 25µl for 45 minutes at 30°C. At the end of the incubation, RNA was phenol-chloroform extracted, precipitated and analyzed on a denaturing gel.

Primer extension:

RNA template was purified from denaturing gels as described above for the de-branching reaction. DNA primer antisense to the 3'splice site and polypyrimidine tract of the second intron of GABA/globin splicing substrate was labeled with ^{32}P-[γ-ATP] and T4 polynucleotide kinase, and gel purified. Around 25 Kcpm labeled oligo-primer was added to around 5 fmol of eluted RNA in 1X annealing buffer (AB) 50 mM Tris pH 8.3, 60 mM NaCl, 10mM DTT in 6µl total reaction volume and annealed by heating to 90°C for 3 min and letting slowly reach 45°C. Primer extension was performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

Immunoprecipitation from splicing reactions:

Immunoprecipitation from *in vitro* splicing reactions was performed essentially as described (Blencowe et al., 1994). *In vitro* splicing reactions containing GABA/globin B substrate, GABA/globin B substrate in presence of 0.06 μM recombinant Nova-1, GABA/globin mutant substrate in presence of 0.06 μM recombinant Nova-1 were scaled up seven times. Rabbit serum anti-mouse Fc was pre-adsorbed to protein A immobilized on Sepharose beads (Sigma). After three washes in IP100 (50 mM Tris 7.45, 100 mM NaCl, 2 mM $MgCl_2$, 0.5 mM DTT, 0,05% (v/v) NP40), equimolar amounts of monoclonal antibodies against Sm domain (Y12; Neomarkers), T7 tag (Novagen) and purified mouse IgG (Pierce) were adsorbed to the protein A beads for 2 hrs at 4°C and washed four times in IP 100. At the end of the four hrs incubation, the splicing reactions were divided in four aliquots. Three aliquots from each reaction were used for immunoprecipitation with each of the indicated antibodies, and RNA from the remaining fraction was directly extracted as the input control. The aliquots used for immunoprecipitation were twice the size of the one from which RNA was directly extracted. After 1.5 hrs incubation at 4°C, the supernatant was removed and the pellet was washed three times in 1 ml IP100. The RNA was extracted from the immunoprecipitated pellets with Tryzol, and analyzed on denaturing PAGE.

Yeast two hybrid assay:

Full-length Nova-1 cDNA was cloned into the LexA vector pBTM116, and transformed into LD40 yeast strain. LD40 yeast are leu-/trp- and contain the lacZ and His+ genes

under the control of LexA promoters. The bait constructs (a kind gift of David Elliot, MRC, Edinburgh, UK) were cloned in PAS vectors (Clontech). Transformations and tests for protein-protein interactions (growth in trp-, leu-, his- medium and production of β-Galactosidase) were performed according to standard protocols (Sambrook et al. 2001).

GST pull-down assay in transfected cells:

In vivo GST pull-down assays were performed by co-transfecting N2a cells in 10 cm diameter plates with constructs encoding the indicated proteins fused to GST subcloned in the PEBG eukaryotic expression vector (a gift of Dr. T Shishido), and vectors encoding T7-Nova. Cells were harvested 2 days later, lysed in LB50: 20 mM Tris 7.5, 50 mM NaCl, 5 mM EDTA, 1% NP40, 1 complete tablet (Roche). Cells were sonicated, debrees removed by centrifugation (14.000 X g, 5 min), and glutathione-sepharose 4B (Pharmacia) beads were added. After overnight incubation, the beads were washed five times with LB50. During the third washing step they were incubated in 23 μg/ml RNAseA for 20 min at 37°C. Proteins recovered in the pellet were analyzed by SDS PAGE and western blot.

Sucrose Gradient centrifugation and Polysome Fractionation:

Analysis of polysomes from cell lines on sucrose gradient was performed using standard protocols (Ruan et al., 1997). For each density gradient analysis of neural polysomes, four spinal cords and hindbrains were dissected. Hypotonic Low Salt Buffer (20 mM Tris 7.4, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1 complete mini EDTA free protease inhibitor cocktail (Roche) for 10 ml of buffer, 100 μg/ml Cycloheximide (CH), Rnasin 20

U/ml) was added to the dissected tissue in a proportion of roughly 0.25 ml of buffer for each 0.1 mg of tissue. The tissue was then homogenized in a motor-driven Teflon-Glass (900 rpm, 10 strokes), and nuclei were removed with a low speed centrifugation step (2,000 X g, 10 min), and the supernatant (S1) was collected. A volume of detergent solution (1.2% NP-40, 0.5 mM DTT, 100 μg/ml CH, 1 complete mini EDTA free protease inhibitors cocktail (Roche), Rnasin 20U/ml) equal to 25% of the S1 volume, and a volume equal to 10% of the sum of S1 and detergent buffer of High Salt Buffer (20mM Tris 7.4, 1.5M KCl, 3mM $MgCl_2$, 0.5mM DTT, 1 complete mini EDTA free, 100μg/ml CH, Rnsain 20U/ml) were added to the supernatant. Subsequently, we separated an ER vesicle-enriched P2 fraction (or "bound" fraction) by centrifugation 20,000 X g for 10 min, from the S2, or "free" fraction. Deoxycholic Acid (DOC) was added to a final concentration of 0.5% (w/v) to S2 and to the resuspended P2, a further centrifugation 20,000 X g for 10 min was performed to eliminate DOC-insoluble material, and the supernatants were loaded on linear 20 to 50% (w/w) sucrose gradients in 20 mM Hepes, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$. For the microarray assays, we did not fractionate separately S2 and P2; we added DOC directly to S1, spun 20,000 X g for 10 min and loaded the resulting "total" supernatant on the sucrose gradients. The gradients were centrifuged 200,000 X *g* in SW 41 rotors (Beckman) for 2 hrs. After the centrifugation, gradients were fractionated using an ISCO fractionator while monitoring the absorbance at 254 nm.

RNA isolation and Oligonucleotide Array Expression Analysis:

For microarrays analysis, pooled spinal cords and hindbrains from four Nova-1-null

animals were used for each replicate. As control, spinal cord and hindbrains from wild type or heterozygotes littermates were processed in parallel. One fifth of each lysate was used to directly extract the unfractionated "Total" RNA. Twelve fractions were collected from each gradient, and RNA obtained from three polysomal fractions, as judged by the UV_{254} absorption profile and by western blot with anti-P0 ribosomal protein, was pooled. The RNA extraction was performed initially by Trizol extraction (Invitrogen), followed by a second purification and DNAse treatment step using the RNeasy kit (Qiagen). The yield of RNA from the pooled polysomal fractions was 15 to 20 μg on average. The synthesis of the probes for microarrays hybridization was performed as described at **http://www.affymetrix.com**. The purified RNA is initially reverse transcribed using a primer containing a 3' end oligo-(dT) and a 5' end T7 promoter sequence. Double stranded cDNA is then transcribed presence of biotinilated UTP and CTPs, producing a biotinilated cRNA probe, which is used for the hybridization. Hybridization and data collection was performed by the Rockefeller University Gene Array Resource Center. Data were initially collected in the Affymetric software MicroArray Suite, and then transferred to Gene Spring software (Silicon Genetics). To allow comparison between chips, the intensity value of each gene was normalized to the mean intensity value of entire chip. Each gene represented on the chips is associated with four sets of intensity values defined by the experimental parameters: wild type total, wild type polysomes, knock out total, knock out polysomes. Each set is made of three values, since the entire procedure was repeated three times. When comparing mRNAs in the polysome fraction between wild type and mutant, we normalized the intensity values in the polysome fraction to corresponding values in the total, to ensure that we were observing changes

that occurred specifically in the polysomes.

Metabolic Phosphorylation assay:

N2a cells growing in 10 cm diameter dishes in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) were transfected with 5 μg of vector DNA encoding T7 tagged Nova-1 using 15 μl of Fugene (Roche) according to the manufacturer's instructions. When cells reached 70% confluence, usually after 40 hrs incubation, the medium was substituted with Phosphate-free DMEM supplemented with Dyalized FBS. After one hour incubation in Phosphate-free medium, inorganic ^{32}P to a final 0.5 mCi/ml was added to the dishes. After 5 hrs incubation at 37°C, the cells were harvested and lysed in 1 ml lysis buffer (1% NP 40, 0.5% DOC, 0.1% SDS, 0.15 M NaCl, 0.01 M Na phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM Na_2OV_4, 1 complete protease inhibitor cocktail (Roche). Nova-1 was immunoprecipiated with 5 μg anti-T7 tag monoclonal antibody (Novagen) after a pre-clearing incubation with a pre-immune mouse serum adsorbed to protein A beads. The pellet was washed five times with lysis buffer. After the second washing step, the pellet was resuspended in TBS containing 2 mg/ml RNaseA and 3,000 U/ml RNase T1, and incubated 15 min at room temperature. After the last washing step, the pellet was resuspended in 2X SDS loading buffer, resolved by electrophoresis on SDS-PAGE, transferred to a nylon membrane and exposed to a radiography film. The identity of the bands observed by autoradiography was confirmed by western blot.

Immunofluorescence of cell cultures:

For immunofluorescence of cell cultures, cells were plated onto chamber slides (Nalge Nunc), transfected where appropriate, and two days later rinsed with PBS, fixed (10 min in 2% paraformaldehyde in PBS at room temperature), washed, permeabilized (10 min in 0.5% NP-40 in PBS at room temperature), washed again, blocked (1 hr at room temperature in PBG: 0.2% gelatin and 0.5% bovine serum albumin in PBS) and stained overnight with primary antibody in PBG at 4°C. After three washes in PBG, slides were incubated with fluorochrome-conjugated secondary antibodies in PBG for 1 hr at room temperature in the dark. Slides were then washed three times in PBG, once with DAPI in PBS, and once in PBS alone. Slides were left at room temperature to dry and then covered with mounting medium (Biomeda) and a coverslip (Fisher) before being sealed with nail polish. The slides were observed under multiple wavelength fluorescence from a mercury lamp (AttoArc), in an upright microscope (Zeiss Axioplan). Pictures were taken with a digital camera (Hamamatsu Orca), using Openlab software (Improvision) and presented with Photoshop software (Adobe).

Chapter 3

1) Role of Nova-1 in the regulation of alternative splicing:

Introduction: *In vitro* RNA selection studies have identified the consensus sequence $(UCAUY)_3$ as a high affinity binding target for Nova-1 recombinant protein (Buckanovich and Darnell, 1997; Jensen et al., 2000b). In an attempt to identify RNA sequences that Nova-1 binds *in vivo*, public databases and a compilation of neuron-specific alternatively spliced exons and their surrounding introns (Stamm et al., 1994) were analyzed. Two candidate target sequences were identified in this way, both in an intronic location close to alternatively spliced exons. Only two potential Nova-1 *in vivo* RNA binding sites were initially identified, one within an intron of the Glycine Receptor α2 subunit (GlyRα2) pre-mRNA (Kuhse et al., 1991), and one in an intron of the Nova-1 pre-mRNA itself (Buckanovich and Darnell, 1997). While these pre-mRNAs were shown to be indeed associated with Nova-1, by co-immuno-precipitation of the RNAs bound to Nova-1 followed by RT-PCR with specific primers, proof that the native Nova protein from mouse brain is able to directly bind the Gly R α2 intronic (UCAUY)3 element was still lacking.

Results: To address this question, a 90 nt intronic RNA surrounding the Gly R α2 (UCAUY)3 element was transcribed in the presence of ^{32}P-UTP, and ultraviolet (UV) cross-linked to proteins in brain extracts. As a control, we also transcribed a mutant Gly R α2 RNA, (UAAUY)3, previously shown not to bind Nova-1 in vitro (Buckanovich and Darnell, 1997) (Fig. 1A). These mixtures were RNase treated, and Nova protein was

immunoprecipitated, run on SDS–PAGE gels, and transferred to nitrocellulose. A ^{32}P-labeled RNA band superimposable on the 55 kDa Nova protein band was seen specifically in immunoprecipitates of extracts cross-linked to the wild-type $(UCAUY)_3$ but not the mutant $(UAAUY)_3$ RNA (Figure 1B). This result demonstrates that native neuronal Nova protein specifically binds to the Gly R α2 intronic $(UCAUY)_3$ RNA.

The location of the Nova-1 binding sites in introns in the proximity of alternatively spliced exons suggested the possibility that Nova-1 could modulate the alternative splicing of its target RNAs. We examined whether Nova-1 is able to directly act on GlyRα2 pre-mRNA to regulate alternative splicing of the E3A/E3B exons. We generated a minigene construct consisting of genomic DNA encoding GlyRα2 E2, E3A, E3B, and E4 and several hundred nucleotides of intronic DNA on either side of these exons (Figure 2A). To allow us to determine whether Nova-1 acts directly on the GlyRα2 intronic (UCAUY)3 element, we also generated a mutant minigene in which this element was mutated to the nonbinding (UAAUY)3 sequence. These constructs were then transfected into three different cell lines along with increasing amounts of a plasmid expressing Nova-1 (pcNova-1). The total amount of DNA transfected in each well was normalized by transfecting appropriate amounts of empty vector DNA. 48 hours after transfection the RNA was extracted, reverse transcribed and amplified by PCR using primers specific to GlyRα2 E2 and GlyRα2 E4. Since the splicing products including exon 3a and exon 3b are exactly the same size, reaction products were digested with restriction enzymes that specifically recognized sites either in E3A (SspI) or E3B (BspMI). We confirmed that these enzymes uniquely recognized E3A or E3B (with >98% specificity; data not shown). Nova-1 transfection mediated an increase in GlyRα2

E3A splicing in each of the three cell lines tested. Figure 2B illustrates the results of splicing assays performed on extracts of a neuroblastoma cell line, N2A, transfected with the wild-type (UCAU) or mutant (UAAU) GlyRα2 minigene in the presence or absence of pcNova-1. A Nova-1 dose-dependent increase in splicing to E3A (and compensatory decrease in E3B) is evident in cells transfected with the wild-type UCAU but not the mutant UAAU GlyRα2 minigene. We demonstrated that increasing amounts of pcNova-1 vector led to increasing amounts of Nova-1 protein by Western blot analysis (Figure 2C).

To confirm these results and analyze splicing to both E3A and E3B, we repeated independent transfection experiments in N2A cells (Figure 2D). In cells transfected with the wild-type UCAU GlyRα2 minigene, Nova-1 reproducibly mediated a two-fold increase of E3A splicing at the expense of E3B splicing.

In N2A cells transfected with the mutant UAAU GlyRα2 minigene, splicing to E3A was unchanged relative to the wild-type minigene (Figure 2D). In the presence of increasing amounts of Nova, an increase in E3B, at the expense of E3A, was seen. While the mechanism underlying this observation is unclear, a possible explanation is that the UAAU mutation allowed detection of a cryptic Nova-1 binding site able to mediate an action on E3B splicing. Taken together, these results demonstrate that the action of Nova to enhance E3A utilization in wild-type UCAU GlyRα2 pre-mRNAs was abolished by mutation in the intronic UCAU Nova-binding element.

We repeated these experiments in HeLa cells and 293T cells, two non-neuronal cell lines that do not express Nova (Figure 2E and Figure 2F; data not shown). pcNova-1 cotransfection with the wild-type GlyRα2 minigene mediated a 2.5-fold increase in E3A splicing in HeLa cells and a 2-fold increase in 293T cells. Some additional effects were

seen. In HeLa cells, the increase in E3A splicing was at the expense of E3B, while in 293T cells the increase in E3A-spliced product was inversely proportional to a decrease in an aberrant spliced product that contained neither E3A nor E3B (E2-E4; data not shown). We also found that in cells transfected with the mutant UAAU GlyRα2 minigene, in the presence of Nova there was no increase in E3A splicing but again an increase in E3B splicing, and in the absence of Nova there was a small but consistent increase in E3A splicing that is unexplained. Taken together, these experiments demonstrate that Nova-1 is able to act directly to enhance the inclusion of GlyRα2 E3A in cell lines and that this action is dependent on the integrity of the intronic UCAU Nova binding site.

The effects of transfected Nova on GlyRα2 minigene alternative splicing are in agreement with the effects of lack of Nova-1 on GlyRα2 splicing observed in Nova-1-null animals (Jensen et al., 2000a). Nova-1 null mutant mice, generated by homologous recombination, are viable and phenotypically indistinguishable from their littermates at birth. Thereafter, they demonstrate progressive motor dysfunction and die an average of 10-15 days after birth. Prior to their death, Nova-1 null mice exhibit an action-induced tremulousness and overt motor weakness, although they maintain normal responses to sensory stimuli. There is no abnormal phenotype in Nova-1 heterozygotes. Histologic examination and TUNEL staining of Nova-1 null mice revealed a 4-fold increase in the number of picnotic cells in the areas of the CNS expressing Nova-1 relative to littermates (Jensen et al., 2000a).

Since GlyRα2 gene expression and physiology in rodent spinal cord and brainstem undergo regulation after the first week of postnatal life (Bechade et al., 1994;

Kuhse et al., 1991; Singer et al., 1998), GlyRα2 splicing was examined at various ages in Nova-1 null mice using RT PCR and RNAse protection assay. These studies demonstrated that the ratio of E3A/E3B splicing was decreased in Nova-1 null mice relative to wild-type littermates by 2-fold. Importantly, splicing defects in Nova-1 null animals were limited to spinal cord and hindbrain, the areas where Nova-1 is normally expressed (Jensen et al., 2000a). These results are consistent with our transfection data, in which Nova-1 acts to enhance the utilization of GlyRα2 E3A, while in the absence of Nova-1 in vivo there is a relative deficit in the ability of neurons to utilize the E3A splice site.

2) *In vitro* assay of Nova action in alternative splicing:

We sought to characterize the mechanism through which Nova achieves regulation of alternative splicing by establishing an *in vitro* assay. Attempts to recapitulate *in vitro* the alternative splicing pattern of GlyRα2 failed, probably due to the size of the four-exon construct and the complexity of the splicing choices (data not shown). We then turned to another target pre-mRNA, which emerged from the analysis of the Nova-1 null animals as a target for Nova-1 regulation. Alternative splicing of the $GABA_A$ receptor γ2 subunit ($GABA_AR\gamma2$) was significantly altered in Nova-1 null mice (Jensen et al., 2000a). The $GABA_AR\gamma2$ transcript is widely expressed in the brain, and the pre-mRNA is alternatively spliced to include the cassette exon 9 preferentially in brainstem and spinal neurons (Zhang et al., 1996). In Nova-1 null animals, the ratio between the exon 9-including and exon 9-excluding forms (called L and S, respectively) was decreased three-fold, suggesting a role for Nova-1 in promoting the inclusion of the alternative exon

(Jensen et al., 2000a).

The effect of Nova-1 on $GABA_AR\gamma2$ pre-mRNA has been further characterized by transfection of minigenes in heterologous cell lines (Dredge and Darnell, in press). For these studies, a modified version of SP64-H$\beta\Delta6$ (Krainer et al., 1984) was employed, in which the length of the middle exon was reduced to 24 nucleotides to attain a roughly 1:1 ratio between the form including and the form excluding the alternative exon when the minigene was transfected in 293T cells. The second intron was also shortened. By inserting progressively shorter sequences from intron 9 of $GABA_AR\gamma2$ in the second intron of this minigene, a sequence of 24 nucleotides was identified, that was able to confer Nova-dependent regulation of the inclusion of the alternative exon (Dredge and Darnell, in press). The 24 nucleotide Nova-dependent intronic splicing enhancer (NISE) contains four UCAU repeats, and their mutation to UAAU completely abolishes the Nova-1 effect on alternative splicing (Dredge and Darnell, in press).

We resolved to utilize the two constructs Globin/GABA 1 and 2 to test the effect of Nova-1 in an *in vitro* splicing assay. Globin/GABA 1, containing 68 nucleotides of the $GABA_AR\gamma2$ sequences, was the construct showing the most prominent effect of Nova-1 in transfection, where the ratio between the long, alternative exon including form (L), and the short final product of splicing (S) increased around ten times in response to Nova co-transfection (Dredge and Darnell, in press). Globin/GABA 2 is the construct containing the minimal 24 nucleotides NISE sequence necessary to enable a Nova-dependent effect on splicing. The ratio between the L product and S product increased around six times in response to Nova-1 co-transfection for the Globin/GABA B construct (Dredge and Darnell, in press).

Nova was purified as a fusion protein containing a six-histidine tag at its C-terminus and a T7 tag at its N-terminus. Three sequential affinity chromatography steps were used in the purification protocol. The protein was initially bound to a Nickel column through its poly-Histidine tag, and then further purified using an immobilized monoclonal antibody against the T7 tag. A final ribohomopolymer poly(G) column was deployed to selectively recover protein still able to bind RNA after the acidic elution from the antibody column. The resulting fusion protein was almost 100% pure by Comassie stain (Fig. 3A) and able to bind its target RNA with high affinity and specificity (Fig. 3B).

We performed *in vitro* splicing assays using HeLa nuclear extracts supplemented with purified recombinant Nova protein and body-labeled RNA. After 4 hrs of incubation the RNA was extracted and analyzed on a denaturing gel. The identity of the bands corresponding to the unspliced RNA, the two alternative products of splicing and exon 1 (Fig. 4A and 5A) was defined by reverse transcription using an antisense exon 3 or 1 primer, PCR amplification, cloning of the products and sequencing. Two substrates were initially tested, one corresponding to the globin/GABA 2 containing 24 nucleotides NISE and a mutated version in which the UCAU repeats were mutated in UAAU (Fig. 4B). The presence of recombinant Nova in the reaction mix reproducibly resulted in a dose-dependent increase of the inclusion of the alternative cassette exon in the final product of splicing (Fig. 4A). The maximal increase is close to 2-fold and is observed with a Nova-1 concentration around 0.12 μM (quantified by Bradford assay), or a molar ratio of Nova-1:RNA of about 100:1 (Fig. 4C). Higher concentrations of Nova-1 in the reaction mix do not result in a further increase in the ratio between the long and the short product, which reaches a plateau and decreases (Fig. 4C and data not shown). Mutation of the GABA

NISE repeats results in the abolition of the ability of Nova-1 to promote inclusion of the alternative exon (Fig. 4C).

In addition to these observations, we noted that a band migrating at an apparent size of around 260 nucleotides appears to dramatically increase in response to increasing concentration of recombinant Nova-1 in the reaction mix (Fig. 4A, indicated with a red star). The size of the band does not correspond to any of the predicted products or singly-spliced intermediates of the splicing reaction. Furthermore, the *in vitro* splicing reaction of globin/GABA 1 substrate, which is completely identical to globin/GABA 2 but for 44 more nucleotides of sequence derived from $GABA_AR\gamma2$ (Fig 5A), results in an identical pattern of bands but for a band that becomes more prominent in a Nova-dependent fashion and migrates at an apparent size of more than 400 nucleotides (Fig. 5B, indicated with a blue star). The lack of correlation between the rate of migration of these two bands and difference in molecular size of the two RNAs suggests that they are lariats, spliced introns whose rate of migration is affected by the presence of a branching site (Ruskin et al., 1984).

The identity of these two Nova-enhanced bands was determined by incubating the RNA eluted from the gel with S100 cytoplasmic extract from HeLa cells. S100 extract contains a 2', 5' phosphodiesterase or de-branching enzyme (Ruskin and Green, 1985). The de-branching activity of S-100 extract induces a shift in the apparent size of the aberrantly migrating bands: the band deriving from splicing of globin/GABA 2 shifts from an apparent size of 260 nucleotides to a size compatible with the second intron of globin/GABA 2, which is 181 nucleotides long (Fig. 6A, compare bands 3 and 4); the band deriving from globin/GABA 1 shifts from an apparent size of more than 400

nucleotides to a size compatible with the second intron of globin/GABA 1, which is 225 nucleotides long (Fig. 6A, compare lanes 7 and 8). This assay also suggests that the bands migrating slower than the unspliced RNA correspond to the lariats including both the introns and the alternative exon that result from the skipping of the alternative exon in the splicing reaction (Fig. 6A, compare lanes 10 to 9 and 12 to 11). As a negative control, RNAs which do not contain lariats, the unspliced precursors, do not undergo any modification of their rate of migration in gel electrophoresis as a consequence of treatment with S100 extract (Fig. 6A, compare lanes 2 to 1 and 6 to 5). The apparent migration rate of lariat-containing RNAs is affected by the ratio between the sizes of the linear and the circularized portions of the molecule, and therefore by the position of the branching point (Grabowski et al., 1984). To determine whether part of the difference in the apparent rate of migration between the lariats observed in the globin/GABA 1 and globin/GABA 2 splicing reactions was due to the utilization of two different branching sites, induced by the insertion of $GABA_AR\gamma 2$ sequence of different length and composition, we mapped the branching point in the two lariat eluted from the gel by primer extension. The primer extension is stopped at the same site in the two lariats, demonstrating that the same branching point is utilized in the two constructs (Fig. 6B). Finally, the de-branched lariat from globin/GABA 2 was eluted from the gel, two RNA linkers were ligated at the 5' and 3' ends, reverse transcription and PCR amplification was performed using primers complementary to the linkers, the product was cloned and sequenced, identifying it as the precisely excised second intron (data not shown).

The dose-dependent increase of the second intron lariat-product in response to Nova-1 protein suggests that the presence of Nova promotes the excision and/or the

stability of the second intron. The two actions (excision stimulation and stabilization) are not mutually exclusive, and it is difficult to discriminate between them, since an increased recruitment of splicing factors dependent on the presence of Nova on the second intron will eventually result in stabilization of the spliced intron. If splicing activation by Nova depends on an enhanced recruitment of splicing factors on the intron Nova binds to, interactions with constitutive splicing factors are expected.

Previous yeast-two-hybrids screenings using Nova as a bait and libraries of embryonic and adult brain cDNAs as preys led to identification of a neuron-enriched PTB-like novel gene (Polydorides et al., 2000) and SRp20 (Yang et al., unpublished), among others, as interacting partners of Nova-1. The interaction with the PTB-like protein has been characterized in detail, and an antagonistic effect of brain PTB on splicing activation mediated by Nova has been demonstrated (Polydorides et al., 2000). The interaction with SRp20, which has been shown in several instances to act as a splicing activator (Cavaloc et al., 1999; Jumaa and Nielsen, 1997; Lim and Sharp, 1998), offers a potential mechanistic explanation for the activating effect of Nova-1 on exon inclusion. SRp20 is part of the large family of the SR proteins (reviewed in Graveley, 2000), and the RS domain, consisting of repeats of Arginine and Serine residues, is found not exclusively in the SR protein family, but also in a number of other nuclear proteins, and it has been characterized as a protein-protein interaction motif (Graveley, 2000). In order to control the specificity of the interaction between SRp20 and Nova, we screened several members of the SR protein family and other SR-related proteins by yeast-two-hybrid assay. Interestingly, some members of the SR proteins family, like SRp20 and 9G8, and the SR-related U2AF65, U1 70K and the U2AF65-interacting protein HUAP

56, were found to interact with Nova, while others, like SC35, ASF/SF2, SRp30c, SRp40 and the SR-related protein U2AF35 did not (Fig. 7A). Nova-1 and the closely related Nova-2 were found to interact with the same set of proteins.

The interaction of these proteins was assessed in mammalian cells by co-transfection assays. 293 T cells were co-transfected with cDNAs encoding two isoforms of Nova-1 generated by alternative splicing (Nova-1 H+ and H-, see in the next sections) tagged with T7 and several proteins tested in the yeast-two-hybrid assay fused with GST. The cell lysate was RNAse treated and the GST fusion proteins were absorbed to GTT-coated beads and the presence of complexes with Nova-1 was verified on western blot with antibodies against the T7 tag. As shown in figure 7B, the interaction of Nova-1 with U2AF65 (lane 3) and U1 70K (lane 4) were confirmed in this assay, the interaction with HUAP 56 was confirmed in a separate set of experiments (data not shown). Surprisingly, the interaction with SRp20 was not confirmed in this system. The interaction with 9G8 was not tested in this system. Furthermore, Nova-1 was shown to interact with itself in transfected cells (lane 1), in agreement with the structural studies in crystals and in solution (Lewis et al., 2000; Ramos et al., 2002), and with HuD, as previously shown (Okano and Darnell, unpublished). The recovery of T7-Nova-1 was overall inefficient (<1% of the total T7 Nova). These data show that Nova-1 is capable of interactions with a subset of splicing factors containing RS domains although, given the limited set of potentially interacting proteins we considered, interactions with other factors are possible.

The demonstrated association of Nova-1 with ninety nucleotides of the intronic sequence of GlyRα2 (Fig. 1) and the dose-dependent increase of the lariat product (Fig. 5A) of globin/GABA constructs suggest that Nova first binds to the intronic sequence of

the regulated pre-mRNA. However, we have not directly verified if Nova-1 binds to the full-length pre-mRNA in the context of assembled spliceosomes, nor if it remains associated with intermediates or products of splicing. To address these issues, the antibody against the T7 tag was used to immunoprecipitate splicing complexes assembled *in vitro* either on globin/GABA B (Fig.8A, lanes 4 and 12) or globin/GABA mutated in the UCAU repeats (Fig.8A, lane 8). The positive control, anti-Sm monoclonal antibody Y12, recognizes the common Sm snRNPs (Lerner et al., 1981), and has been shown to immunoprecipitate pre-mRNAs, intermediates and products of various RNAs engaged in spliceosomes *in vitro* (Black et al., 1985; Chabot and Steitz, 1987; Chou et al., 1999; Grabowski et al., 1985). As expected, Y12 immunoprecipitates the unspliced pre-mRNA as well as intermediates and products of the splicing reactions of globin/GABA wild type and mutated (Fig. 8A, lanes 3, 7, 11). The protein A beads adsorbed with purified mouse IgG precipitate trace amounts of various RNA species (Fig. 8a, lanes 2, 6, 10). The anti-T7 antibody, which in the absence of exogenous T7 tagged-Nova-1 does not precipitate RNAs above background levels (lane 4), efficiently precipitates various RNA species in the presence of recombinant Nova-1 (lanes 8 and 12). The precipitation of unspliced pre-mRNA was nearly as efficient as with Y12 (compare lane 11 and 12) and the precipitation of intron 2 lariat product was more than three times more efficient in the wild type RNA reaction than in the mutant (compare lanes 8 and 12). These results indicate that Nova-1 is bound to the full-length pre-mRNA in the course of the splicing reaction. The immunoprecipitation of splicing intermediates indicates that Nova-1 is present in the pre-mRNA complexes containing the spliceosome, and it is not dissociated from its substrate during spliceosome assembly and the splicing reaction. Interestingly, a

sizable amount of the long product of splicing is recovered in the Nova-1 pellets. While the ratio between the long and the short product in the pellet of the Y12 immunoprecipitation is exactly equal to the ratio between the two products in the total reaction (compare lanes 11 to 9 and 7 to 5, and Fig. 8b), there is an enrichment of the long form of almost two times in the pellet of the anti-T7 immunoprecipitation (compare lanes 12 to 9, and Fig.8b); the mutation of the Nova binding site in the second intron abolishes such enrichment (compare lanes 8 to 5, and figure 8b).

3) Discussion:

The results shown demonstrate that Nova is capable of affecting alternative splicing of artificial constructs in a way consistent with the results obtained *in vivo* in Nova-1-null mutant mice. Nova-1 present in brain extracts specifically cross-links the sequence (UCAUY)3 but not the mutant sequence (UAAUY)3, consistent with previous co-immunoprecipitation experiments demonstrating that Nova-1 protein binds GlyRα2 pre-mRNA in vivo (Buckanovich and Darnell, 1997). In cotransfection assays, GlyRα2 pre-mRNAs harboring this (UCAUY)3 sequence show a Nova-1 dose-dependent increase in utilization of the alternatively spliced E3A, while mutant pre-mRNAs harboring the (UAAUY)3 point mutant do not show this effect. In Nova-1 null mice, there is a reciprocal 2-fold decrease in the ratio of GlyRα2 E3A relative to E3B splicing (Jensen et al., 2000a). These data provide converging lines of evidence that Nova-1 regulates GlyRα2 splicing by directly binding to the pre-mRNA in neurons.

Potential mechanisms for Nova action in alternative splicing regulation: The abolition of the effect of Nova on splicing by mutations of the Nova binding site both in

transfected cells and *in vitro* supports the notion that the binding of Nova-1 to its substrates is required for regulation of alternative splicing. Furthermore, the observed ability of recombinant Nova-1 to recapitulate *in vitro* the regulation of alternative splicing shows that at least part of the action of Nova is post-transcriptional and limited to the splicing step. In identified pre-mRNAs in which Nova-1 promotes the inclusion of an alternatively spliced exon, GlyRα2 and $GABA_AR\gamma2$, the Nova binding site is situated in an intron 80 nucleotides upstream of a 3'splice site. While in the GlyRα2 case Nova binds to the intron preceding the exon whose inclusion is promoted by Nova, in the $GABA_AR\gamma2$ pre-mRNA Nova binds to the intron downstream of the regulated exon. Thus, in the first example Nova binds an intron whose 3' splice site undergoes regulated splicing, while in the case of the second intron of $GABA_AR\gamma2$ (and of GABA/Globin constructs) the 3' splice site is constitutively spliced and the 5' splice site is alternatively utilized (compare schemes in figures 2A and 4B). In both cases Nova-1 promotes the utilization of the alternative splice site more proximal to its intronic binding site.

The search for basic components of the splicing machinery interacting with Nova resulted in factors that participate in the recognition of both 5' and 3' splice sites. U1 70K is a component of U1 snRNP, whose snRNA anneals to the 5' splice site consensus sequence, and U2AF65 recognizes the polypyrimidine tract, close to the 3' end of the intron. These data suggest that Nova binds to specific sequences in the intron and recruits splicing factors through protein-protein interaction, stabilizing their interactions with the most proximal splicing sites and ultimately promoting the excision of the shorter version of the intron it is bound to. Consistent with this idea, in the only *in vitro* model available of a minigene regulated by Nova-1, the lariat product of the intron bound by Nova is

increased in a dose-dependent manner. After four hours of incubation in nuclear extract, the splicing products visualized in the urea PAGE are most probably the ones that were protected from the nucleases present in the extract because they were engaged in protein-RNA complexes. The increase of the lariat product in response to Nova may result from two mechanisms: a) increased production (by increased splicing) and stabilization by the recruited U2/U5/U6 snRNPs or b) protection from nucleases through multimerization of Nova-1 from the initial high affinity binding site to cover the rest of the excised intron, analogously to the described behavior of Rev (Malim and Cullen, 1991), therefore independently of spliceosome recruitment. The second hypothesis does not seems very probable, since the interaction between Nova and its target RNA has always been found to be restricted to the UCAU repeats, when characterized with various RNAse protection techniques, even at very high protein/RNA ratios (Dredge and Darnell, unpublished). Furthermore, the excised lariat is precipitated also by anti Sm-antibody, suggesting that it is part of a complex with snRNPs. Therefore, although we have not directly demonstrated an increased level of spliceosomal complex formation in response to Nova-1, the simplest explanation for the increased levels of lariat product is that Nova stimulates the excision of the intron through recruitment of components of the spliceosome.

While the enhanced recruitment of the spliceosome to the intron bound by Nova is a probable mechanism through which Nova regulates alternative splicing of target pre-mRNAs, alternative scenarios cannot be ruled out at present. A construct made of the first and second (alternative) exons of the globin/GABA minigene fused together, followed by the second intron and the third exon, failed to show any enhanced splicing in response to increasing level of Nova in transfection and *in vitro* (data not shown). This lack of effect

may be due to the high efficiency with which this construct is spliced constitutively, probably due to the close match to the consensus sequence of both the splice sites, polypyrimidine tract and branch point. A second competing 5'splice site is required for a Nova effect to be observed in this artificial construct. In both the GABA/Globin artificial construct and in the $GABA_AR\gamma2$ pre-mRNA, the 3' splice site of the first intron is weaker then the 3'splice site of the second, due to one and four purines, respectively, interrupting the polypyrimidine tract. An alternative way Nova could promote the inclusion of the cassette exon is by favoring the utilization of the weaker acceptor. This could be achieved by inhibiting the utilization of the constitutive 3'splice site, a mechanism opposite to the recruitment of components of the spliceosome we discussed above. A slower kinetic of the pathway leading to the exclusion of the alternative exon would unbalance the reaction in favor of the inclusion of the cassette exon. The described interaction of Nova with PTB and brPTB, a brain enriched gene similar to PTB (Polydorides et al., 2000), offers a possible mechanism for such inhibitory effect. However, this second hypothetical mechanism is difficult to reconcile with the GlyRα2 splicing regulation, where a slower kinetic of the utilization of the 3'splice site closer to the Nova binding site would lead to an increased inclusion of exon 3B, an opposite effect to what we observe in the presence of Nova.

Interestingly, we observed that some Nova is also associated with the spliced product. Furthermore, there is specifically an enrichment of the long, exon-including form in the Nova pellet. While we have not directly tested if Nova is able to bind the exonic sequences of our construct, there are no obvious consensus Nova-binding sites in

the exons, nor they are created by the fusion of the exonic sequences by splicing. Therefore, the association of Nova-1 with the spliced product could result from interactions with the same components of the splicing machinery that Nova-1 helped recruit to the intron. The spliced products are indeed precipitated also by the anti-Sm antibody in our assay, suggesting that they are not completely disengaged from the splicing machinery. An alternative, albeit not incompatible explanation, is that Nova remains associated to the splicing product through interactions with components of the exon junction complex. The observed interaction between Nova and UAP56 provides a possible mechanism for such interaction. UAP56, a DEAD motif containing helicase, has been described as a factor coupling splicing and RNA export. UAP56 is required for interaction between U2 snRNP and the branchpoint (Fleckner et al., 1997), and it is eventually necessary for the recruitment of the mRNA export factor Aly to the spliced mRNP (Luo et al., 2001; Strasser and Hurt, 2001). Further experiments, in particular the purification of the mRNP through centrifugation on linear density gradient, are required to verify whether Nova is associated with the spliced RNA in the context of a mature mRNP. The "tagging" with Nova molecules of mRNA resulting from alternative splicing regulated by Nova could offer an interesting mechanism to couple nuclear events of RNA processing to the downstream steps of export, localization and translation.

Figure 1: Nova-1 protein from CNS lysates binds the predicted site in GlyRα2 intron.

(A) The 90 nt of GlyRα2 pre-mRNA sequence surrounding the Nova-1 binding consensus site. The mutations of the site are indicated in light blue. (B) An extract of mouse brainstem was incubated with the 90 nt ^{32}P-labeled RNAs shown in A., encoding either the GlyR α2 UCAU intronic sequence (lanes 1, 2) or mutant UAAU RNA (lanes 3, 4). Samples were UV cross-linked, RNase treated, and immunoprecipitated with either rabbit anti-Nova serum (lanes 1, 3) or pre-immune serum (lanes 2, 4), run on SDS–PAGE gels and transferred to a filter for Western blot analysis with POMA antiserum. The same filter was also exposed directly to X-ray film to detect ^{32}P-labeled RNA (Autoradiography). The arrow indicates the molecular size of Nova-1. The strong reactivity present underneath the Nova-1 bands (lanes 1–4) is due to secondary antibody cross-reactivity with rabbit immunoglobulin G. The ~70 kDa band visible in lanes 1 and 3 is a protein related to Nova-2 that immunoprecipitates specifically with POMA antisera.

A.

5'–AUCAUGCAGUUCUGGUUUAAUUUUUUUUCUUUGCAGUCUCAUCAUCAUCAUUUUCAU
UUUGUUUACAUCGCUAACCACAGAUGUUGAUGGAGCU–3'

B.

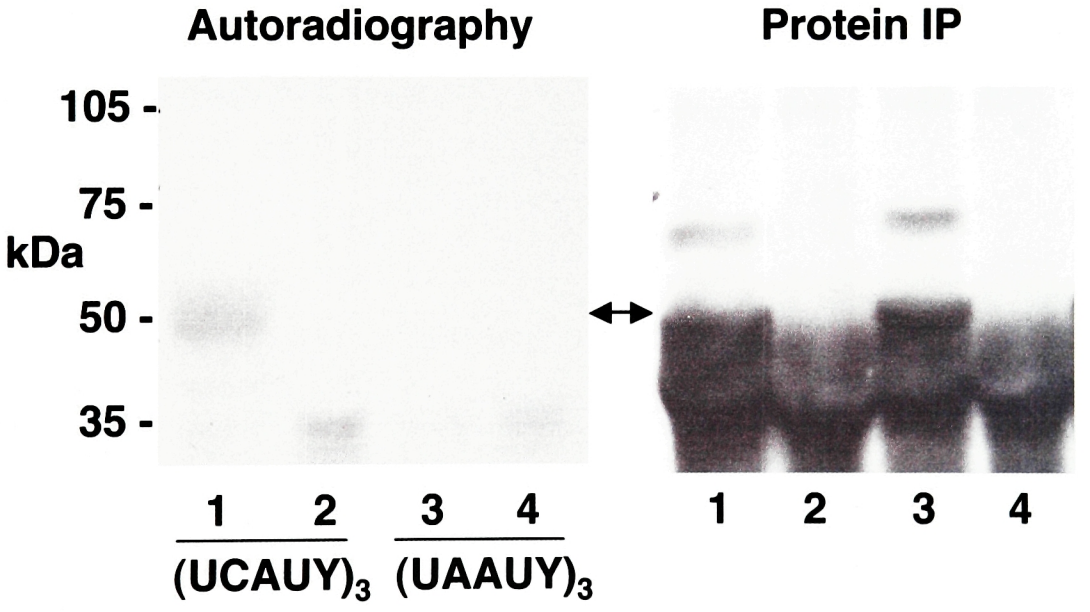


Figure 2: Nova-1 Enhances GlyRα2 E3A Splicing in Heterologous Cell Lines.

(A) The GlyRα2 minigene used for splicing assays in transiently transfected cells contains the complete GlyRα2 E2, a shortened intron 2, the complete genomic sequence surrounding E3A and E3B, a shortened intron 3, and 233 nucleotides at the 5' end of E4. The UCAU Nova-1 binding site in intron 2 is indicated by the schematic hairpin loop, and the UAAU mutant minigene is indicated by an X.

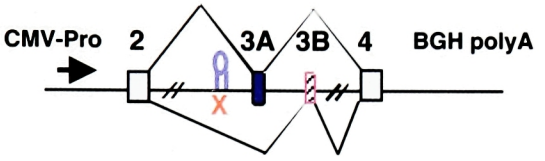
(B) Nova-1-dependent effects on E3A splicing of the wild-type UCAU and mutant UAAU GlyRα2 minigenes. RNA from N2A cells transfected with the wild-type or mutant GlyRα2 minigene and the indicated amount of pcNova-1 plasmid were analyzed as in Figure 3A.

(C) Western blot using anti-T7 antibody showing the titration of T7-tagged Nova protein levels after N2A cells were cotransfected with the GlyRα2 minigene and the indicated amounts of pcNova-1.

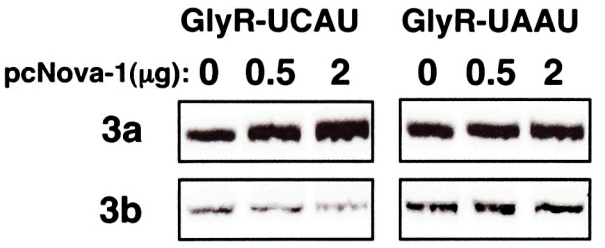
(D) Nova increases the ratio of E3A/E3B splicing from wild-type GlyRα2 pre-mRNA, but this effect is abrogated in the GlyRα2 mutant UAAU pre-mRNA. Three independent transfections were performed in which splicing products (E3A or E3B) were quantitated as in Figure 3A. Increasing amounts of pcNova-1 were cotransfected with either the wild type or mutant GlyRα2 minigene, as indicated. Results are displayed as a ratio of E3A/E3B. The total amount of spliced product is unchanged as a result of Nova titration; the increase in E3A usage is accompanied by a compensatory decrease in E3B utilization (data not shown). Results shown are the average of three independent transfections, and error bars represent standard deviation.

(E and F) Nova facilitates E3A splicing in HeLa (E) and 293T (F) cells specifically in wild-type UCAU GlyRα2 transcripts. Western blot confirmed that HeLa and 293T cells did not express Nova and that Nova transfection led to linear increases in Nova protein (data not shown). Results are the average of two (E) and four (F) independent transfections.

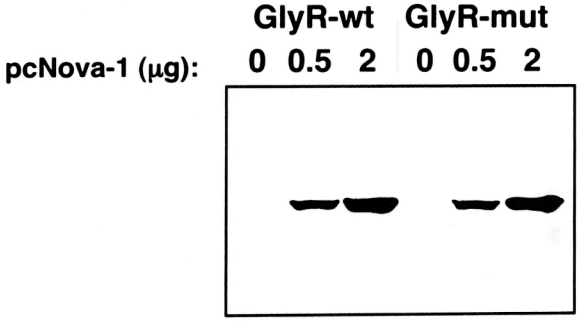
A.



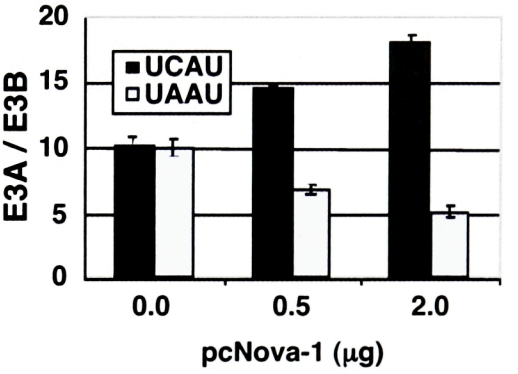
B.



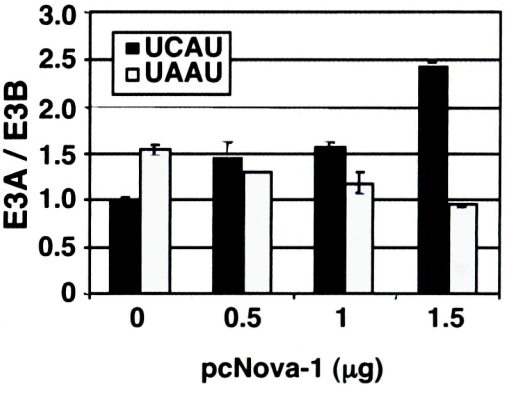
C.



D. N2A



E. HeLa



F. 293T

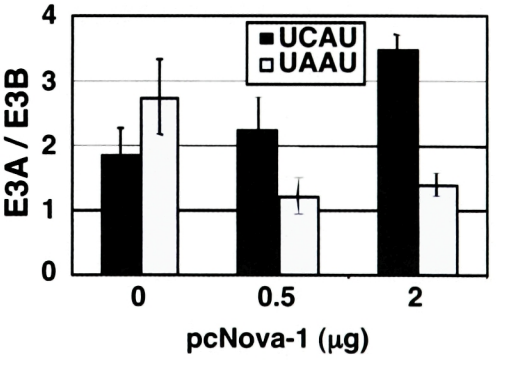
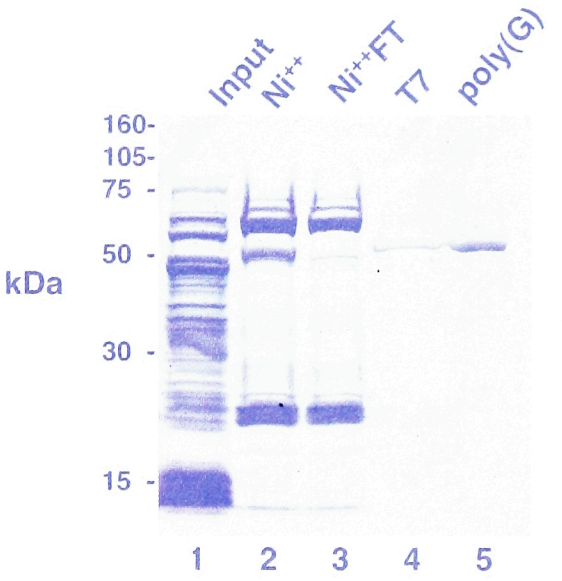


Figure 3: Scheme of purification of recombinant Nova-1:

(A) Recombinant Nova-1 fused to a C-terminal poly-Histidine tag and a N-terminal T7 tag was purified from transformed *E.Coli* through three affinity steps: Ni^{++} column, followed by anti T7 affinity column, and a ribohomopolymer column. Lane 1: an aliquot of the bacterial lysate. Lane 2: the eluted material from the Ni^{++} column; lane 3: the flow-through from the Ni^{++} column. An almost pure Nova was obtained by anti-T7 affinity chromatography (lane 4), and it was further purified and concentrated by the binding to ribohomopolymer affinity step (lane 5).

(B) Nitrocellulose filter binding assays were performed using Nova-1 fusion protein eluted from the ribohomopolymer column in 0.25 M NaCl and dialyzed against buffer D. GABA= *in vitro* transcribed RNA corresponding to the 150 nucleotides region of $GABA_AR\gamma2$ intron 8 surrounding the Nova-dependent intronic splicing enhancer (Dredge and Darnell, in press). Globin= RNA derived from human β-Globin which spans a 150 nucleotides region of exon 1 and intron 1, and contains no YCAY motifs.

A.


Input
Ni++
Ni++FT
T7
poly(G)
kDa
160-
105-
75 -
50 -
30 -
15 -
1
2
3
4
5

B.

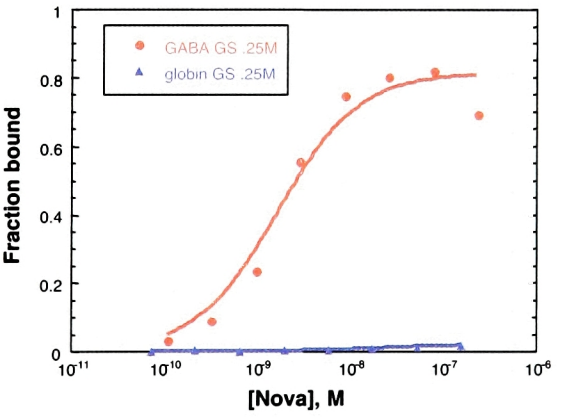

GABA GS .25M
globin GS .25M
Fraction bound
1
0.8
0.6
0.4
0.2
0
10-11
10-10
10-9
10-8
10-7
10-6
[Nova], M

Figure 4: Nova-1 regulates alternative splicing *in vitro*:

(A) ^{32}P-labeled RNA products and intermediates of a splicing reaction in HeLa nuclear extract displayed on 8% denaturing gel. The reaction was carried out for 4 hrs in the presence of 0 (lanes 1, 2, 6), 0.06 (lanes 3 and 7), 0.12 (lanes 4 and 8) and 0.25 μM (lanes 5 and 9) recombinant Nova. The substrates, indicated on the top of the gel as WT and mut, are GABA/Globin 2 and GABA/Globin 2 mut (see panel B), body labeled with ^{32}P-αUTP. The identity of the indicated bands was established by RT PCR, cloning and sequencing. The red star indicates the lariat characterized in detail in figure 6.

(B) GABA/Globin 2 substrate. The indicated cytidine residues were mutated to adenosine in the GABA/Globin 2 mut substrate.

(C) Quantification of three different experiments performed on different days with different preparations of the substrates. The concentration of recombinant Nova is plotted in the *x*-axis; the ratio between the long and the short form normalized for the ratio observed in absence of added Nova is plotted in the *y*-axis. The red bars represent the ratio between the long and the short products in reaction containing the wild type substrate, the blue bars represent the same ratio in reactions containing the mutant substrate. The error bars represent standard deviations.

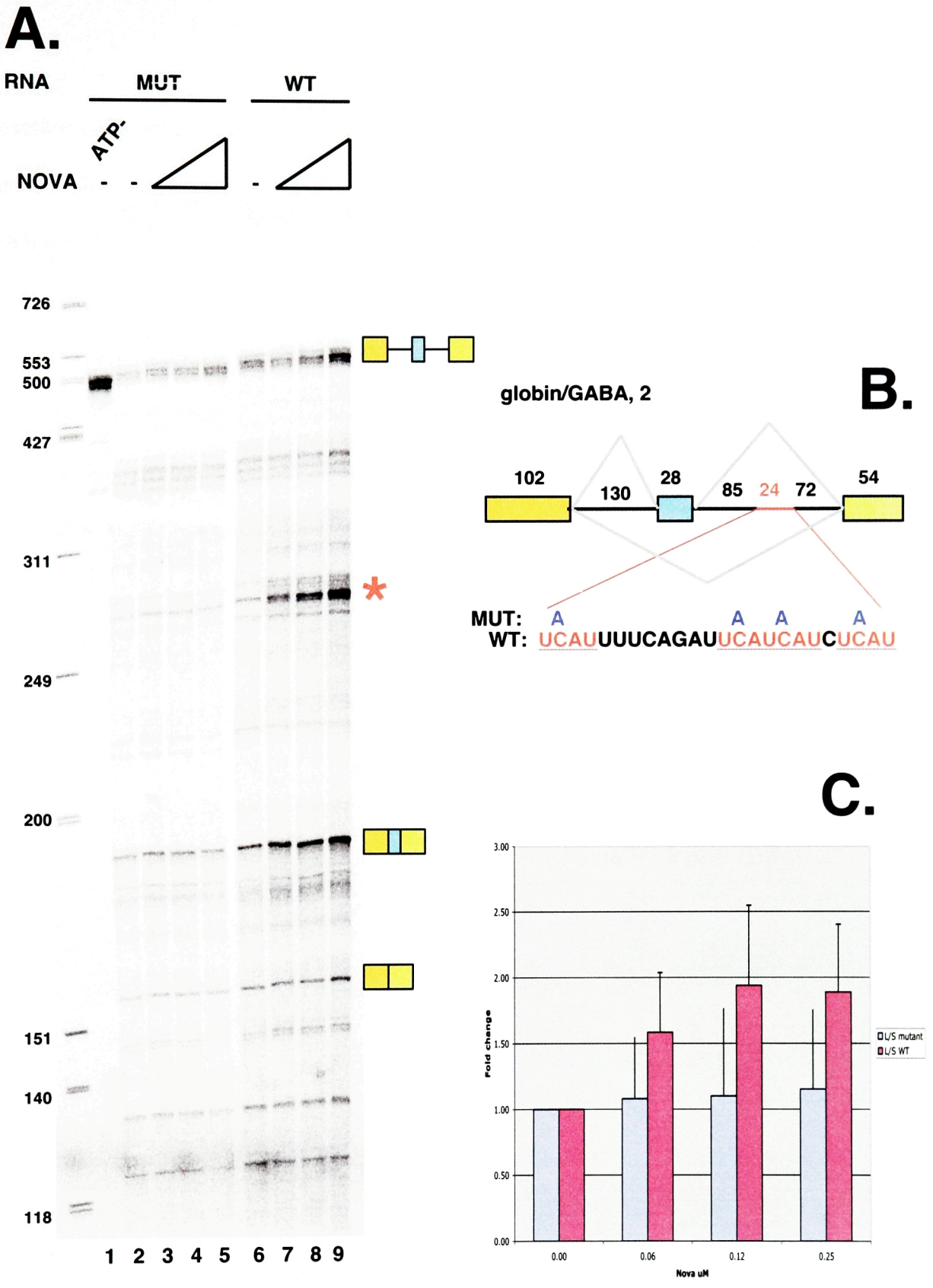
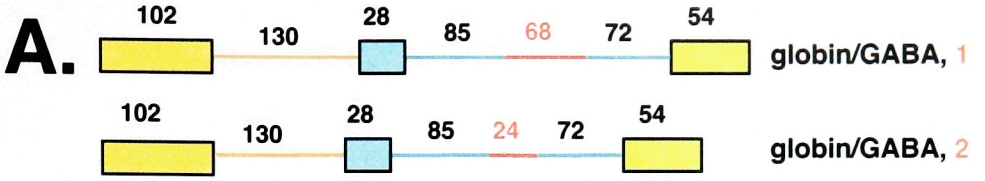

A.
RNA
MUT
WT
ATP-
NOVA
726
553
500
427
311
249
200
151
140
118
1 2 3 4 5 6 7 8 9
globin/GABA, 2
B.
102
130
28
85
24
72
54
MUT: A A A A
WT: UCAUUUUCAGAUUCAUCAUCUCAU
C.
Fold change
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
0.06
0.12
0.25
Nova uM
U/S mutant
U/S WT

Figure 5: Formation of Prominent Nova-dependent products of splicing in vitro:

(A) Schematic of the substrates used for *in vitro* splicing reactions. GABA/Globin 1 and GABA/Globin 2 only differ for 44 nucleotides of $GABA_AR\gamma2$ intron 8 inserted in a globin-based backbone.

(B) In vitro splicing reactions as in figure 4A using GABA/Globin 1 and GABA/Globin 2 as substrates. The stars indicate the bands from which RNA was eluted and analyzed in de-branching assay (see figure 6). The color code of the stars is the same as in figure 6.


A.
102
130
28
85
68
72
54
globin/GABA, 1
102
130
28
85
24
72
54
globin/GABA, 2

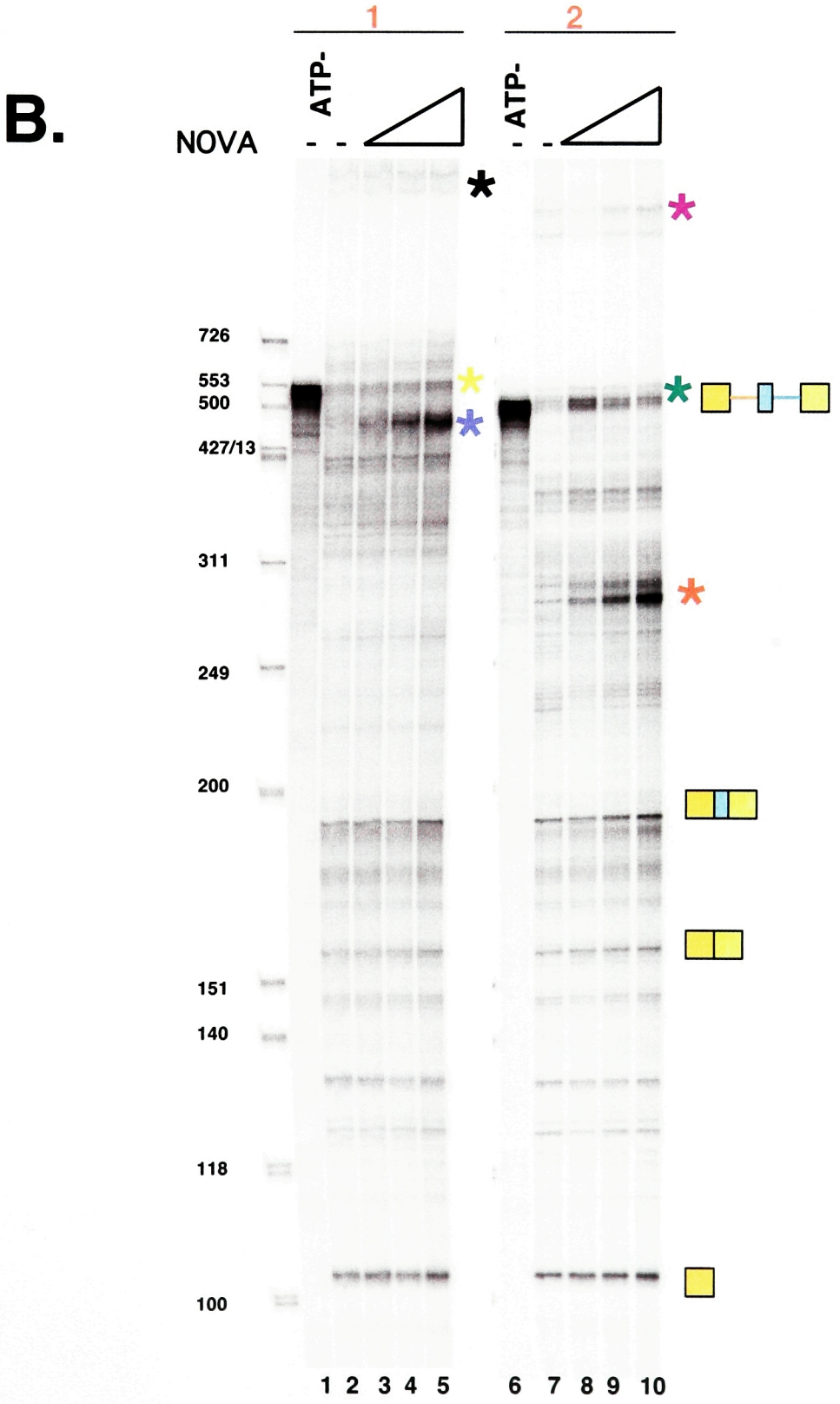
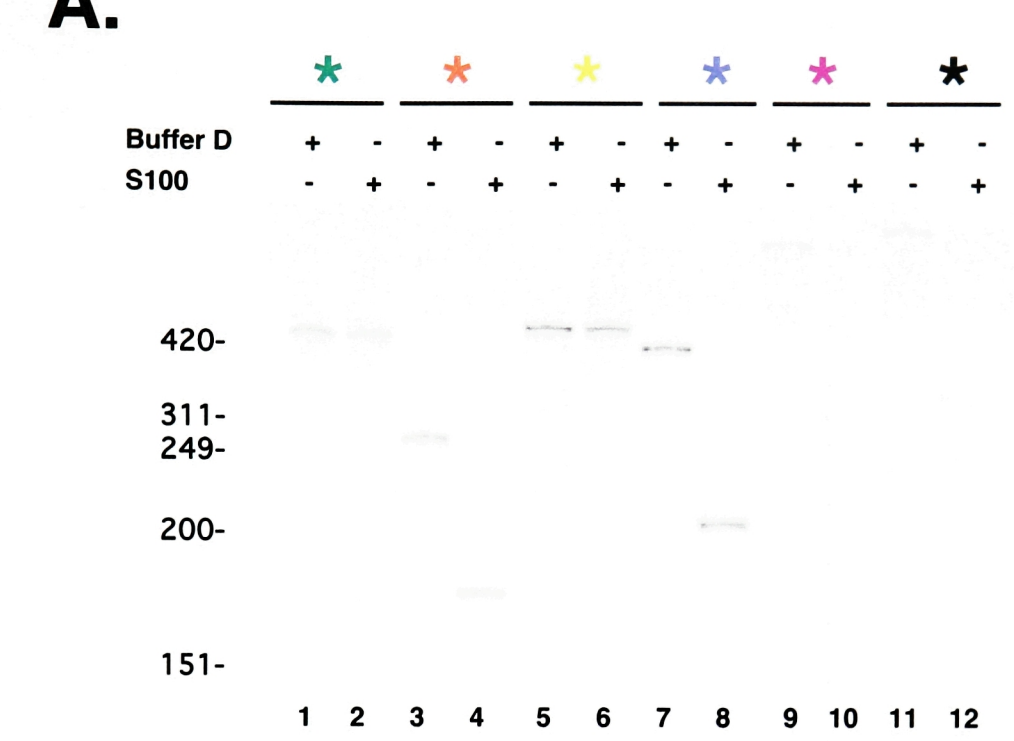

B.
NOVA
1
2
- ATP-
- ATP-
726
553
500
427/13
311
249
200
151
140
118
100
1 2 3 4 5
6 7 8 9 10

Figure 6: Characterization of Lariat Products:

(A) Debranching assay in HeLa cell S100 extract. The indicated bands (color code as in figure 5B) were incubated in S100 extract (lanes 2, 4, 6, 8, 10, 12) or buffer D (lanes 1, 3, 5, 7, 9, 11). lanes 1 and 2: unspliced RNA 2, lanes 3 and 4: lariat product 2, lanes 5 and 6: unspliced substrate 1, lanes 7 and 8: lariat product 1, lanes 9 and 10: long lariat (cassette exon-skipping) 2, lanes 11 and 12: long lariat 1.

(B) Mapping of the branch site by primer extension. A reverse transcription reaction was primed by a ^{32}P-labelled oligonucleotide complementary to the 3'splice site and polypyrimidine tract of the second intron. The presence of a branching site interrupts the reverse transcription. Dideoxynucleotides were included in the RT mix in lanes 1, 2, 3, 4 to produce a sequencing ladder. Lane 5, lariat product from 2 substrate, lane 6, unspliced 2 substrate, lane 7, unspliced 1 substrate, lane 8, lariat product from 1 substrate.


A.
Buffer D
S100
420-
311-
249-
200-
151-
1 2 3 4 5 6 7 8 9 10 11 12

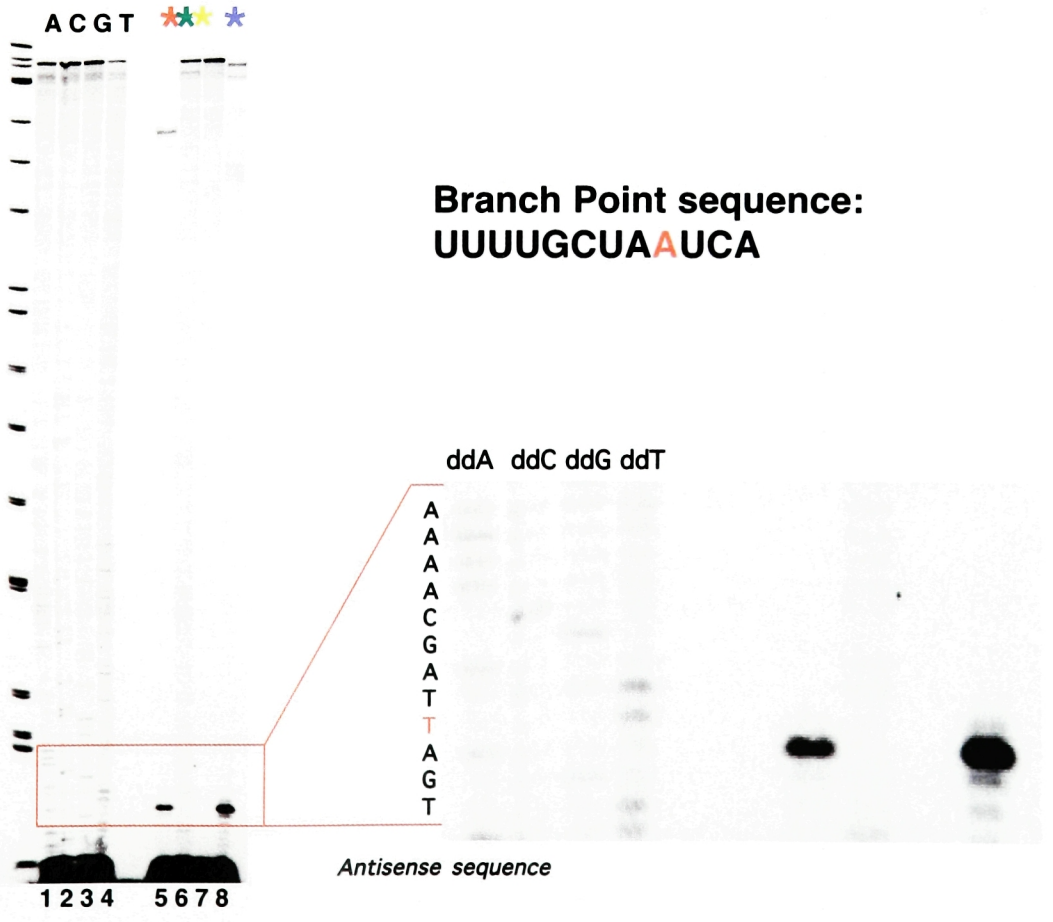

B.
A C G T
Branch Point sequence:
UUUUGCUAAUCA
ddA ddC ddG ddT
A A A A A C G A T T A G T
Antisense sequence
1 2 3 4 5 6 7 8

Figure 7: Nova-1 interacts with component of the splicing machinery.

(A) Nova-1 and Nova-2 interactions with splicing factors. Nova-1 and Nova-2 bait constructs were used to screen a group of splicing factors prey constructs (a kind gift of David Elliot, Medical Research Council, Edinburgh). Colonies were screened for growth in medium lacking Histidine and X-gal assay.

(B) Co-immunoprecipitation assay from 293T cells co-transfected with constructs encoding Nova-1, HuD, U2AF65, U1 70K, SRp40, SRp20 fused to a N-terminal GST, and constructs encoding two isoforms of Nova-1 (with and without the 24 amino acid insertions encoded by the alternative exon H) fused to a flag tag. After cell-lysis, the protein complexes were precipitated with Glutathione-coated beads, washed several times and analyzed by western blot with anti-flag monoclonal antibody, but for the top panel, which was probed with anti-GST antibody.

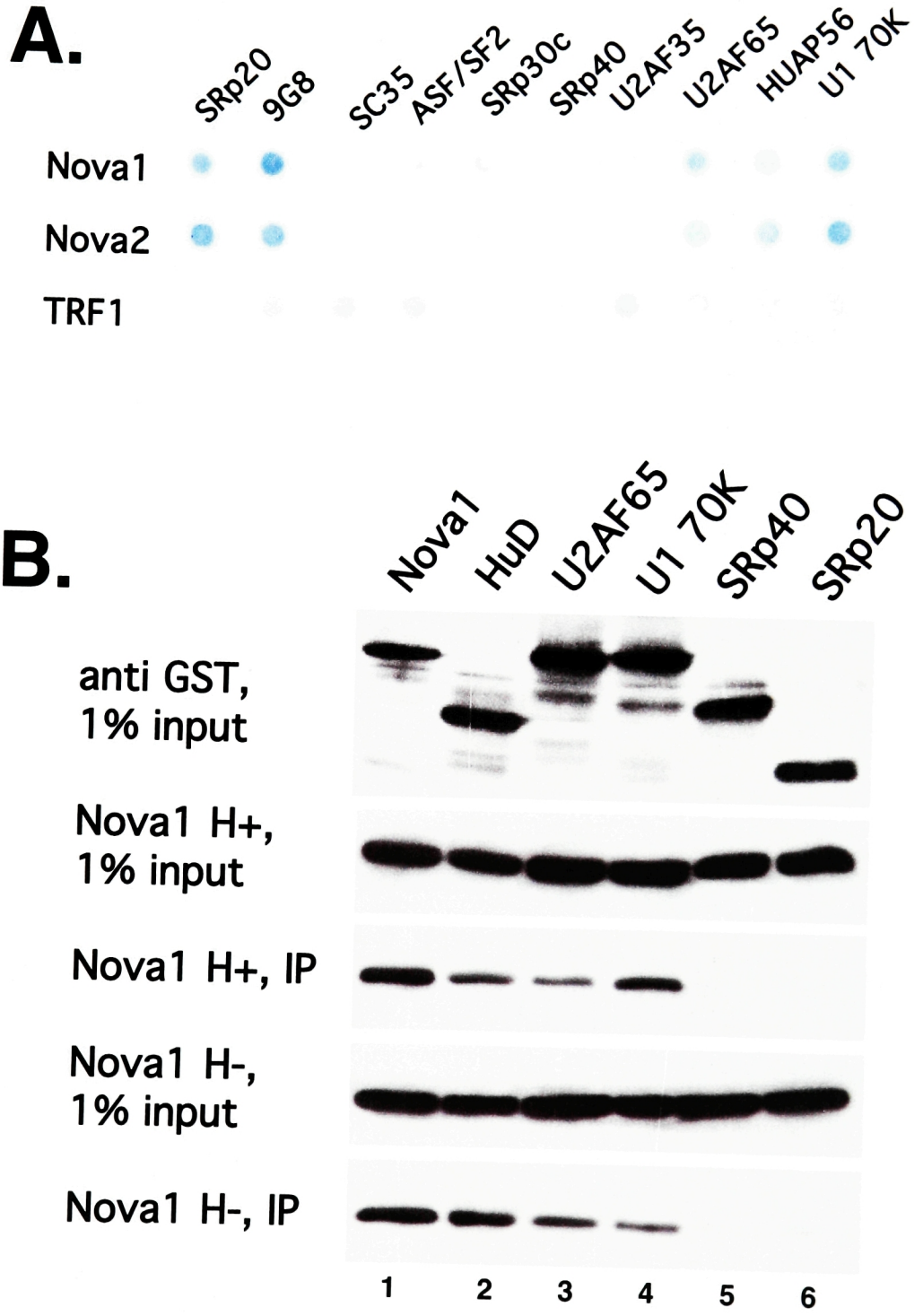
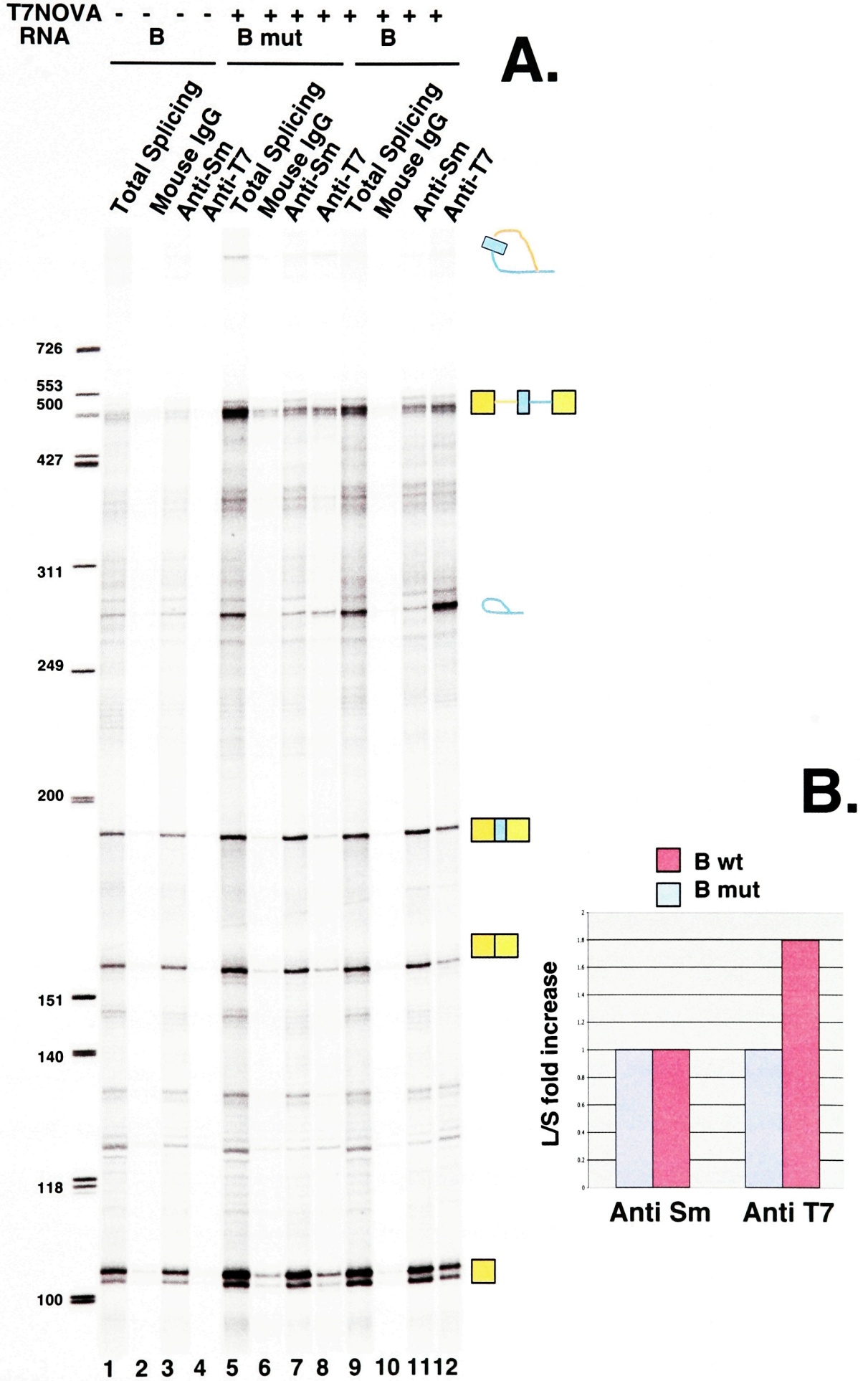

A.
SRp20
9G8
SC35
ASF/SF2
SRp30c
SRp40
U2AF35
U2AF65
HUAP56
U1 70K
Nova1
Nova2
TRF1
B.
Nova1
HuD
U2AF65
U1 70K
SRp40
SRp20
anti GST,
1% input
Nova1 H+,
1% input
Nova1 H+, IP
Nova1 H-,
1% input
Nova1 H-, IP
1
2
3
4
5
6

Figure 8: Immunoprecipitation from *in vitro* splicing reactions:

(A) Splicing reactions employing GABA/Globin construct 2 (lanes 1-4 and 9-12) and GABA/Globin construct 2 mutant (lanes 5-8) were incubated for 4 hrs at 30°C. At the end of the incubation, the indicated antibodies immobilized on protein A-beads were added, and the reactions were rocked for 1 hr. After three washes, the bound RNA was extracted and analyzed on a denaturing gel.

(B) Enrichment of long product of splicing in the Nova pellet. The ratio long product/short product were measured in the immunoprecipitated pellets with anti T7 and anti Sm, and normalized to the same ratio in the input.


A.
T7NOVA RNA
- - - - + + + + + + + +
B
B mut
B
Total Splicing
Mouse IgG
Anti-Sm
Anti-T7
726
553
500
427
311
249
200
151
140
118
100
1 2 3 4 5 6 7 8 9 10 11 12
B.
B wt
B mut
L/S fold increase
Anti Sm
Anti T7

Chapter 4

Introduction: Role of KH domain-containing RNA binding proteins in translation:

It has been known for some time that several hnRNP proteins and SR proteins shuttle between the nucleus and the cytoplasm (Caceres et al., 1998; Pinol-Roma and Dreyfuss, 1992). These findings, and the discovery of the exon junction complex, primed a series of studies aiming to demonstrate a connection between the nuclear history and the fate of messages in the cytoplasm (Dreyfuss et al., 2002; Keene and Tenenbaum, 2002; Matsumoto et al., 1998; Shyu and Wilkinson, 2000; Tenenbaum et al., 2000). Nova has been shown to be present both in the nucleus and in the cytoplasm in brain sections and in primary neurons in culture (Buckanovich et al., 1993; Buckanovich et al., 1996; Yang et al., 1998), and a Leucine-rich region resembling a NES domain is present in both Nova-1 and Nova-2.

Nova is closely related to three shuttling hnRNPs, hnRNP K, E1 and E2, which have been shown to play a role in the cytoplasmic fate of their RNA targets. HnRNP K represses the translation of 15-lipoxygenase (LOX), a key enzyme involved in mitochondrial membrane breakdown during erythroid cell differentiation. The expression of this enzyme, which is potentially extremely toxic in normal cells, must be tightly regulated during the course of erythroid cell maturation, while the cell is still relying on its mitochondria for energy, before it is released in the peripheral blood to become reticulocyte and erythrocyte. Such control is achieved through the binding of hnRNP K and E1, 2 to an UC-rich element (CCUCUU repeats) in the 3'UTR of the LOX message, called Differentiation-Control Element (DICE). The binding of hnRNP K to the 3'UTR

masks the message from the translational apparatus. In particular, hnRNP K bound to DICE has been shown to inhibit the assembly of the two subunits of the ribosome at the 5' end of the mRNA, in a process that presumably involves the circularization of the message (Ostareck et al., 2001; Ostareck et al., 1997). Interestingly, hnRNP E (also called αCP) is also implicated in stabilization and, ultimately, increased expression, of a different message, α-globin, through the binding to a C-rich motif present in the 3'UTR. The protein is found to be part of the RNP "α-complex", which is compatible with efficient polysome loading and active translation (Ji et al., 2003). Other KH-domain containing proteins have been implicated in translational control and have been found to be associated with polysomes. FMRP, the product of the gene responsible for most Fragile X chromosome Mental Retardation cases, is associated with polysomes in several cell lines (Khandjian et al., 1996), while some of the cellular FMRP seems to be part of pre-polysomal mRNPS (Feng et al., 1997). More recent studies have defined a restricted subset of FMRP-targeted mRNAs and further demonstrated that the absence of FMRP results in an increase and a decrease of two distinct subsets of mRNAs in the actively translating polysome fraction (Brown et al., 2001; Darnell et al., 2001). The results of Brown et al. suggest that FMRP controls some aspect of translation, although the precise mechanism of such regulation is entirely undefined at the moment. Other, less well characterized KH-domain containing proteins, like SCP 160 in *S. cerevisiae* and CRD-BP, a protein that binds to the c-*myc* coding region, have also been shown to be associated with polysomes (Doyle et al., 1998; Lang et al., 2001)

A clue on the potential cytoplasmic role of Nova came from the results of a "transcribed sequence selection" (TSS) experiment in which recombinant Nova was used

to select target sequences out of a pool of transcribed RNAs (Jensen and Darnell, unpublished). Among other target sequences isolated with this approach, there was a distinct population of transcribed RNAs containing a 50 bp stretch of RNA present in domain IV of 28S ribosomal RNA. Domain IV is one of the most exposed sequences found in 28S rRNA and in the intact 60S ribosomal subunit, as determined by chemical probing and X-ray diffraction crystallography (Ban et al., 2000; Holmberg et al., 1994a; Holmberg et al., 1994b), and it is therefore likely to be accessible to protein binding *in vivo*. Immunocytochemical studies performed in brain slices and in primary neurons have shown that Nova distributes in the cytoplasm in rather discrete granules that are heterogeneous in size. Partial co-localization of Nova and P0 ribosomal protein in the cytoplasm and dendrites was observed (Triller and Darnell, unpublished).

Nova distribution on linear density gradients:

To gain insight into the function of Nova in the cytoplasm, we fractionated post-mitochondrial cytoplasmic extracts on linear density gradients. We analyzed cytoplasmic extract from N2A and P19, two cell lines that express Nova, and from spinal cords and hindbrain of mice at different ages. The profile of sedimentation of Nova-containing structures shows some variability between cell lines and tissue, and between extracts from tissues at different ages. In N2a cells (Fig. 9) and in P19 cells differentiated into a neuron-like phenotype with retinoic acid (not shown), 50% of Nova was found in the first two fractions of the gradient, probably corresponding to protein not engaged in large complexes. 70% of the total content of Nova, as estimated by densitometry, sediments in fractions lighter then those containing the 80S ribosomal monomer (fractions 1 to 8), and

only very small amounts are seen in the fractions corresponding to polyribosomes. The treatment of the extracts with 30 mM EDTA, which results in the disruption of polysomes (right panel of figure 9), did not change the profile of sedimentation of Nova, nor did treatment with RNAse A (not shown). These data show that in the cytoplasm of the analyzed cell lines, most Nova is not engaged in the formation of large complexes. As a control, the same blots were re-probed with a monoclonal antibody against FMRP, a protein known to be largely associated with polysomes in various cell lines (Feng et al., 1997; Khandjian et al., 1996). As shown in figure 9, a considerable fraction of FMRP (36% as estimated by densitometry) associates with polyribosomes (fractions 13 through 18); the disruption of the polysomes by 30 mM EDTA treatment results in a sizable shift of FMRP to the upper half of the gradient (figure 9, right panel).

Most of the observations that suggested co-localization of Nova and P0 ribosomal protein were obtained in primary neurons, and increased co-localization and more discrete Nova-positive granules were observed after three to four weeks in culture (Triller and Darnell, unpublished). Therefore, we analyzed the distribution of Nova on linear density gradients in neural tissue from animals at three (P3), fourteen (P14) and thirty days (P30) of age. The tissue was obtained from spinal cord and hindbrain, where Nova-1 is the predominant Nova species. After homogenization, nuclei and non-lysed cells were removed by a low speed centrifugation step. The supernatant was further fractionated with a higher speed centrifugation step (20,000*Xg*, 10 min) to obtain a pellet enriched in vesicles of the rough endoplasmic reticulum, as shown by the staining with an antibody against Riboferin I (Fig. 10C) a transmembrane glycoprotein specifically associated with the rough endoplasmic reticulum (Kelleher and Gilmore, 1997; Silberstein et al., 1992).

Nova is more than three times more abundant in the supernatant than in the pellet obtained in this centrifugation step (Fig. 10C). Vesicles were solubilized with deoxycholic acid, and residual insoluble structures were discharged through a further centrifugation step. The supernatants were then fractionated on linear density gradients.

When the supernatant of the 20,000Xg centrifugation (S) was analyzed by linear density gradient, a large fraction of Nova (around 40% of the total, by densitometry) sediments in the first two fractions, corresponding to the lysate layered on the top of the gradient (Fig.10A). This fraction probably corresponds to monomeric Nova, or Nova not engaged in large complexes. 78% of the total amount of Nova sediments above the 80S ribosome (Fig.10A). The treatment with 30 mM EDTA does not result in a major change of the Nova profile of sedimentation (Fig. 10A, right panel), although it does cause a shift of the ribosomes from the bottom part of the gradient to the upper part, following the disruption of the polysomes (Fig. 10A, P0 lane, right panel). When we analyzed the Pellet (P) of the 20,000Xg centrifugation, a smaller fraction of Nova was found in the top fractions of the gradient, not surprisingly, since the majority of the proteins that are not part of large complexes are expected to be found in the S fraction. Around 50% of the Nova reactivity is found in the fractions above the 80S ribosome monomer, and 20% sediments in the lower part of the gradient, in the fractions where polysomes are found (Fig.10B). Again, the treatment with 30 mM EDTA does not dramatically modify the overall distribution of Nova on the gradient.

The analysis of extracts from fourteen day old animals shows a similar profile, with around 60% of Nova found in fractions above the 80S, and 20% of Nova in the polysomal fractions, in both supernatant and pellet fractions (data not shown).

In one month old animals, a fraction of Nova variable between 17 and 30% of the total (in the supernatant and pellet fractions, respectively), is found to sediment in the lower part of the gradient (fractions 13 to 18, polysomal fractions). EDTA treatment shifts 77% of the pool of Nova associated with polysomes (from 17.4% of the total reactivity for Nova by densitometry to 3.9% in the "free" polysomes fraction) to the upper part of the gradient, coincidentally with the expected shift of the ribosomes detected with the anti-P0 serum (Fig. 11A and C).

As a comparison, we stained the same blots with a human polyclonal antibody against Hu (Okano and Darnell, 1997). The pattern of sedimentation of Hu proteins in gradients of Accudenz and Glycerol has been characterized in various tumor cell lines of neural origin (Antic and Keene, 1998). While some of the cytoplasmic Hu is found in a free state, a variable amount is engaged in the formation of large heterogeneous ribonucleoprotein complexes (α complexes), and around twenty per cent of the total is associated with polysomes (β complexes)(Antic and Keene, 1998). In our tissue extracts, the distribution of Hu reactivity matches quite closely the distribution of Nova. At P3 most of Hu is part of large, heterogeneous complexes, that coincide with the α complexes described in previous studies (Antic and Keene, 1998), and the treatment with EDTA only marginally modifies the pattern of sedimentation. At P30 more than twenty percent of the Hu sediments in the denser part of the gradient and the treatment with EDTA determines a shift to less dense fraction, coincidentally with the disruption of polysomes (Fig. 11A to B).

Changes in the level of expression of genes in the absence of Nova:

In an attempt to expand our understanding of the variations in gene expression underlying the Nova null phenotype, mRNAs from mutant animals and their wild-type or heterozygote littermates were purified and utilized to probe microarrays. The position of a given mRNA in a linear density gradient can be attributed to the number of associated ribosomes. The number of ribosomes per mRNA reflects the translational efficiency of the mRNA, unless the ribosomes are stalled or translation is inhibited in the elongation phase. An mRNA that is poorly or not translated will typically accumulate in the mRNP or "monosome" (80S) fraction of the gradient; translationally active mRNAs will sediment in the polysome fraction (reviewed by Carter, M.S. et al., 2000). To specifically identify differences at the translational level, we compared absolute levels of mRNAs and abundance of mRNA specifically in the polysome fraction. For each experiment, post-mitochondrial supernatant obtained from four pooled spinal cords and hindbrains was fractionated on a linear 20 to 50% sucrose gradient and the mRNA associated with each fraction was purified (Fig. 12A). One fifth of the total volume of extract was not fractionated; mRNA was directly purified as the "total" fraction. The purified mRNA from the input and polysome fractions was utilized to generate cDNA first, using an oligo(dT) containing the T7 promoter sequence as primer, which allowed a further transcription step in presence of biotinylated nucleotides to generate the cRNA used to probe the microchips (Fig. 12B). We probed the three Affymetrix Murine Genome Array U74 chips covering approximately 36,000 full-length mouse cDNAs and EST clusters from the UniGene database (www.affymetrix.com). The experiment was repeated three times, resulting in three intensity values for each gene represented on the

chips for the total (mutant and wild type) and three intensity values for the polysomes fraction (mutant and wild type).

We first verified the efficacy of our fractionation procedure by comparing the abundance of mRNAs in the total fraction (independently of the genotype) with the polysome fraction. We performed a *t*-test analysis between the pool of six values of the total fraction (three wild type and three mutant) and the corresponding six values of the polysome fraction for each gene represented in the chips, using GeneSpring 5.0 software (Silicon Genetics) (Fig. 13). A number of genes appear to be more abundant in the total than in the polysome fraction. Ten cDNAs, among which glyceraldehyde-3-phosphate dehydrogenase and beta actin, are enriched in the polysomes, consistently with a high rate of translation. A number of cDNAs encoding ribosomal proteins are two to four fold more abundant in the total fraction than in the polysomes, consistently with previous findings (Table I)(reviewed in Meyuhas and Hornstein, 2000). mRNAs encoding ferritin, whose expression is also regulated at the level of translation, were similarly distributed.

To compare differences specifically in the polysome fractions between wild-type and Nova-null mice, the set of values corresponding to the polysome-associated mRNAs was normalized to the corresponding set of values obtained with the total probe. A *t*-test analysis detected statistical differences between the two sets of polysome-associated mRNAs normalized to their corresponding input values. The intensity of sixty-seven genes that showed equivalent level in the total fraction was statistically different between wild-type and mutant polysomes ($p<0.05$) (Fig. 14). These genes were grouped into two groups based on whether they were decreased or increased in the polysomes of Nova-1 null mice. Thirty-nine messengers were found to be less represented in the pool of the

polysome-associated mRNAs from Nova-null mice (table II), while twenty eight were increased (table III).

Discussion:

Several RNA binding proteins are found both in the nucleus and in the cytoplasm and play different roles in RNA metabolism in the two compartments (reviewed in (Dreyfuss et al., 2002). In an attempt to understand the function of the cytoplasmic pool of Nova, we began the characterization of the cytoplasmic complexes that contain the protein. Our results show that an amount of Nova variable between cell lines and tissues from animals of different ages is involved in large heterogeneous complexes, which are stable in presence of EDTA. Only in extract from animals older than three weeks, around one fifth of the total amount of Nova detected by western blot sediments in the same fractions as polyribosomes formed by four or more ribosomes, and it is shifted to less dense fractions in response to EDTA treatment. The size of these later complexes and their dependence on divalent cations suggest that they represent Nova associated with polyribosomes.

The large heterogeneous complexes containing Nova, not dependent on divalent cations, as shown by their resistance to EDTA treatment, are reminiscent of the α-complexes containing FMRP, Hu or αCP (Antic and Keene, 1998; Feng et al., 1997; Ji et al., 2003). These complexes are described as mRNPs, but their exact composition and the relationship between proteins and RNA within them are largely unknown. It is postulated that mRNAs are tightly packed with protein components of the mRNPs, since the complexes are completely or partially resistant to RNAse treatment (Ch'ng et al., 1990;

Feng et al., 1997; Herrera et al., 1988), and very prolonged treatments with RNAse are required for their disruption (Antic and Keene, 1998). Treatments with RNAse A sufficient to disrupt the polyribosomes did not result in shift of the Nova complex to less dense fractions (data not shown), suggesting that either the Nova-positive complexes we observed do not contain RNA, or that the RNA contained in them is not exposed to the RNAse action. Since we have evidence that Nova is associated with poly(A)+ RNA in the cytoplasm (Triller and Darnell, Dunne and Darnell, unpublished) we are inclined to consider the second explanation as more probable, although the possibility that Nova can be engaged in large complexes not containing RNA can not be disregarded.

Most of the studies aimed to characterize the cellular structures RNA binding proteins are engaged in, such as mRNPs and polyribosomes, have been conducted in immortalized cell lines, rather than in extracts from differentiated tissues. Given the complex relationships between mechanism of gene expression, cell cycle and differentiation, it is not surprising that RNA binding proteins are part of complexes of different size and functional state across different cell lines and tissues. The differences in the Nova sedimentation pattern we observed between neuroblastoma cells and spinal chord tissue, and between spinal chord tissues of different ages, may underlie changing functional roles of Nova in various physiological states. The heterogeneous structures in which most of Nova is engaged could represent sites of storage or transport of mRNAs whose translation requires a tight regulation in time or in space. The later association of some Nova with polyribosomes could be due to the activation of the translation of some of the mRNAs Nova is bound to, possibly as a consequence of some maturation event in the nervous system.

Hu is a family of four RNA binding proteins found in the nucleus and cytoplasm. HuA(also called R) is ubiquitously expressed, while HuB, HuC, and HuD are exclusively expressed in neurons. A role in regulation of alternative splicing have been demonstrated for their *Drosophila* homolog ELAV1 (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001). Hu family members bind poly(A)+ RNA in the cytoplasm (Gallouzi et al., 2000; Gao and Keene, 1996). Described cytoplasmic Hu functions include stabilization of messages containing AU-rich elements (ARE) in their 3'UTR, and stimulation of translation of specific messages (Antic et al., 1999; Brennan and Steitz, 2001; Fan and Steitz, 1998). Hu has consistently been found to be engaged in heterogeneous mRNPs in several cell lines, and to be associated with polysomes in a proportion variable on the cell type (Antic and Keene, 1998; Gallouzi et al., 2000; Gao and Keene, 1996). We report the first analysis of the Hu sedimentation in CNS extracts. Interestingly, the pattern of sedimentation of Hu in spinal chord and hindbrain extracts matches closely the one of Nova: Hu sediments with large heterogeneous structures, presumably mRNPs, throughout the development time points we analyzed, and some Hu is found in the polysome fractions, most visibly in extracts from one month or older animals. Hu has been found to form with Nova an RNAse-resistant complex (Okano and Darnell, unpublished, and Fig. 7B). It is therefore possible that in spinal chord cytoplasmic extracts, Hu and Nova are present in the same mRNP and regulate the expression of an overlapping set of messages.

We utilized the fractionation of cytoplasmic extracts from neural tissue on linear density gradients to begin to identify genes whose translational state is changed in the absence of Nova-1. While a similar approach has been utilized to analyze extracts from S.

cerevisiae and a number of replicating cell lines (Arava et al., 2003; Brown et al., 2001; Galban et al., 2003; Grolleau et al., 2002; Johannes et al., 1999; Zong et al., 1999), this is the first such analysis on extracts from fully differentiated neural tissue. This strategy is of obvious relevance for the study of aspects of translation that are thought to be peculiar of mature neurons, such as dendritic translation. The reliability of our data set was validated by the finding that several mRNAs encoding ribosomal proteins were found decreased in the polysome fraction compared to the total. The synthesis of many mammalian ribosomal proteins has been shown to be selectively regulated in a growth-dependent manner at the translational level. The corresponding mRNAs are characterized by the presence of a 5'-terminal oligopyrimidine tract (5'TOP) and therefore are referred to as TOP mRNAs. The proportion of TOP mRNAs actively engaged in protein synthesis, and therefore associated with polysomes, is significantly lower than that characteristic of other ubiquitous mRNAs in cells that cease to divide, as most of the cells in the spinal cord, than in actively replicating cells (reviewed by Meyuhas and Hornstein, 2000).

We identified sixty-seven genes whose distribution in the polysomes is increased or decreased in response to the ablation of Nova. Although further experiments are required to verify that the observed shift of mRNAs in the linear gradient reflects changes of protein synthesis, the identity of some of the messengers identified with this approach provides some clues on cellular functions that are altered as a direct or indirect consequence of the deletion of Nova. The described functions of a group of six genes, four of which are decreased in absence of Nova, and two increased, can be loosely referred to the mechanisms connecting receptors and cytoskeleton in the post-synaptic

densities through clustering molecules and the signalling cascades mediated by ras monomeric GTPases. CNK (connector enhancer of ksr) has been originally described in *Drosophila* as a modifier of *Kinase suppressor of ras (ksr)*, a required component within the RAS/MAPK cascade (Therrien et al., 1998)). CNK is a large protein containing a variety of potential protein-protein interaction modules: a sterile alpha motif (SAM) domain, a PDZ domain, a pleckstrin homology domain (PH), a so-called CRIC domain (Conserved Region in CNK) two proline-rich stretches that fit the consensus for SH3 domain binding site, and several potential tyrosine phosphorylation sites (Therrien et al., 1998). It has been proposed to act as a multiadaptor molecule by binding and probably inhibiting RAF (MAPKKK) while enhancing Ras signaling in *Drosophila* (Therrien et al., 1999). Studies in rat have shown that Maguin, the rat homolog of CNK, interacts with Postsynaptic density PSD-95 (SAP 90), a major component of the post-synaptic densities with a role in clustering receptors and channels and in signal transduction (Yao et al., 1999). Interestingly, the level of mRNA encoding DAP-1 (GKAP), a highly brain-enriched protein also known to form a complex with PSD-95 (Kim et al., 1997; Satoh et al., 1997), is decreased in the polysome fraction of the Nova-null animals. mRNAs encoding Rho GTPase activating protein 6 and ras-j were also decreased in the polysomes in the absence of Nova. Members of the Ras family of monomeric GTPases play a major role in the signal transduction from receptors associated with post-synaptic density. SynGAP is associated with PSD-95 in many glutamatergic synapses, citron (a rho/rac effector protein), and synaptic gp130 Ras GAP have been the detected as part of post-synaptic densities (Kennedy, 1998; Kennedy, 2000). Furthermore, regulation of remodeling of the actin cytoskeleton by Rho GTPases is essential for the formation of

post-synaptic specializations, like dendritic spines, and yet another protein encoded by one of the mRNAs whose distribution is modified in the absence of Nova, filamin, plays a crucial role in the modulation of the functional proprieties of filamentous actin in dendritic spines (van der Flier and Sonnenberg, 2001). The simultaneous alteration of the distribution on polysomes of messengers encoding functionally related proteins in response to the absence of Nova suggests that disruption of organization of post-synaptic densities or dendritic spines, through alteration of the clustering complexes or the ras-mediated pathways may be implicated in aspects of the Nova-1-null phenotype.

Simultaneous changes of abundance in the polysomes are also observed for a group of three mRNAs encoding metalloproteases, and three mRNAs encoding transcription factors. No obvious functional relationship is evident among the members of these two groups.

Figure 9: Analysis of cytoplasmic extracts from N2a cells on linear density gradients:

A cytoplasmic extract from N2a cells was analyzed by sedimentation on a linear density gradient.

For (A) and (B), the top panel shows the absorption profile of a 20%–50% (w/w) sucrose gradient at 254 nm, with major ribosomal peaks indicated; the bottom panel shows the distribution of Nova, FMRP and ribosomal protein P0 in correlation to the sedimentation profile. Fraction numbers are indicated underneath the corresponding lanes.

(A) The cytopasmic extract was prepared and sedimented in the presence of 5 mM $MgCl_2$.

(B) Polysomes and ribosome monomers are disrupted by EDTA.

Polysome analysis from N2a cells

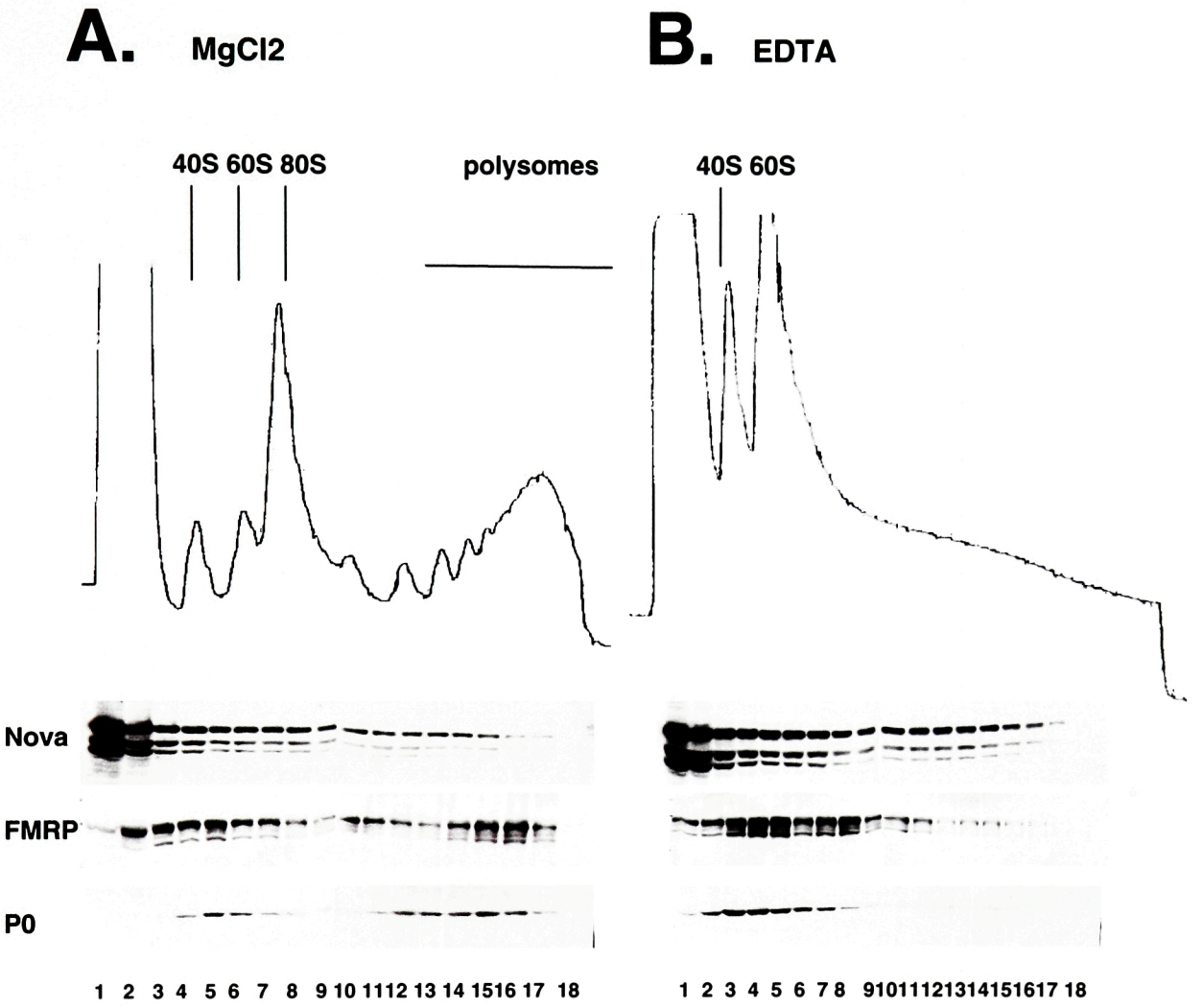


Figure 10: Analysis of polysomes from P3 spinal cords and hindbrains.

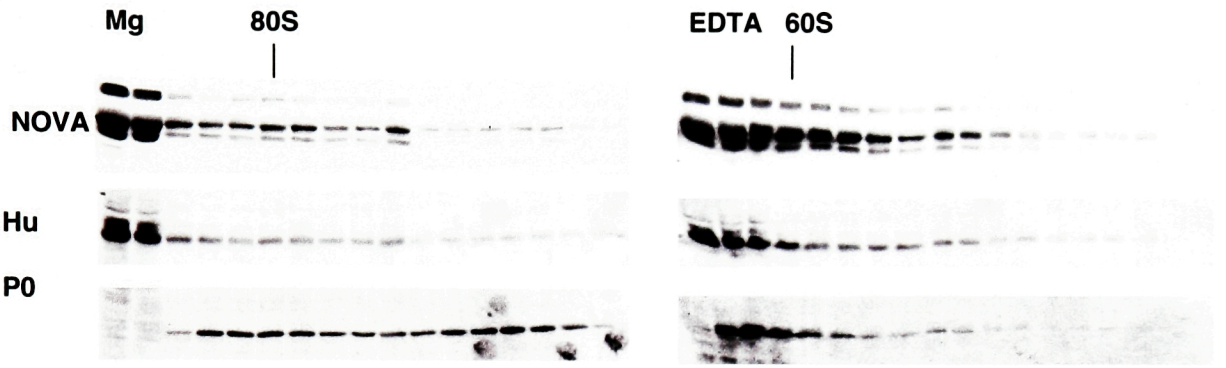
Spinal cords and hindbrains from P3 animals were homogenized and a cytoplasmic extract was extracted. After eliminating nuclei with a low speed centrifugation, the extract was fractionated by centrifugation at 20,000 *X g* yielding a supernatant ("free") fraction and a pellet enriched in rough endoplasmic reticulum. The pellet was solubilized ("bound") with deoxycholic acid, and both fractions were analyzed by centrifugation on a linear density gradient.

(A) Western blot analysis of the collected fractions of the sucrose gradient where the "free" fraction was loaded. Antibodies against Nova, Hu, P0 were employed.

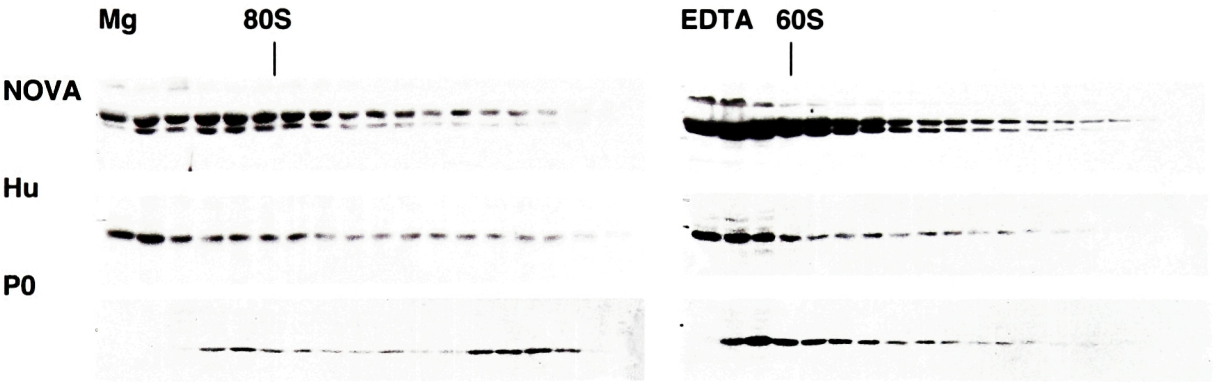
(B) Western blot analysis of the collected fractions of the sucrose gradient where the "bound" fraction was loaded. Antibodies against Nova, Hu, P0 were employed.

(C) An aliquot of the "free" and "bound" fraction was analyzed by western blot with antibody against ribophorin, a marker of the endoplasmic reticulum, and antibody against Nova.

A. free polysomes from P3 spinal cord and hindbrain



B. bound polysomes from P3 spinal cords and hindbrain



C.

Figure 11: Analysis of polysomes from P30 spinal cords and hindbrains.

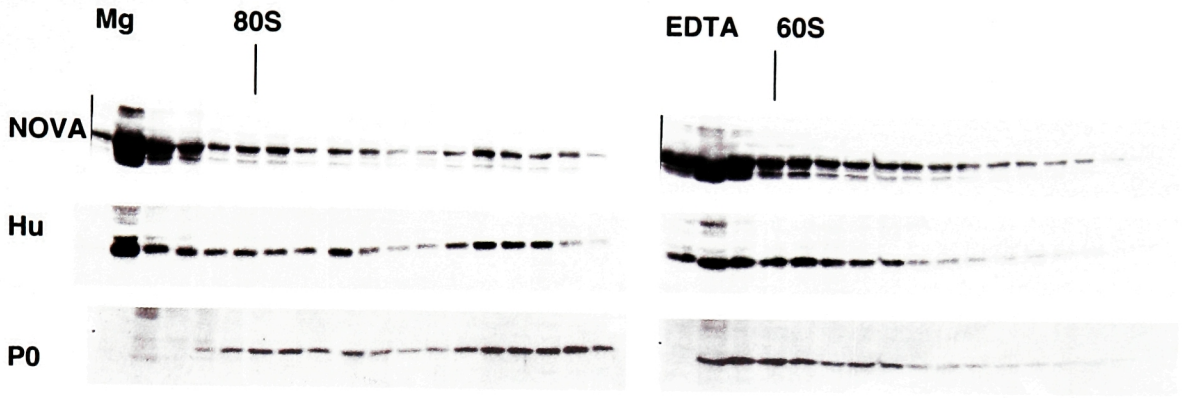
Extracts from spinal cords and hindbrains of P30 animals were prepared as in figure 11 and analyzed on linear density gradient as described.

(A) Analysis of the "free" polysomes.

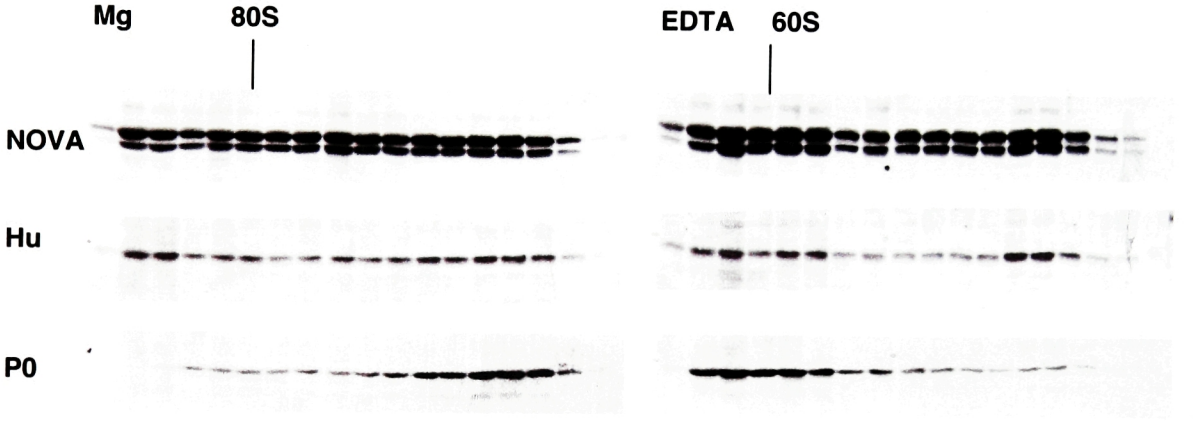
(B) Anlysis of the "bound" polysomes.

(C) Quantification by densitometry of the bands reactive to Nova in western blot of linear density gradient fractions shown in (A). The blue bars indicate the values of the Mg fractions, the red bars indicate the EDTA fractions.

A. free polysomes from P30 spinal cord and hindbrain



B. Bound polysomes from P30 spinal cord and hindbrain



C.

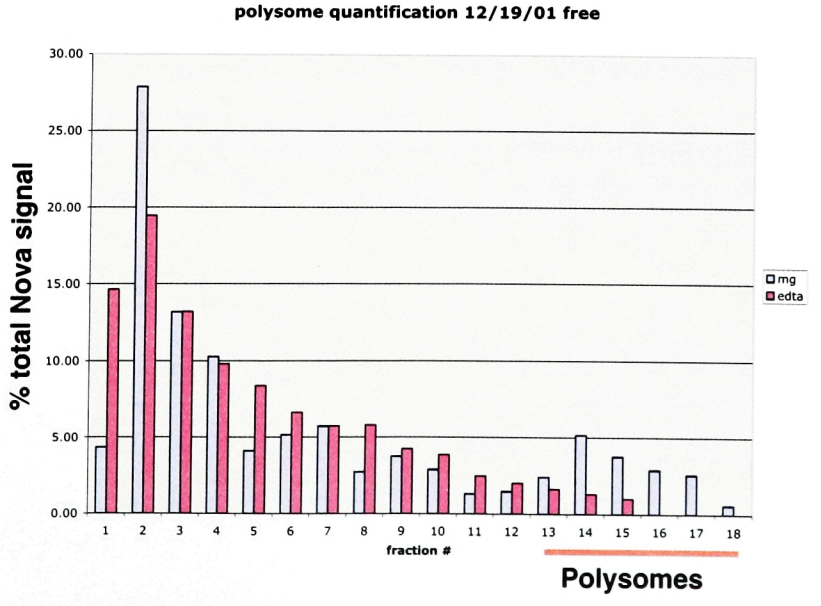


Figure 12: Scheme of the analysis of the polysome-associated mRNAs in wild type and Nova-null mice:

(A) Sucrose gradient fractionation of total cytoplasmic extracts from P7 spinal cord and brainstem. The top panel shows the absorption profile of the sucrose gradient. The polysome fractions are indicated. The bottom four panels show the distribution of Nova and P0 ribosomal protein by Western analysis of the fractions from two sucrose gradients on which extracts from wild type and Nova-1 null tissues were loaded. The residual bands reacting with the anti-Nova antibody in the KO row correspond to Nova-2. Fraction numbers are indicated below the corresponding lanes, and fractions 9–11, used for microarray analysis, are underlined.

(B) Synthesis of the biotinilated probes for Affymatrix microchips hybridization. The RNA collected from the indicated fractions or from the total was reverse transcribed using primers containing a stretch of oligo(dT) at their 3' end and a T7 promoter sequence at their 5'end. The cDNA resulting from this reaction is then transcribed using T7 promoter in presence of Biotin, producing the biotinilated cRNA probe that is used for the hybridization of the microarrays.

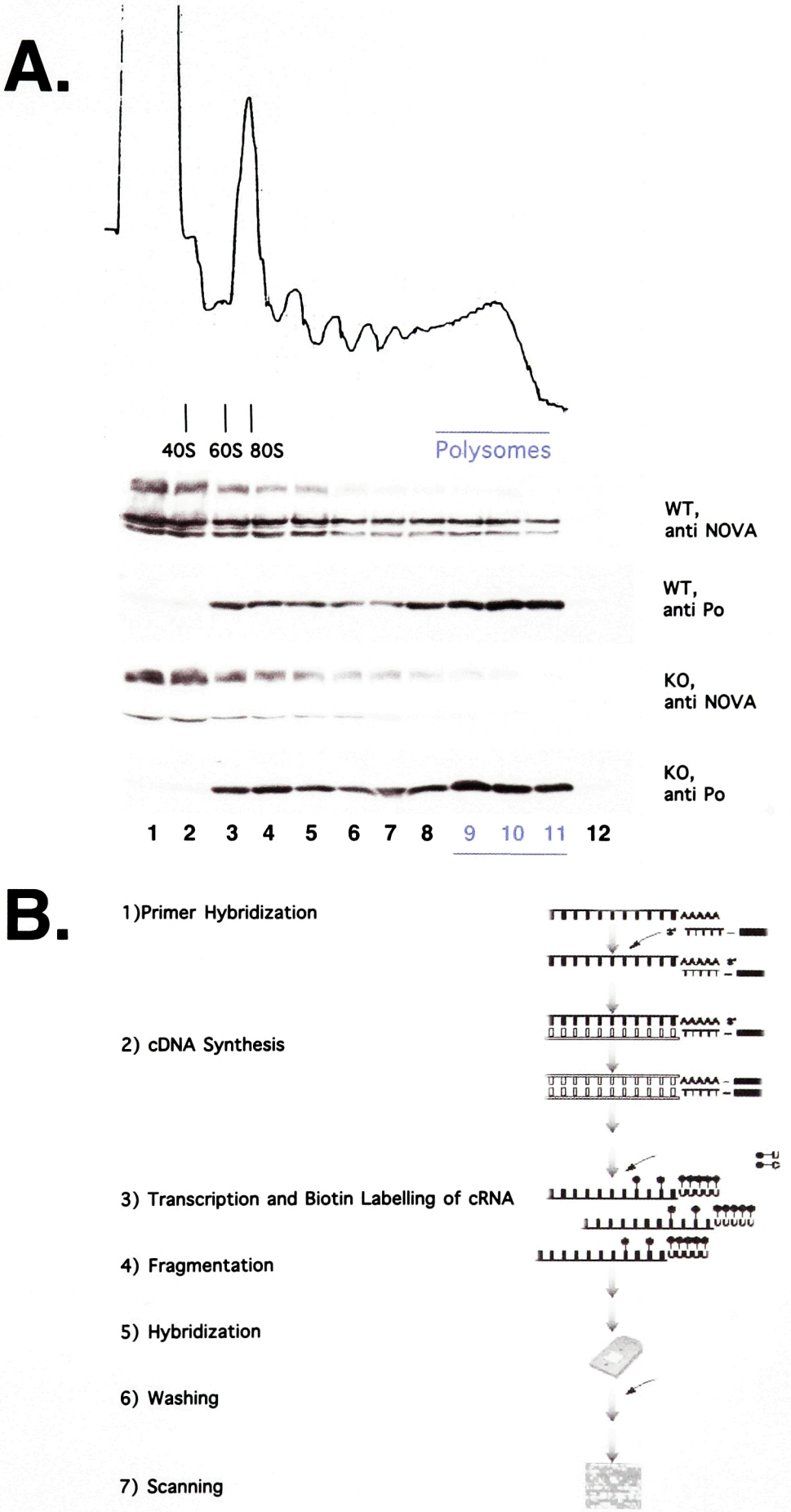
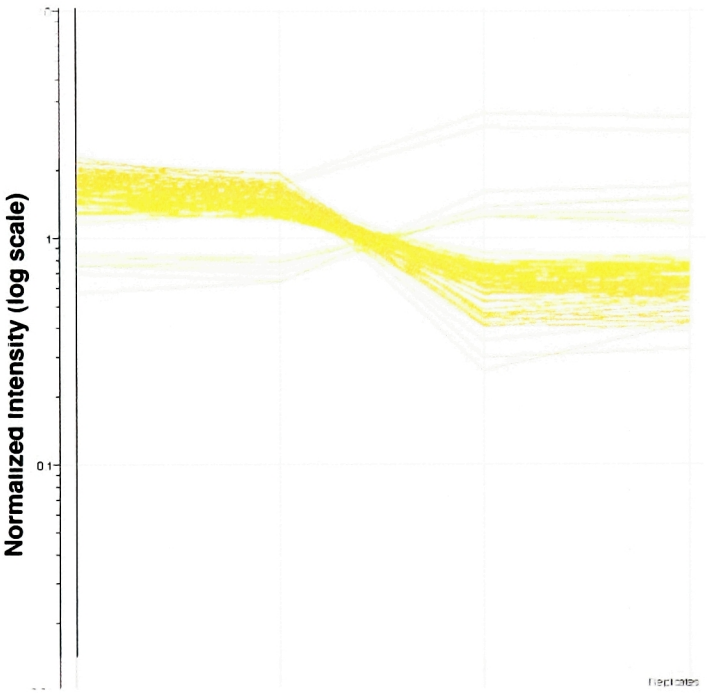

A.
40S
60S
80S
Polysomes
WT, anti NOVA
WT, anti Po
KO, anti NOVA
KO, anti Po
1 2 3 4 5 6 7 8 9 10 11 12
B.
1)Primer Hybridization
2) cDNA Synthesis
3) Transcription and Biotin Labelling of cRNA
4) Fragmentation
5) Hybridization
6) Washing
7) Scanning

Figure 13: Comparison between the total and polysome fraction (validation of the fractionation procedure):

Four sets of intensity values were obtained for each gene from the hybridization of microarrays. Each set is defined by Genotype and Fraction (WT tot, KO total, WT polysomes, KO polysomes), and contains three intensity values (replicates) per gene. In the comparison between total and polysome fractions, shown in this figure, the sets were pooled by Fraction. Therefore, two sets (Total and Polysome) with six values of intensity each to each gene correspond. A *t*-test was run between the Total and the Polysome sets for each gene. The graph shows the intensities of those genes for which the two sets of values (total and polysome) were found to be different ($p<0.001$). The y-axis represent the intensity values expressed in logarithmic scale, normalized by the mean intensity of each chip. The x-axis lists the four experimental conditions. For each gene, a colored line joins the four intensity values (each of which is the mean of three replicates) across the four experimental conditions.



Replicates: WT Total KO Total WT Polysomes KO Polysomes

Table I:

List of genes that show different relative intensity in the Polysome fraction and the Total ($p<0.001$). Yellow rows indicate genes encoding ribosomal proteins. Light blue rows indicate genes encoding ferritin.

LESS ABUNDANT IN THE POLYSOME FRACTION THAN IN THE TOTAL	T/P
protein tyrosine phosphatase 4a3	6.12
Mus musculus, clone IMAGE:1379146, mRNA, partial cds	5.35
ribosomal protein S12	4.58
growth arrest specific 5, encodes U75 and U76 snoRNAs	4.46
t-complex testis expressed 1, dynein light chain (microtubule associated transport)	4.45
ribosomal protein L41	4.39
RIKEN cDNA 3200001M24 gene	4.31
epimorphin	4.27
unknown;	4.13
synapsin I actin binding synaptic vesicle	4.07
translocase of inner mitochondrial membrane 9 homolog (yeast)	4.04
RIKEN cDNA 2400008I04 gene	3.96
hexokinase 1, deficiency causes hemolytic anemia	3.94
ribosomal protein S26	3.78
RIKEN cDNA 2900010M23 gene	3.62
thymosin, beta 10	3.62
ESTs, Weakly similar to RIKEN cDNA 2010000G05	3.54
Mus musculus, Similar to chromosome 9 open reading frame 16, clone MGC:19388	3.34
ribosomal protein L27a	3.29
RIKEN cDNA 3110005M08 gene	3.27
ribosomal protein L28	3.15
ESTs	3.13
DiGeorge syndrome critical region gene 6, development, schizophrenia	3.12
cyclin I	3.07
ribosomal protein S28	3.04
Mouse gene for ferritin H subunit.	3.04
neuronatin, exp in neonates>adults, expressed in VP cells of rat supraoptic nucleus	3.04
SEC61, gamma subunit (S. cerevisiae)	3.02
vesicle transport through interaction with t-SNAREs 1b homolog	2.99
RIKEN cDNA 4922501H04 gene	2.97
thymosin beta 4	2.87
ribosomal protein, large, P1	2.86
ribosomal protein L31	2.83
Mus musculus gene for ribosomal protein L27A, complete cds.	2.82
RIKEN cDNA 2310022M17 gene	2.82
ribosomal protein S8	2.81
small nuclear ribonucleoprotein polypeptide G	2.75
RIKEN cDNA 1010001M12 gene	2.71
Mus musculus, guanine nucleotide binding protein (G protein), gamma 5, clone MGC:	2.69
ferritin heavy chain	2.69
Mus musculus, ribosomal protein L23a, clone IMAGE:4988735, mRNA, partial cds	2.66
Purkinje cell protein 4	2.62
ribosomal protein S11	2.61
S100 calcium binding protein A1	2.61
GTP cyclohydrolase 1	2.57
RIKEN cDNA 2410089B13 gene	2.55
Mus musculus ferritin L-subunit gene exons 1-4, complete cds.	2.50
ESTs	2.50
RIKEN cDNA 2310040A07 gene	2.49
RIKEN cDNA 1110060M21 gene	2.48
gamma-aminobutyric acid receptor associated protein	2.47
RIKEN cDNA 2810004N20 gene	2.46
monocyte to macrophage differentiation-associated	2.45
deformed epidermal autoregulatory factor 1 (Drosophila)	2.42
DNA segment, Chr 2, ERATO Doi 217, expressed	2.40
Cluster Incl AF100956:Mus musculus major histocompatibility locus class II region	2.39
RIKEN cDNA C330016H24 gene	2.39
ribosomal protein S17	2.37
RIKEN cDNA 4933439F18 gene	2.36
RIKEN cDNA 1810037I17 gene	2.35
ESTs	2.30
Mus musculus, Similar to hypothetical protein FLJ21079,	2.29
67kD, ribosomal protein	2.28
Mouse ribosomal protein L30 gene, complete cds.	2.28
RIKEN cDNA 2900074C18 gene	2.27
RIKEN cDNA 3110038K10 gene	2.26
cadherin 11, alternatively spliced	2.25
Mus musculus, clone IMAGE:4504748, mRNA	2.24
RIKEN cDNA 0610010E21 gene	2.23
acidic ribosomal phosphoprotein PO	2.19
RIKEN cDNA 2410088K19 gene	2.18
RIKEN cDNA 2210013O21 gene	2.18
ribosomal protein L13	2.18
mitochondrial ribosomal protein L52	2.16
ESTs	2.16
ribosomal protein S2	2.12
expressed sequence AI463719	2.10
RIKEN cDNA C530005M16 gene	2.10
protein kinase inhibitor, gamma	2.09
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2	2.07
ESTs, Highly similar to NUMM_MOUSE NADH-ubiquinone oxidoreductase 13 kDa-A	2.02
RIKEN cDNA 2010003O14 gene	2.00
MORE ABUNDANT IN THE POLYSOME FRACTION THAN IN THE TOTAL	
protein kinase C, lambda	0.70
RIKEN cDNA 1110007C02 gene	0.69
NADH dehydrogenase flavoprotein 1	0.66
ESTs	0.61
actin, beta, cytoplasmic	0.60
RIKEN cDNA 2210013K02 gene	0.59
glyceraldehyde-3-phosphate dehydrogenase	0.54
expressed sequence AW048948	0.54
RIKEN cDNA 2410015A15 gene	0.51
expressed sequence AI481191	0.38

Fig. 14: Comparison between the polysome fractions of WT and Nova-1-null animals: For each gene, two sets of intensity values were compared: WT polysome and KO polysome. Each set contained three values, corresponding to the number of replicates. The intensity values in the polysome data sets were normalized to the corresponding values in the total data sets. The graph, with the same conventions as in figure 14, represents exclusively the genes that shown significant difference (*t*-test, $p<0.05$).

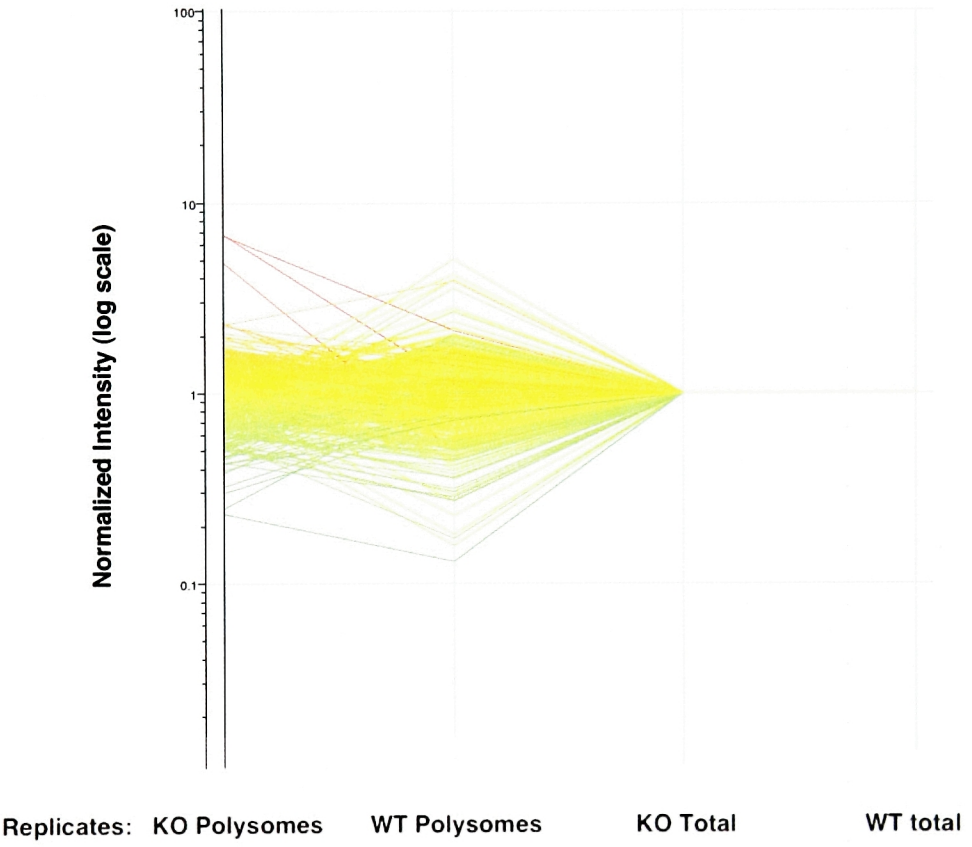

Normalized Intensity (log scale)
100
10
1
0.1
Replicates:
KO Polysomes
WT Polysomes
KO Total
WT total

Table II:

List of Genes whose intensity is at least two times higher in the Wild Type polysome fraction normalized to Total than in the Nova-null polysome fraction ($p<0.05$).

Entries corresponding to genes with a described function related to post-synaptic densities, ras/rho signaling pathways and dynamic organization of the cytoskeleton in neurites are colored in light blue. Transcription factors are labeled in purple. Metalloproteases are labeled in yellow.

Table III:

List of Genes whose intensity is at least two times higher in the Nova-1-null mutants polysome fraction normalized to Total than in the Wild Type polysome fraction ($p<0.05$). Color convention are the same as in Table II.

TABLE II: Decreased in the Polysome Fraction of KO

Name	(WT P/WT T)/(KO P/KO T)	p-value
ESTs(unknown)	6.13	0.00159
EST(unknown) transcription factor?	5.29	0.00332
Mouse CNK (connector enhancer of ksr)	4.79	0.04817
RICKEN cDNA	4.3	0.02798
Damage-induced neuronal endopeptidase (DINE)	4.16	0.03258
RICKEN cDNA	4.01	0.04257
cell division cycle 45 homolog -like	3.92	0.00594
Rho GTPase activating protein 6	3.53	0.01020
ESTs	3.42	0.04506
RICKEN cDNA	3.38	0.01594
ESTs	3.35	0.04348
insulin like peptide?	3.19	0.03302
endothelin converting enzyme 2	3.12	0.02224
RICKEN cDNA	3.07	0.02317
RICKEN cDNA	2.95	0.03850
ESTs	2.63	0.04425
Ngfi-A binding protein 1(NAB1)	2.63	0.01900
helicase-like transcription factor	2.56	0.00563
ADAMTS15	2.55	0.04784
ras homolog gene family, member J	2.5	0.02647
ESTs	2.49	0.01853
ESTs	2.44	0.02088
melastatin 1	2.43	0.04842
ESTs	2.43	0.01588
Fas-activated serine/threonine kinase	2.41	0.04615
acid phosphatase 2, lysosomal	2.4	0.03946
RICKEN cDNA	2.36	0.03320
RICKEN cDNA	2.32	0.04149
RICKEN cDNA	2.3	0.00626
ESTs	2.28	0.00620
RICKEN cDNA	2.26	0.01563
nPAS 1	2.19	0.04362
RICKEN cDNA	2.19	0.00720
SPLASH (PLA2IID),phospholipase A2	2.19	0.02959
ESTs(unknown)	2.19	0.02530
RICKEN cDNA	2.18	0.02309
similar to cleavage stimulation factor subunit 3	2.15	0.02142
structural protein of Rauscher oncovirus(p10)	2.05	0.00987
DAP-1	2.04	0.02370

TABLE III: Increased in the Polysome Fraction of KO

GENBANK	Name	(KOP/KO T)/(WTP/WT T)	p-value
AV085172	selenium binding protein 2	9.92	0.00102
AV330090	(PNKP) polynucleotide kinase 3'- phosphatase	5.87	0.03178
AI465103	VACM(vasopressin activated Calcium mobilizing)	4.73	0.03356
AV092700	epithelial membrane protein 3	4.51	0.03013
AV294462	unknown	3.91	0.02058
AV250850	RIKEN cDNA	3.89	0.04504
AA623482	Similar to PDZ protein interacting with TC10	3.44	0.00351
X00686	Mouse gene for 18S rRNA.	3.19	0.03115
AV378373	Filamin	3.12	0.03880
AW049330	EST unknown	2.95	0.00885
AW123239	EST unknown	2.92	0.00041
AA919877	Putative membrane protein	2.76	0.04070
U72059	chloride channel regulator Icln(pseudogene)	2.64	0.00409
AV094422	EST unknown	2.43	0.03592
AW122825	EST unknown	2.42	0.03813
AI854147	brain and acute leukemia (BALC) cytoplasmic	2.4	0.00534
AW045678	RIKEN cDNA	2.4	0.04961
AW045965	RIKEN cDNA	2.36	0.00679
U62674	Cluster Incl	2.35	0.04245
AI837599	neural stem cell derived neuronal survival protein	2.3	0.03956
AI848299	RIKEN cDNA	2.29	0.03593
AW048995	clone IMAGE	2.27	0.02920
AW120523	EST unknown	2.14	0.00425
AA839260	EST unknown	2.09	0.00918
AI850861	EST unknown	2.08	0.02885
AI152936	RIKEN cDNA	2.07	0.04229
AI585778	EST unknown	2.07	0.00915
AI850923	EST unknown	2.02	0.00999

Chapter 5:

Role of phosphorylation in control of splicing and translation in the neural system:

Phosphorylation is arguably the most frequent reversible post-translational modification in the nervous system. Innumerable examples of reversible change of the proprieties of a protein due to addition of a phosphate group to the hydroxyl groups of Serine, Threonine or Tyrosine have been described. In neurons, like in all cell types, phosphorylation of proteins is massively employed in signal transduction pathways that convey information from the extra-cellular environment to the inside of the cell. Despite the enormous complexity of the characterized signal transduction pathways identified in the nervous system, one can find a few themes common to the vast majority of them (reviewed in Siegelbaum et al., 2000). In the most schematic form, all signal transduction pathways in the CNS are initiated by an external signal or first messenger, which binds a receptor. Protein phosphorylation plays a major role in the modulation of the synaptic functions in response to the stimulation metabotropic receptors. The complex of ligands and receptor activates downstream events through the activation of another set of molecules. Despite the great variety of molecules involved in this step, pathways of signal transduction acting through phosphorylation can be grouped in two large groups: those initiated by tyrosine kinase receptors and those initiated by G protein-coupled receptors. The receptor tyrosine kinases bind peptide ligands, mostly cytokine and growth factors. The activation of tyrosine kinase pathways results mostly in long term changes in neuronal function through changes in gene expression. G protein-coupled receptors constitute a large family of integral proteins characterized by the presence of seven

transmembrane domains and by the functional coupling with guanine nucleotide-binding proteins (G proteins). G proteins, in turn, activate or inhibit enzymes often referred to as primary effectors, which produce second messengers. Second messengers transducing signals from G protein-coupled receptors in neurons are essentially cyclic AMP, which activates Protein kinase A, Inositol triphosphate, Diacyl Glycerol and Ca^{2+}, which activate protein kinase C, and, through the activation of phospholipase A2, Arachidonic Acid and its metabolites. The second messengers, in turn, trigger biochemical cascades, often involving the phosphorylation of numerous other substrates, the activation of other signaling cascades, or the mobilization of Ca^{2+}ions from intracellular stores. The physiologic outcome of the activation of a signal transduction pathway is often the result of the interaction with many other pathways that can converge or antagonize each other. LTP, for example, has been shown to be affected by the protein kinase A, calcium calmodulin kinase IV and MAP kinase cascades, all which converge in the phosphorylation of CREB (reviewed in Barco et al., 2003).

Phosphorylation is known to play important roles in the regulation of pre-mRNA splicing and regulation of mRNA translation. I already mentioned the role of mTOR in synaptic plasticity (Tang et al., 2002), and the role of phosphorylation of eEF2 in the increase of αCaMK II synthesis in response to stimulation of the NMDA receptor (Scheetz et al., 2000). Changes in alternative splicing have been observed in numerous instances in intact animals in response to behavioral pattern and physiological stimulation as diverse as stress, nutritional status, cocaine and ethanol abuse (reviewed in Stamm, 2002). In few cases, however, has the activation of a specific signaling pathway directly been shown to be involved in the regulation of alternative splicing choices. Notably for

the neural functions, a pyrimidine-rich element has been identified, that is necessary and sufficient to confer regulation by Ca^{2+}/Calmodulin-dependent protein kinase IV (CaMK IV). The element is found in the STREX exon of the BK potassium transcript, and the activation of CaMK IV inhibits its inclusion, thereby likely decreasing the Ca^{2+} sensitivity and opening probability of the channel (Xie and Black, 2001).

Nova 1 is a phosphoprotein:

The alternative splicing of the seventy-two nucleotides long exon H is known to generate two isoforms of Nova-1 (Buckanovich et al., 1993). Exon H encodes a domain, that we will call domain H, characterized by its high content of serine and threonine residues (twelve), five of which are preceded or followed by prolines, suggesting that they may be phosphorylation sites (Fig. 15B) (Kemp and Pearson, 1990). Furthermore, Nova-1 recognition motifs have been described in the intron downstream of exon H, and in the exon H itself, suggesting that the alternative splicing of exon H may be regulated by Nova itself (Buckanovich and Darnell, 1997). These features suggest that exon H may encode a regulatory domain, whose inclusion is object of further regulation. To verify the first part of this hypothesis we tested whether Nova-1 is phosphorylated *in vivo* and whether differences in the level of phosphorylation exist between the two isoforms. Vectors encoding the two isoforms were transfected into neuroblastoma N2a cells, radioactive inorganic phosphate was added to the medium, and the protein purified by immunoprecipitation was analyzed by autoradiography for the incorporation of phosphate. The isoform containing domain H is robustly phosphorylated, more than ten times above the level of the form without the domain (Fig. 15A and 15D). Analysis of the

sequence of the domain H shows that six residues match the consensus sequence for phosphorylation sites by two protein kinases, protein kinase C (PKC) and Glycogen Synthase Kinase 3 (GSK 3) (Fig. 15B). We substituted alanine residues for some of the serine and threonine residues in various combinations by site-directed mutagenesis to try to identify the phosphorylation sites. The phosphorylation level was unchanged when the sites 5 and 6 were mutated, and was reduced to the level of the form not containing domain H when sites 2 and 4 or 2, 5 and 6 were mutated, suggesting that site 2 is necessary for phosphorylation (Fig. 15C). The mutation of site 2 alone to alanine was sufficient to reduce the intensity of phosphorylation to the level of the form not containing domain H (Fig. 15D). These data suggest that the second serine of domain H is the only site of phosphorylation.

We have not been able to observe functional differences between the two isoforms of Nova-1, nor between the forms generated by site-directed mutagenesis and the wild type. Both the isoforms generated by alternative splicing are engaged in the same set of known protein-protein interactions (Fig. 7B), and they have a similar effect in promoting the inclusion of exon 3a of the Glycine receptor minigene (Fig. 16A). Furthermore, the mutation of the second Serine to Glutamate, which mimic a phosphate group because of its negative charge, has the same effect on splicing of the Glycine receptor minigene as the mutation to Alanine, which cannot be phosphorylated (Fig. 16B). Furthermore, the intracellular distribution of the two isoforms differing for the inclusion of the domain H does not show any major difference (Fig. 16C).

Discussion:

We established that Nova 1 is a phosphoprotein, and that the phosphorylation site is in an alternatively spliced exon. A weakly labelled band corresponding to the isoform not containing the alternative exon is observed in some of the experiments, indicating that much less utilized phosphorylation sites exist in the protein outside domain H. It is possible that the second serine of exon H is not the only phosphorylated site, but a site whose phosphorylation is required for the phosphorylation of a second site, a mode of phosphorylation that has been shown for Glycogen Synthetase Kinase 3 (GSK3) substrates, for example (reviewed in (Biondi and Nebreda, 2003). We have not been able to detect a functional difference in the ability to regulate splicing nor in the intracellular localization of the isoforms differing for the alternative inclusion of exon H, nor between the mutants. We do not know at present what the functional role the phosphorylation of Nova-1 is. The H domain primary sequence, which is rich in purine, serine, threonine, matches the consensus for PEST regions as evaluated by a web-based algorithm, PESTFind (http://www.icnet.uk/LRITu/projects/pest/). It is well established that PEST motifs serve as proteolytic signals to target the proteins for degradation by the 26S proteasome, in a process that involves phosphorylation-dependent ubiquitination (Rechsteiner and Rogers, 1996). Although we have not directly measured the half-life of the two isoforms in a pulse-chase experiment, we have no indication that the isoform containing exon H is less stable than the one not containing it. PEST sequences have also been demonstrated not to affect protein stability in a few instances (Chen and Clarke, 2002). Bicoid (Bcd), a homeodomain transcription factor, is also a translational regulator of *caudal* in *Drosophila*. The binding of a Bcd-binding region (BBR) in the 3'UTR of

caudal through the homeodomain is not sufficient to achieve such regulation: a PEST region in Bicoid is required for the translational repression of *caudal* (Niessing et al., 1999). Furthermore, deletion of a region that includes a PEST domain in CPEB abrogates tubulin binding *in vitro* and centrosome localization *in vivo*, suggesting that PEST domain may mediate the interaction of CPEB with the cytoskeleton (Groisman et al., 2000). Analogously, one can envision a scenario in which the H domain exerts an inhibitory effect on translation of the mRNA bound to Nova, and phosphorylation could exert some regulatory effect, for example releasing the inhibition in response to the activation of a signaling pathway. The interaction to the cytoskeleton, as in the CPEB example, is also an interesting hypothetical scenario for the function of the H domain, since RNA granules reactive to anti-Nova antibodies have been described in dendrites, presumably carrying mRNA to intracellular destinations (Darnell and Triller, unpublished).

Figure 15: Nova-1 is phosphorylated in a domain encoded by cassette exon H:

(A) Comparison between the level of phosphorylation of Nova-1 H+ and Nova-1 H- in N2a cells. Cells were transfected with constructs encoding the two isoforms tagged with T7, and inorganic ^{32}P was added to the medium. After 5 hrs incubation, cells were harvested, lysated and and immunoprecipitated. The pellet was treated with RNase A for 15 minutes at room temperature between the second and third washing steps. The precipitated material was resolved on a SDS PAGE, transferred to a nylon membrane and exposed to a radiography film (upper panel). Subsequently, the membrane was probed with anti body against T7 tag (lower panel).

(B) Sequence of the domain encoded by exon H (H domain). The residues colored in red and numbered 1 to 6 are the phosphorylation sites predicted by Prosite (http://us.expasy.org/prosite/) and Scansite (http://scansite.mit.edu/).

(C) Identification of the phosphorylation site by site-directed mutagenesis. Metabolic phosphorylation assay was performed as in (A). Expression constructs encoding Nova-1 H+ (lane 1), Nova-1 H- (lane 2), and point mutants in which the indicated residues were mutated to Alanine (lanes 3 to 6) were transfected. The numbers above the lanes refer to the numeration of the phosphorylation sites in Fig. 16B.

(D) Constructs encoding Nova-1 H+ (lane 1), Nova-1 H- (lane 2) and a Nova-1 H+ in which the Serine of site 2 was mutated to Alanine were utilized for the transfection/metabolic labeling assay as in (A).

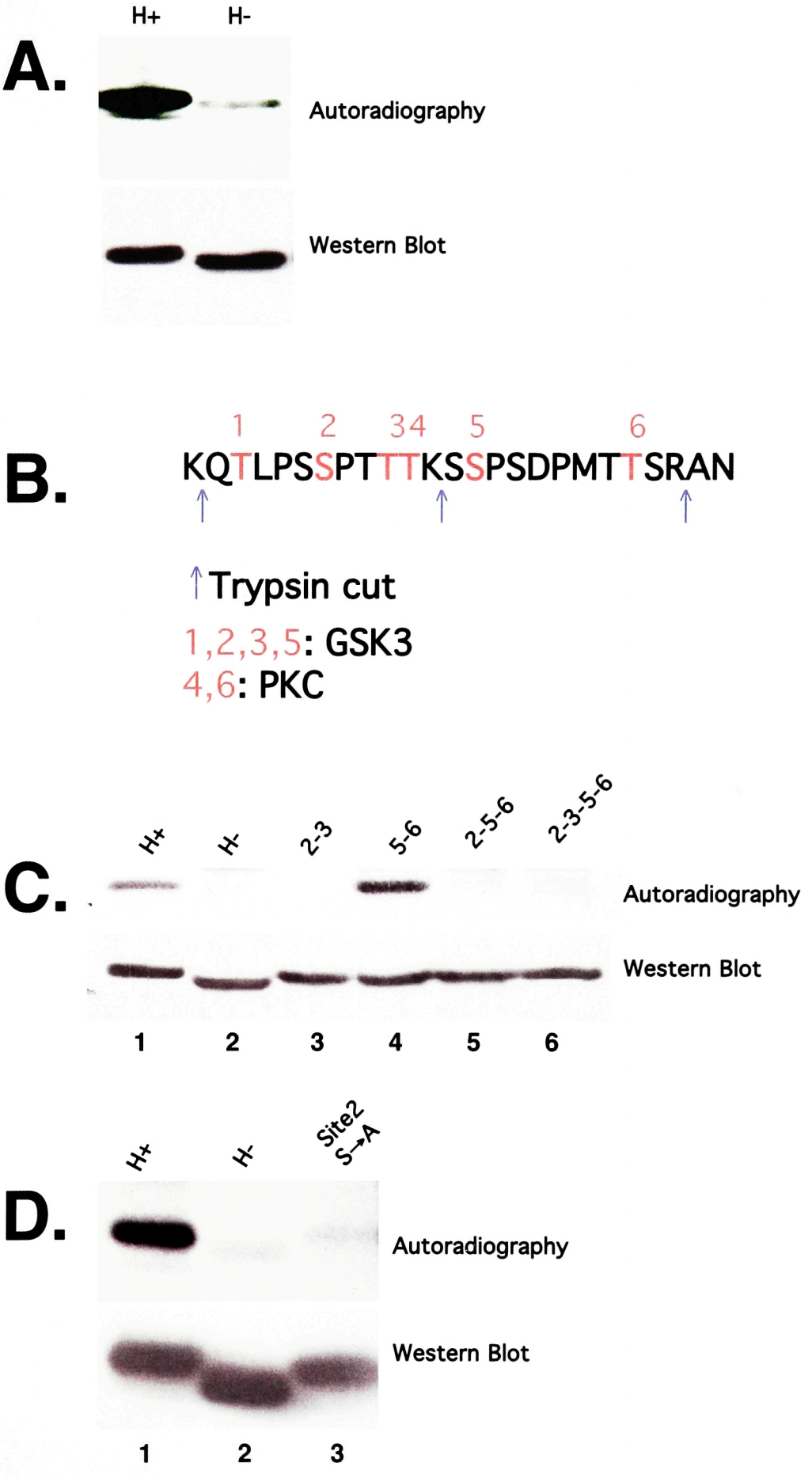

A.
H+
H-
Autoradiography
Western Blot
B.
1 2 34 5 6
KQTLPSSPTTTKSSPSDPMTTSRAN
Trypsin cut
1,2,3,5: GSK3
4,6: PKC
C.
H+
H-
2-3
5-6
2-5-6
2-3-5-6
Autoradiography
Western Blot
1 2 3 4 5 6
D.
H+
H-
Site2 S→A
Autoradiography
Western Blot
1 2 3

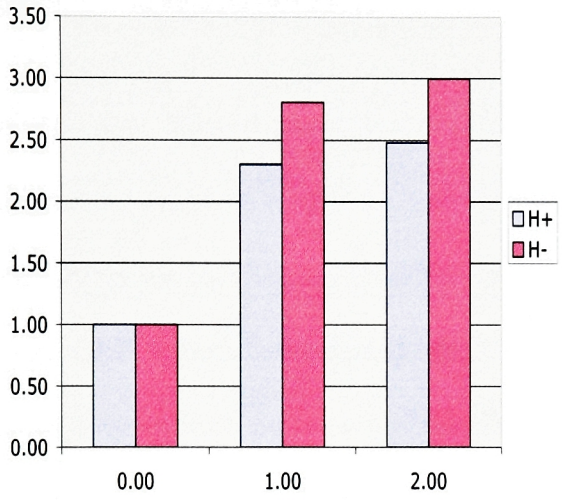
Figure 16: The state of phosphorylation of site 2 does not affect the properties of Nova-1 in splicing and localization:

(A) Co-transfection splicing assay of GlyRα2 minigene in 293 T cells. Increasing amount of DNA constructs encoding Nova-1 H+ and Nova-1 H- were co-transfected with a fixed amount of vector encoding GlyRα2 minigene. The ratio of the spliced isoforms was assessed by RT PCR like in figure 2.

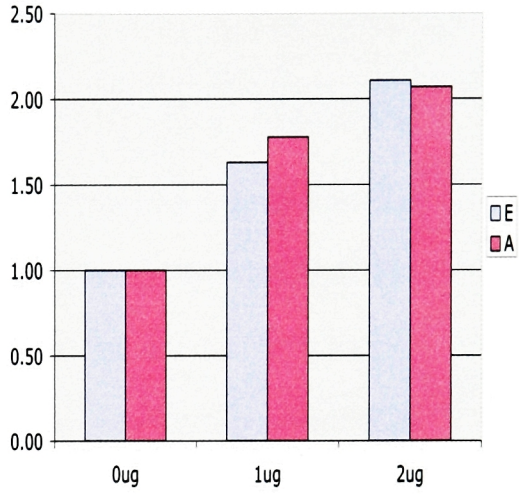
(B) The same co-transfection assay was employed by co-transfecting increasing amount of vector encoding Nova-1 carrying a mutation of the site 2 either to Alanine or to Glutamate with GlyRα2 minigene.

(C) Intracellular localization of the two Nova-1 H+ and H- isoforms was assessed by immunocytochemistry in transfected N2a cells.

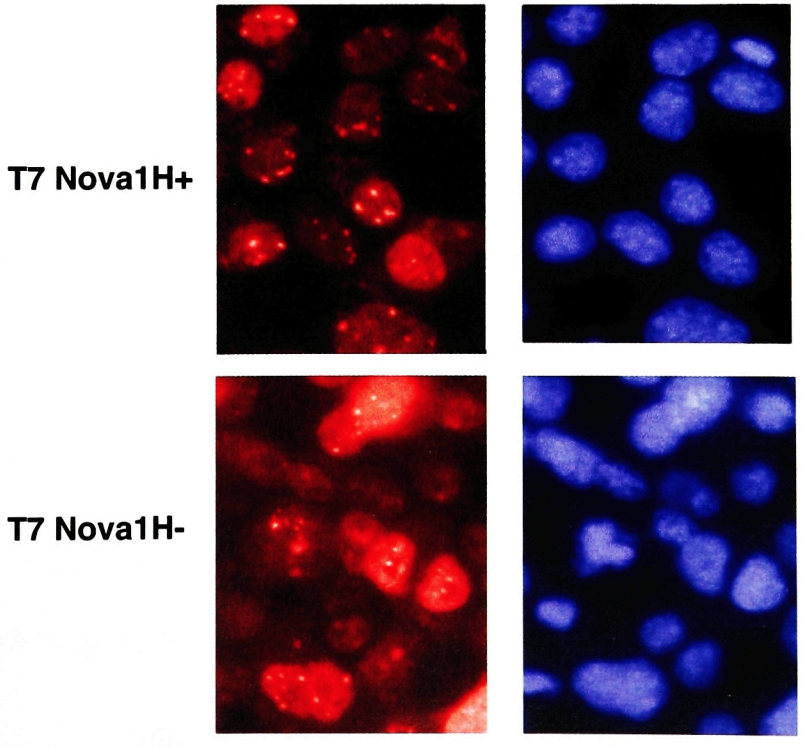
A. H+ vs. H-



B. S → A vs. S → E



C.



Chapter 6: Discussion

We demonstrated that Nova-1 regulates alternative splicing in several experimental systems. The analysis of Nova-1-null mutant animals had shown that the splicing of a candidate target RNA for Nova-1, *GlyRα2* pre-mRNA, was mis-regulated specifically in the areas of the CNS where Nova-1 is normally expressed. The alternative exon 3A, which is preceded by an intronic sequence matching the consensus for Nova binding, is under-utilized in the Nova-null animals. Consistent with this, a reciprocal increased utilization of exon 3A was observed upon co-transfection of Nova with a *GlyRα2* minigene in three different cell lines. The mutation of the candidate Nova-binding intronic sequence abolished the Nova-dependent effect. Furthermore, we demonstrated by UV cross-linking and *in vitro* splicing assay that the effect of Nova on alternative splicing is at the RNA level. Nova protein from brain extracts specifically cross-links the sequence (UCAUY)3 but not the mutant sequence (UAAUY)3. Purified Nova-1 fusion protein added to HeLa nuclear extract promotes the splicing of a cassette exon of an artificial pre-mRNA derived from $GABA_AR\gamma 2$ pre-mRNA, another gene whose splicing was found to be mis-regulated in the Nova-null mutants.

Nova-1 was the first described tissue-specific regulator of alternative splicing in vertebrates (Jensen et al., 2000a). Few other such factors have been described since then. A largely brain-restricted protein similar to PTB called brain PTB (brPTB) or neural PTB (nPTB), was discovered in a search for proteins interacting with Nova and independently

biochemically purified (Markovtsov et al., 2000; Polydorides et al., 2000). A family of six RNA binding proteins called CELF (CUBP and Etr-1-like factors) or Brunol (Bruno-like) have been recently described, some of whose members are exclusively expressed in one or two tissues (Ladd et al., 2001; Suzuki et al., 2002). ET3, a member of this family, increases the splicing of exon 5 of chicken cardiac Troponin by interacting with intronic elements called muscle-specific enhancers (MSE) (Charlet et al., 2002; Ladd et al., 2001). Another member of this family, NAPOR1, is enriched in restricted areas of the central neural system, and the pattern of splicing of *N*-methyl-D-aspartate (NMDA) receptor pre-mRNA correlates with its distribution. Experiments in transfected cells show a NAPOR1-dependent effect on a NMDA minigene splicing, and identifies particular intronic elements required for such regulation (Zhang et al., 2002). The RNA–binding protein Fox-1, whose expression is restricted to brain, heart and skeletal muscle in vertebrate, has recently been shown to regulate the alternative splicing of several genes containing its binding sequence UGCAUG (Jin et al., 2003). A few other tissue-restricted splicing regulators have been identified in invertebrates, for instance the neural-specific ELAV as well as the ovarian-specific Half Pint in *Drosophila* (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001; Van Buskirk and Schupbach, 2002).

The first well-characterized cascade of regulated alternative splicing, the sex determination pathway in *Drosophila,* consists of a series of binary choices between a regulated and a default pathway. Female-specific factors (Sex-lethal, Transformer) instruct the regulated, female-specific pattern of splicing; in absence of such factors, the pre-mRNAs are processed along the male-specific pathway, apparently the default pattern (reviewed by Wang et al., 1997). The observation that the tissue-specific exons in

vertebrate are most often poor splice substrates because of their small size and poor match to the consensus splice sequences prompted the idea that for vertebrates the exon skipping in the inappropriate cell types is also a default pathway (Black, 1991; Dominski and Kole, 1991; Dominski and Kole, 1992; Xu et al., 1993). The discovery of the antagonistic effects of ASF/SF2 and hnRNPA1 on the 5' splice site choice (Mayeda and Krainer, 1992) revealed a general feature of alternative splicing regulation in vertebrates: the choice between alternative pathways of splicing is the result of the interplay of activating and inhibiting factors. Most of the studied cell-type specific events have revealed complex mechanisms in which activating factors not only promote splicing, but also counteract inhibitory mechanisms. For example, in the case of the exon N1 of c-src splicing, one of the best-characterized instances of tissue-specific alternative splicing, several activators have been identified. KSRP, hnRNP H and hnRNP F promote the inclusion of exon N1 by binding the Downstream Control Sequence (DCS), while ASF/SF2 exerts its activating role interacting with the exon itself. These activators are required not only to overcome the intrinsic weakness of exon N1, but also to counteract the inhibitory role of PTB, which binds on both sides of the alternative exon, possibly forming a RNA looping complex that masks exon N1 to the splicing machinery (reviewed in Black, 2003). brPTB (or nPTB) is a weaker inhibitor of the inclusion of exon N1 *in vitro* than PTB thus providing a possible mechanism for the tissue specificity of exon N1 inclusion (Markovtsov et al., 2000). Analogously, the inclusion of exon 5 of cardiac Troponin (cTNT) is controlled by a balance of the positive action of ETR-3 --a member of the CELF family-- and the inhibitory action of PTB. The proportion of exon inclusion doubles when fibroblasts are transfected with a dominant negative form of

PTB, and returns to baseline when a dominant negative form of CELF is co-transfected (Charlet et al., 2002). The data on Nova-1 co-transfection with brPTB suggest a similar scenario, at least in the case of regulation of alternative splicing of *GlyRα2*, where brPTB antagonizes the effect of Nova (Polydorides et al., 2000). While we have not yet tested the effect of co-transfection of PTB or brPTB on the Nova-dependent activation of $GABA_AR\gamma 2$ splicing, previous studies have demonstrated an inhibitory effect of PTB on the splicing of this substrate (Ashiya and Grabowski, 1997). The interactions of Nova-1 with U2AF65 and U1 70K suggest that Nova can also regulate alternative splicing through a direct effect on recruitment of the spliceosome (Fig. 17A). In the case of the regulation of *GABA γr2* pre-mRNA splicing by Nova, one can hypothesize that the interaction with U1 70K is instrumental to recruit the entire U1snRNP to the 5' splice site most proximal to the intronic Nova binding site. TIA1, a splicing activator acting through an intronic enhancer, has been shown by psoralen-mediated UV cross-linking assays to increase the recruitment of U1 snRNP to a weak 5'splice site through its interaction with U1-C (Forch et al., 2000; Forch et al., 2002). Future *in vitro* studies will employ a similar approach to verify a similar effect on U1 recruitment by Nova through U1 70K.

Some features of the Nova-1-null mutants, like tremors and motor dysfunctions are consistent with functional failure of inhibitory responses in the CNS, as one would expect from impairment of the Glycinergic and GABAergic pathways. Several observations show that the $GABA_AR\gamma 2$ subunit is a very important component of the $GABA_A$ receptor. Mutations of the $GABA_AR\gamma 2$ subunit have been documented in association with various forms of familial epilepsy (Baulac et al., 2001; Bianchi et al.,

2002; Bowser et al., 2002; Harkin et al., 2002; Marini et al., 2003; Wallace et al., 2001). The targeted disruption of the entire $GABA_AR\gamma2$ subunit in mice results in animals that are normal at birth, but subsequently show retarded growth, sensor-motor dysfunctions and reduced life span. Consistent with previous studies identifying $GABA_AR\gamma2$ subunit as the target of benzodiazepines, the mutant mice are insensitive to this family of drugs (Gunther et al., 1995). However, present knowledge concerning the differences in physiologic proprieties of $GABA_AR\gamma2L$ and S isoforms does not provide a satisfactory explanation for the dramatic phenotype of Nova-1-null mutant mice. The alternative exon 9 of $GABA_AR\gamma2$ encodes a stretch of eight amino acids that are thought to contribute to the intracellular loop between the third and the fourth putative transmembrane domains of $GABA_AR\gamma2$ (Pritchett et al., 1989). Mice resulting from the specific deletion of the alternative exon 9 show only slightly altered responses in tests designed to measure the level of anxiety, and higher sensitivity to benzodiazepines compared to their wild type littermates (Homanics et al., 1999; Quinlan et al., 2000). Even less is known about the features of the two isoforms of $GlyR\alpha2$ generated by the inclusion of exon 3A or 3B: the two exons encode twenty-three aminoacid long extracellular domains that differ by only two amino acids (Kuhse et al., 1991), and no functional differences have been described.

It is probable that the phenotype of Nova-1-null mice results from defects of the regulation of other genes at the level of splicing beside *GlyRα2* and *$GABA_AR\gamma2$*, and possibly, given the high degree of integration of the mechanism of gene expression, at the level of export, localization of the messages and translation. Cytoplasmic and dendritic distribution of Nova has indeed been demonstrated by immunocytochemistry both in cultures of primary neurons and in ventral horn spinal cord neurons (Triller and Darnell,

unpublished). To gain some insight in the function of the cytoplasmic Nova, we began the characterization of the cytoplasmic structures Nova interacts with, by analyzing cytoplasmic extracts in linear density gradients. Rather large cytoplasmic structures, sedimenting around 80S, are observed in extracts of animals from P3 to P30. Unlike translating polysomes, these structures are resistant to treatment with high concentration of EDTA. We are inclined to consider these structures mRNPs, similar to structures of similar size characterized as reactive to antibodies against FMRP, Hu and αCP (Antic and Keene, 1998; Feng et al., 1997; Ji et al., 2003). Further experiments are required to unambiguously establish the nature of these structures. Poly(A) specific fractionation by Chromatography on Oligo(dT)-cellulose (Dreyfuss et al., 1984a; Dreyfuss et al., 1984b) and a more stringent treatment with RNAse will verify their RNA content. An amount of Nova that increases with age, up to around twenty-five percent at P30, sediments in the denser fractions of the gradient, and shifts to the upper part of the gradient in presence of EDTA, a behavior consistent with association with polysomes.

In an attempt to achieve a broader picture of the changes in gene expression levels underlying the phenotype of the Nova-null mutants, we utilized the RNA purified from the polysomal fractions of the gradient to interrogate DNA microarray. We compared the information obtained with the levels of gene expression in the input material. The overall number of sequences that show variations between wild type and mutants is rather small compared to the variations observed comparing polysome fractions from lymphoblastoid cell lines expressing normal or non-functional FMRP (Brown et al., 2001). As in Brown et al., 2001, we observed both increased and decreased abundance of specific messages in the polysomal fraction. Little information is available about the biological proprieties of a

quite large portion of the sequences that show modified levels in polysomes in Nova-null mice. At least six sequences have known biological proprieties that can be related to a role in growth cone functions, post-synaptic architecture and related signaling pathways. Two of them are increased and four decreased in absence of Nova, exclusively in the polysome fraction. We also found three metalloproteases to be decreased in the polysome-associated pool of mRNAs in absence of Nova. Metalloproteases have a demonstrated role in neurites growth during development of the CNS, among many other functions (reviewed in (McFarlane, 2003; Yong et al., 2001).

While the decreased levels of these messages in the polysomes purified from Nova-1-null mice is consistent with a positive role of Nova-1 in the regulation of their translation, many issues remain open for future investigation. The data obtained from the microarrays only provide a picture of the final outcome of potentially many events consequent to the absence of Nova-1. Although the decreased level of messages exclusively in the polysomes hint at a role of Nova in translational regulation, the modality of such action is unclear. One first point to be addressed is the validation of consistent changes in the level of the proteins encoded by the messages whose abundance is altered. Another important question is whether the altered level of messages are due to a direct effect of Nova through interaction with the proposed target RNAs. Although sets of potential Nova binding sites are identifiable by inspection of the sequences, no evidence of a physical interaction is presently available.

Most of the described mechanisms of translational regulation by RNA binding proteins in eukaryotes involve an inhibitory effect that is removed upon the delivery of an appropriate signal. Among the best studied examples, hnRNP K, E1 and E2 are known to

inhibit the initiation of translation of *15 LOX* mRNA by binding the 3'UTR (Ostareck et al., 2001; Ostareck et al., 1997). Iron regulatory proteins (IRP) regulate translation of ferritin by inhibiting initiation through an interaction with the 5' UTR of the message; upon increase of the Iron levels which are bound by IRP, a conformational change of the proteins induces the release of the inhibition (reviewed in (Hentze and Kuhn, 1996). Nova could conceivably inhibit translation with analogous mechanisms at the level of initiation. On the contrary, the decreased levels of mRNAs in the Nova-null polysomes suggest a positive effect of Nova on their translation. While mechanisms of general activation of cap dependent translation through mTOR protein kinase are well characterized, few examples of translational activation of subset of messages by sequence-specific RNA binding proteins are known. In *Drosophila*, Aubergine has been shown to enhance oskar translation in the ovary (Wilson et al., 1996). Motifs with translational enhancer activity have been identified in plants (Yamamoto et al., 1995). The RNA-trafficking sequence (RTS) originally identified as a determinant for transport of *Myelin Basic Protein (MBP)* mRNA is a vertebrate translational enhancer. hnRNP A2 acts on RTS as a *trans*-activator (Kwon et al., 1999). The finding of simultaneous increase of some messages and decrease of others in the polysome fraction in response to the abolition of Nova is seemingly paradoxical. Nonetheless, translational control factors seem to act in a combinatorial fashion in many instances. Analogous to well-documented principles of transcriptional regulation, distinct protein-protein interactions between RNA binding proteins are thought to discriminate among various RNA substrates and yield specific biological outcomes (reviewed by Wickens, M., et al., 2000). HnRNP E1 acts with hnRNP K to repress 15 LOX translation, by binding the DICE element (Ostareck et

al., 1997), but it has additional roles as well. In fact, HnRNP E1 with hnRNP E2 is also part of the α-complex that promote the stability of α-globin mRNA by binding to CU-rich sequences in the 3'UTR, ultimately promoting the expression of this gene (Kiledjian et al., 1995; Wang et al., 1995). Therefore, by interacting with two different mRNAs as part of two different protein complexes, hnRNP E1 exerts two opposite effects on the expression of two different genes in the same cell type. Furthermore, the mRNAs analyzed on microarrays were purified from spinal cord, which is constituted by a heterogeneous population of neuronal subtypes: inhibitory and activating actions of Nova on translation could conceivably not take place simultaneously in the same cell. Given the similar sedimentation pattern on linear density gradient, and the demonstrated physical interaction, Hu proteins are possible functional partners of Nova in the formation of complexes with a positive role on translation. Increased translation of *neurofilament M* mRNA has been described in response to over-expression of HuB, in absence of stabilization of the message (Antic et al., 1999). The translation of a number of other messages has been shown to be increased in response to Hu proteins, but the mechanism of such effect is not known (Keene, 2001).

A noticeable feature of the mechanisms of translational control in early *Drosophila* and *C.elegans* development is the strict coordination of mRNA localization and translation (Johnstone and Lasko, 2001). In several instances, the translation of particular mRNAs in an inappropriate location in the cell could have damaging consequences; therefore mRNA is complexed with proteins that keep it masked from the translational apparatus during transport and mediate interactions with cytoskeleton and motor proteins. One possible interpretation of the observed decrease of certain RNA in

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the polysomes of Nova-1-null animals is the loss of a hypothetical localizing effect. One could envisage a scenario where in absence of Nova its mRNA targets do not reach a location in the cell where they are usually translated. If, in order to be translated, these mRNAs are required to be localized in the dendrites or in the growth cone, and the absence of Nova impaired such spatial targeting, one would expect a diminished engagement in the polysomes in the Nova-null mice. An obvious implication of this model is that the mRNAs that we found decreased in the polysomes should be normally localized in discrete domains of the cytoplasm.

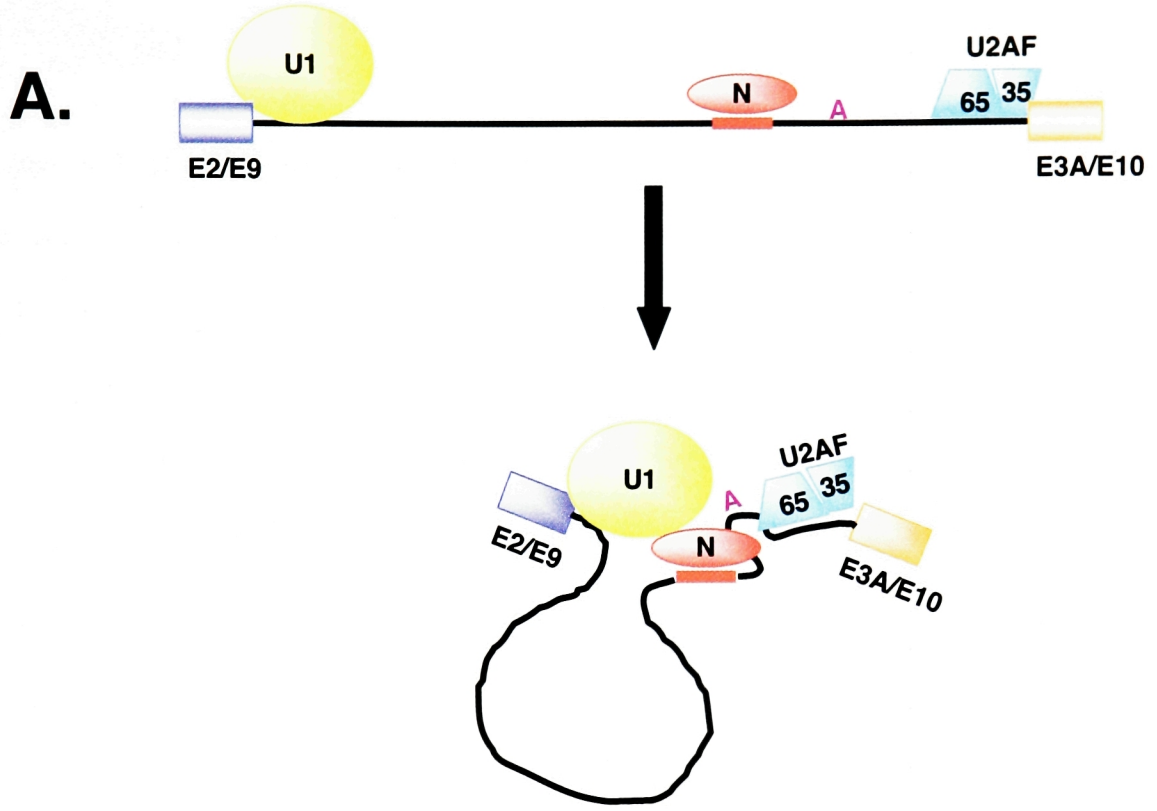
The observation of splicing defects and translational alterations in the Nova-null mutants makes it tempting to consider a direct connection between the nuclear and cytoplasmic events regulated by Nova-1. However, we have not observed alterations of the polysome distribution of GABAR and GlyR mRNA in absence of Nova. The knowledge of translational alteration in Nova-null mutants puts us in the position to tackle the hypothesis of coupling between the nuclear and cytoplasmic events regulated by Nova from the cytoplasmic end of the mRNA's journey. If that is the case, one would expect changes in the pattern of alternative splicing of those mRNAs whose level is altered in the polysomes. Recent investigations conducted in our laboratory have led to the isolation of a number of RNAs based on their physical association with Nova. The splicing of some of these new targets is modified in absence of Nova (Ule and Jensen, unpublished). Interestingly, a number of them encode proteins involved in clustering of receptors, post-synaptic structures and growth cones, similar to the mRNAs we have found altered in the polysomes. However, no overlapping RNAs between the two screenings have emerged at present. The data emerging from diverse approaches are

beginning to draw a rather complex picture of the functional role of Nova in the regulation of gene expression. We have shown that Nova affects the pattern of alternative splicing of an as yet unknown number of genes, and we provide initial observations suggesting a role in the regulation of cytoplasmic events of gene expression (Fig.17b). Functionally related genes encoding receptors, components of the post-synaptic densities, growth cones and elements of related signal transduction pathways are regulated by Nova at different steps of their expression pathway.

Figure 17: Functions of Nova in RNA metabolism:

(A) Nova acts in the nucleus as a regulator of alternative splicing. Our data suggest that Nova binds to intronic enhancers and promotes the utilization of the most proximal splice sites of the intron it is bound to. This observations and the interactions with U1 70K and U2AF65 suggest that Nova recruits components of the basic splicing machinery to the closest splice sites.

(B) Changes in the relative abundance of messengers specifically in the polysome fraction in absence of Nova-1 suggest that the pehnotype of Nova-1-null mice is at least partially imputable to alterations of gene expression at the level of translation. Since Nova has been observed in discrete foci in the dendrites, coincident with reactivity for component of the ribosome, one can speculate a functional role in regulation of peculiar aspect of translation in neurons, like coupling of translation to neural activity in phenomena of synaptic plasticity. Consistently with this hypothesis, several messengers whose association with polysomes is altered in absence of Nova-1 encode proteins with functions related to the maturation or function of synaptic contacts.


A.
U1
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A
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E2/E9
E3A/E10
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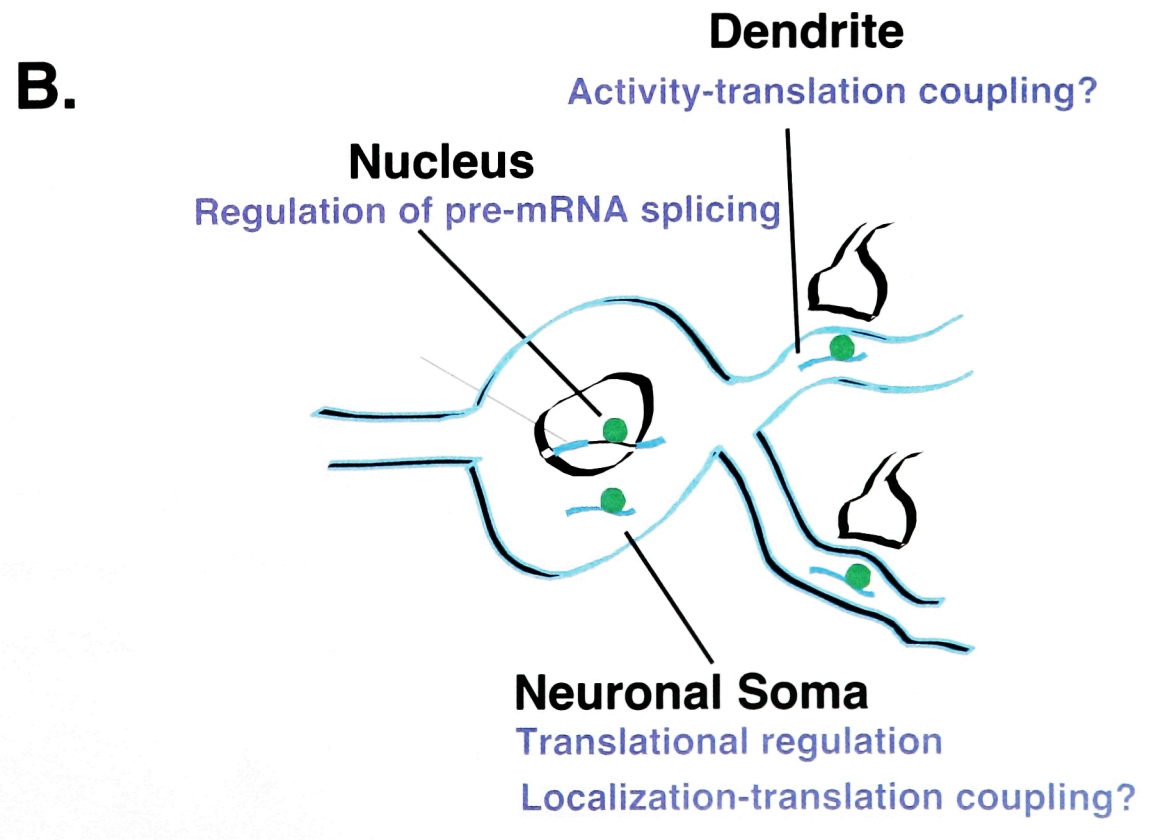
E3A/E10


B.
Dendrite
Activity-translation coupling?
Nucleus
Regulation of pre-mRNA splicing
Neuronal Soma
Translational regulation
Localization-translation coupling?

Reference List

Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C., and Schuman, E. M. (2001). Dynamic visualization of local protein synthesis in hippocampal neurons. Neuron *30*, 489-502.

Akamatsu, W., Okano, H. J., Osumi, N., Inoue, T., Nakamura, S., Sakakibara, S., Miura, M., Matsuo, N., Darnell, R. B., and Okano, H. (1999). Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. Proc Natl Acad Sci U S A *96*, 9885-9890.

Albert, M. L., Darnell, J. C., Bender, A., Francisco, L. M., Bhardwaj, N., and Darnell, R. B. (1998). Tumor-specific killer cells in paraneoplastic cerebellar degeneration. Nat Med *4*, 1321-1324.

Anderson, N. E., Budde-Steffen, C., Rosenblum, M. K., Graus, F., Ford, D., Synek, B. J., and Posner, J. B. (1988). Opsoclonus, myoclonus, ataxia, and encephalopathy in adults with cancer: a distinct paraneoplastic syndrome. Medicine (Baltimore) *67*, 100-109.

Antic, D., and Keene, J. D. (1998). Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus. J Cell Sci *111 (Pt 2)*, 183-197.

Antic, D., Lu, N., and Keene, J. D. (1999). ELAV tumor antigen, Hel-N1, increases translation of neurofilament M mRNA and induces formation of neurites in human teratocarcinoma cells. Genes Dev *13*, 449-461.

Arava, Y., Wang, Y., Storey, J. D., Liu, C. L., Brown, P. O., and Herschlag, D. (2003). Genome-wide analysis of mRNA translation profiles in Saccharomycescerevisiae. Proc Natl Acad Sci U S A *100*, 3889-3894.

Ashiya, M., and Grabowski, P. J. (1997). A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart. Rna *3*, 996-1015.

Bachi, A., Braun, I. C., Rodrigues, J. P., Pante, N., Ribbeck, K., von Kobbe, C., Kutay, U., Wilm, M., Gorlich, D., Carmo-Fonseca, M., and Izaurralde, E. (2000). The C-terminal domain of TAP interacts with the nuclear pore complex and promotes export of specific CTE-bearing RNA substrates. Rna *6*, 136-158.

Bagni, C., Mannucci, L., Dotti, C. G., and Amaldi, F. (2000). Chemical stimulation of synaptosomes modulates alpha -Ca2+/calmodulin-dependent protein kinase II mRNA association to polysomes. J Neurosci *20*, RC76.

Ball, N. S., and King, P. H. (1997). Neuron-specific hel-N1 and HuD as novel molecular markers of neuroblastoma: a correlation of HuD messenger RNA levels with favorable prognostic features. Clin Cancer Res *3*, 1859-1865.

Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000). The complete atomic structure of the large ribosomal subunit at 2.4 A resolution. Science *289*, 905-920.

Banks, R. E., Dunn, M. J., Hochstrasser, D. F., Sanchez, J. C., Blackstock, W., Pappin, D. J., and Selby, P. J. (2000). Proteomics: new perspectives, new biomedical opportunities. Lancet *356*, 1749-1756.

Barco, A., Pittenger, C., and Kandel, E. R. (2003). CREB, memory enhancement and the treatment of memory disorders: promises, pitfalls and prospects. Expert Opin Ther Targets *7*, 101-114.

Bashaw, G. J., and Baker, B. S. (1997). The regulation of the Drosophila msl-2 gene

reveals a function for Sex-lethal in translational control. Cell *89*, 789-798.

Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., Baulac, M., Brice, A., Bruzzone, R., and LeGuern, E. (2001). First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet *28*, 46-48.

Bechade, C., Sur, C., and Triller, A. (1994). The inhibitory neuronal glycine receptor. Bioessays *16*, 735-744.

Berget, S. M. (1995). Exon recognition in vertebrate splicing. J Biol Chem *270*, 2411-2414.

Berglund, J. A., Fleming, M. L., and Rosbash, M. (1998). The KH domain of the branchpoint sequence binding protein determines specificity for the pre-mRNA branchpoint sequence. Rna *4*, 998-1006.

Beyer, A. L., and Osheim, Y. N. (1988). Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. Genes Dev *2*, 754-765.

Bianchi, M. T., Song, L., Zhang, H., and Macdonald, R. L. (2002). Two different mechanisms of disinhibition produced by GABAA receptor mutations linked to epilepsy in humans. J Neurosci *22*, 5321-5327.

Bilodeau, P. S., Domsic, J. K., Mayeda, A., Krainer, A. R., and Stoltzfus, C. M. (2001). RNA splicing at human immunodeficiency virus type 1 3' splice site A2 is regulated by binding of hnRNP A/B proteins to an exonic splicing silencer element. J Virol *75*, 8487-8497.

Biondi, R. M., and Nebreda, A. R. (2003). Signalling specificity of Ser/Thr protein kinases through docking site-mediated interactions. Biochem J *Pt.*

Black, D. L. (1991). Does steric interference between splice sites block the splicing of a short c-src neuron-specific exon in non-neuronal cells? Genes Dev *5*, 389-402.

Black, D. L. (2003). Mechanisms of Alternative Pre-Messenger RNA Splicing. Annu Rev Biochem.

Black, D. L., Chabot, B., and Steitz, J. A. (1985). U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell *42*, 737-750.

Blencowe, B. J., Nickerson, J. A., Issner, R., Penman, S., and Sharp, P. A. (1994). Association of nuclear matrix antigens with exon-containing splicing complexes. J Cell Biol *127*, 593-607.

Bowser, D. N., Wagner, D. A., Czajkowski, C., Cromer, B. A., Parker, M. W., Wallace, R. H., Harkin, L. A., Mulley, J. C., Marini, C., Berkovic, S. F., *et al.* (2002). Altered kinetics and benzodiazepine sensitivity of a GABAA receptor subunit mutation [gamma 2(R43Q)] found in human epilepsy. Proc Natl Acad Sci U S A *99*, 15170-15175.

Brennan, C. M., Gallouzi, I. E., and Steitz, J. A. (2000). Protein ligands to HuR modulate its interaction with target mRNAs in vivo. J Cell Biol *151*, 1-14.

Brennan, C. M., and Steitz, J. A. (2001). HuR and mRNA stability. Cell Mol Life Sci *58*, 266-277.

Brett, D., Pospisil, H., Valcarcel, J., Reich, J., and Bork, P. (2002). Alternative splicing and genome complexity. Nat Genet *30*, 29-30.

Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. (1988). Introns increase transcriptional efficiency in transgenic mice. Proc Natl Acad Sci U S A *85*, 836-840.

Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., Jin,

X., Feng, Y., Wilkinson, K. D., Keene, J. D., *et al.* (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell *107*, 477-487.

Buckanovich, R. J., and Darnell, R. B. (1997). The neuronal RNA binding protein Nova-1 recognizes specific RNA targets in vitro and in vivo. Mol Cell Biol *17*, 3194-3201.

Buckanovich, R. J., Posner, J. B., and Darnell, R. B. (1993). Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. Neuron *11*, 657-672.

Buckanovich, R. J., Yang, Y. Y., and Darnell, R. B. (1996). The onconeural antigen Nova-1 is a neuron-specific RNA-binding protein, the activity of which is inhibited by paraneoplastic antibodies. J Neurosci *16*, 1114-1122.

Budde-Steffen, C., Anderson, N. E., Rosenblum, M. K., Graus, F., Ford, D., Synek, B. J., Wray, S. H., and Posner, J. B. (1988). An antineuronal autoantibody in paraneoplastic opsoclonus. Ann Neurol *23*, 528-531.

Caceres, J. F., and Kornblihtt, A. R. (2002). Alternative splicing: multiple control mechanisms and involvement in human disease. Trends Genet *18*, 186-193.

Caceres, J. F., Screaton, G. R., and Krainer, A. R. (1998). A specific subset of SR proteins shuttles continuously between the nucleus and the cytoplasm. Genes Dev *12*, 55-66.

Caceres, J. F., Stamm, S., Helfman, D. M., and Krainer, A. R. (1994). Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science *265*, 1706-1709.

Caputi, M., Mayeda, A., Krainer, A. R., and Zahler, A. M. (1999). hnRNP A/B proteins

are required for inhibition of HIV-1 pre-mRNA splicing. Embo J *18*, 4060-4067.

Carmo-Fonseca, M. (2002). The contribution of nuclear compartmentalization to gene regulation. Cell *108*, 513-521.

Carson, J. H., Cui, H., and Barbarese, E. (2001). The balance of power in RNA trafficking. Curr Opin Neurobiol *11*, 558-563.

Cartegni, L., Chew, S. L., and Krainer, A. R. (2002). Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet *3*, 285-298.

Cartegni, L., and Krainer, A. R. (2002). Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat Genet *30*, 377-384.

Carter, M. S., Kuhn, K. M. and Sarnow, P. (2000) in Translational Control of Gene Expression (Sonenberg, N., Hershey, J. W. B. and Mathews, M. B., eds), pp. 615–636, Cold Spring Harbor Press, Cold Spring Harbor, NY

Casadio, A., Martin, K. C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C. H., and Kandel, E. R. (1999). A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell *99*, 221-237.

Cavaloc, Y., Bourgeois, C. F., Kister, L., and Stevenin, J. (1999). The splicing factors 9G8 and SRp20 transactivate splicing through different and specific enhancers. Rna *5*, 468-483.

Ch'ng, J. L., Shoemaker, D. L., Schimmel, P., and Holmes, E. W. (1990). Reversal of creatine kinase translational repression by 3' untranslated sequences. Science *248*, 1003-1006.

Chabot, B., and Steitz, J. A. (1987). Multiple interactions between the splicing substrate and small nuclear ribonucleoproteins in spliceosomes. Mol Cell Biol *7*, 281-293.

Charlet, B. N., Logan, P., Singh, G., and Cooper, T. A. (2002). Dynamic antagonism between ETR-3 and PTB regulates cell type-specific alternative splicing. Mol Cell *9*, 649-658.

Chen, E. Y., and Clarke, D. M. (2002). The PEST sequence does not contribute to the stability of the cystic fibrosis transmembrane conductance regulator. BMC Biochem *3*, 29.

Chomczynski, P., Mackey, K., Drews, R., and Wilfinger, W. (1997). DNAzol: a reagent for the rapid isolation of genomic DNA. Biotechniques *22*, 550-553.

Chou, M. Y., Rooke, N., Turck, C. W., and Black, D. L. (1999). hnRNP H is a component of a splicing enhancer complex that activates a c-src alternative exon in neuronal cells. Mol Cell Biol *19*, 69-77.

Cook, P. R. (1999). The organization of replication and transcription. Science *284*, 1790-1795.

Cote, C. A., Gautreau, D., Denegre, J. M., Kress, T. L., Terry, N. A., and Mowry, K. L. (1999). A Xenopus protein related to hnRNP I has a role in cytoplasmic RNA localization. Mol Cell *4*, 431-437.

Cramer, P., Caceres, J. F., Cazalla, D., Kadener, S., Muro, A. F., Baralle, F. E., and Kornblihtt, A. R. (1999). Coupling of transcription with alternative splicing: RNA pol II promoters modulate SF2/ASF and 9G8 effects on an exonic splicing enhancer. Mol Cell *4*, 251-258.

Cramer, P., Pesce, C. G., Baralle, F. E., and Kornblihtt, A. R. (1997). Functional

association between promoter structure and transcript alternative splicing. Proc Natl Acad Sci U S A *94*, 11456-11460.

Cullen, B. R. (2000). Nuclear RNA export pathways. Mol Cell Biol *20*, 4181-4187.

Dabeva, M. D., and Warner, J. R. (1993). Ribosomal protein L32 of Saccharomyces cerevisiae regulates both splicing and translation of its own transcript. J Biol Chem *268*, 19669-19674.

Dalmau, J., Furneaux, H. M., Cordon-Cardo, C., and Posner, J. B. (1992a). The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. Am J Pathol *141*, 881-886.

Dalmau, J., Graus, F., Rosenblum, M. K., and Posner, J. B. (1992b). Anti-Hu--associated paraneoplastic encephalomyelitis/sensory neuronopathy. A clinical study of 71 patients. Medicine (Baltimore) *71*, 59-72.

Daneholt, B. (1997). A look at messenger RNP moving through the nuclear pore. Cell *88*, 585-588.

Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell *107*, 489-499.

Darnell, R. B. (1996). Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain. Proc Natl Acad Sci U S A *93*, 4529-4536.

Darnell, R. B. (1998). Immunologic complexity in neurons. Neuron *21*, 947-950.

Darnell, R. B., and Posner, J. B. (2003). Observing the invisible: successful tumor immunity in humans. Nat Immunol *4*, 201.

Dirksen, W. P., Li, X., Mayeda, A., Krainer, A. R., and Rottman, F. M. (2000). Mapping the SF2/ASF binding sites in the bovine growth hormone exonic splicing enhancer. J Biol Chem *275*, 29170-29177.

Dominski, Z., and Kole, R. (1991). Selection of splice sites in pre-mRNAs with short internal exons. Mol Cell Biol *11*, 6075-6083.

Dominski, Z., and Kole, R. (1992). Cooperation of pre-mRNA sequence elements in splice site selection. Mol Cell Biol *12*, 2108-2114.

Doyle, G. A., Betz, N. A., Leeds, P. F., Fleisig, A. J., Prokipcak, R. D., and Ross, J. (1998). The c-myc coding region determinant-binding protein: a member of a family of KH domain RNA-binding proteins. Nucleic Acids Res *26*, 5036-5044.

Dreyfuss, G., Adam, S. A., and Choi, Y. D. (1984a). Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. Mol Cell Biol *4*, 415-423.

Dreyfuss, G., Choi, Y. D., and Adam, S. A. (1984b). Characterization of heterogeneous nuclear RNA-protein complexes in vivo with monoclonal antibodies. Mol Cell Biol *4*, 1104-1114.

Dreyfuss, G., Kim, V. N., and Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. Nat Rev Mol Cell Biol *3*, 195-205.

Eperon, I. C., Makarova, O. V., Mayeda, A., Munroe, S. H., Caceres, J. F., Hayward, D. G., and Krainer, A. R. (2000). Selection of alternative 5' splice sites: role of U1 snRNP and models for the antagonistic effects of SF2/ASF and hnRNP A1. Mol Cell Biol *20*, 8303-8318.

Expert-Bezancon, A., Le Caer, J. P., and Marie, J. (2002). Heterogeneous nuclear

ribonucleoprotein (hnRNP) K is a component of an intronic splicing enhancer complex that activates the splicing of the alternative exon 6A from chicken beta-tropomyosin pre-mRNA. J Biol Chem *277*, 16614-16623.

Fakan, S., Leser, G., and Martin, T. E. (1984). Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. J Cell Biol *98*, 358-363.

Fakan, S., Leser, G., and Martin, T. E. (1986). Immunoelectron microscope visualization of nuclear ribonucleoprotein antigens within spread transcription complexes. J Cell Biol *103*, 1153-1157.

Fan, X. C., and Steitz, J. A. (1998). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. Embo J *17*, 3448-3460.

Feng, Y., Absher, D., Eberhart, D. E., Brown, V., Malter, H. E., and Warren, S. T. (1997). FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. Mol Cell *1*, 109-118.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W., and Luhrmann, R. (1995). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell *82*, 475-483.

Fleckner, J., Zhang, M., Valcarcel, J., and Green, M. R. (1997). U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction. Genes Dev *11*, 1864-1872.

Fong, Y. W., and Zhou, Q. (2001). Stimulatory effect of splicing factors on transcriptional elongation. Nature *414*, 929-933.

Forch, P., Puig, O., Kedersha, N., Martinez, C., Granneman, S., Seraphin, B., Anderson, P., and Valcarcel, J. (2000). The apoptosis-promoting factor TIA-1 is a regulator of alternative pre-mRNA splicing. Mol Cell *6*, 1089-1098.

Forch, P., Puig, O., Martinez, C., Seraphin, B., and Valcarcel, J. (2002). The splicing regulator TIA-1 interacts with U1-C to promote U1 snRNP recruitment to 5' splice sites. Embo J *21*, 6882-6892.

Fornerod, M., Ohno, M., Yoshida, M., and Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. Cell *90*, 1051-1060.

Furger, A., O'Sullivan, J. M., Binnie, A., Lee, B. A., and Proudfoot, N. J. (2002). Promoter proximal splice sites enhance transcription. Genes Dev *16*, 2792-2799.

Galban, S., Fan, J., Martindale, J. L., Cheadle, C., Hoffman, B., Woods, M. P., Temeles, G., Brieger, J., Decker, J., and Gorospe, M. (2003). von Hippel-Lindau protein-mediated repression of tumor necrosis factor alpha translation revealed through use of cDNA arrays. Mol Cell Biol *23*, 2316-2328.

Gall, J. G. (2001). A role for Cajal bodies in assembly of the nuclear transcription machinery. FEBS Lett *498*, 164-167.

Gall, J. G., Bellini, M., Wu, Z., and Murphy, C. (1999). Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. Mol Biol Cell *10*, 4385-4402.

Gallouzi, I. E., Brennan, C. M., Stenberg, M. G., Swanson, M. S., Eversole, A., Maizels, N., and Steitz, J. A. (2000). HuR binding to cytoplasmic mRNA is perturbed by heat shock. Proc Natl Acad Sci U S A *97*, 3073-3078.

Gallouzi, I. E., and Steitz, J. A. (2001). Delineation of mRNA export pathways by the use

of cell-permeable peptides. Science *294*, 1895-1901.

Gao, F. B., and Keene, J. D. (1996). Hel-N1/Hel-N2 proteins are bound to poly(A)+ mRNA in granular RNP structures and are implicated in neuronal differentiation. J Cell Sci *109 (Pt 3)*, 579-589.

Gardiol, A., Racca, C., and Triller, A. (1999). Dendritic and postsynaptic protein synthetic machinery. J Neurosci *19*, 168-179.

Gatfield, D., and Izaurralde, E. (2002). REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export. J Cell Biol *159*, 579-588.

Ge, H., and Manley, J. L. (1990). A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. Cell *62*, 25-34.

Gebauer, F., Merendino, L., Hentze, M. W., and Valcarcel, J. (1997). Novel functions for 'nuclear factors' in the cytoplasm: the Sex-lethal paradigm. Semin Cell Dev Biol *8*, 561-566.

Gebauer, F., Merendino, L., Hentze, M. W., and Valcarcel, J. (1998). The Drosophila splicing regulator sex-lethal directly inhibits translation of male-specific-lethal 2 mRNA. Rna *4*, 142-150.

Gorlich, D., and Kutay, U. (1999). Transport between the cell nucleus and the cytoplasm. Annu Rev Cell Dev Biol *15*, 607-660.

Grabowski, P. J. (1998). Splicing regulation in neurons: tinkering with cell-specific control. Cell *92*, 709-712.

Grabowski, P. J., Padgett, R. A., and Sharp, P. A. (1984). Messenger RNA splicing in vitro: an excised intervening sequence and a potential intermediate. Cell *37*, 415-427.

Grabowski, P. J., Seiler, S. R., and Sharp, P. A. (1985). A multicomponent complex is

involved in the splicing of messenger RNA precursors. Cell *42*, 345-353.

Graveley, B. R. (2000). Sorting out the complexity of SR protein functions. Rna *6*, 1197-1211.

Gregory, S. G., Sekhon, M., Schein, J., Zhao, S., Osoegawa, K., Scott, C. E., Evans, R. S., Burridge, P. W., Cox, T. V., Fox, C. A., *et al.* (2002). A physical map of the mouse genome. Nature *418*, 743-750.

Groisman, I., Huang, Y. S., Mendez, R., Cao, Q., Theurkauf, W., and Richter, J. D. (2000). CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. Cell *103*, 435-447.

Grolleau, A., Bowman, J., Pradet-Balade, B., Puravs, E., Hanash, S., Garcia-Sanz, J. A., and Beretta, L. (2002). Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics. J Biol Chem *277*, 22175-22184.

Gunther, U., Benson, J., Benke, D., Fritschy, J. M., Reyes, G., Knoflach, F., Crestani, F., Aguzzi, A., Arigoni, M., Lang, Y., and et al. (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A *92*, 7749-7753.

Guzowski, J. F., Lyford, G. L., Stevenson, G. D., Houston, F. P., McGaugh, J. L., Worley, P. F., and Barnes, C. A. (2000). Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci *20*, 3993-4001.

Hanamura, A., Caceres, J. F., Mayeda, A., Franza, B. R., Jr., and Krainer, A. R. (1998). Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. Rna *4*, 430-444.

Harkin, L. A., Bowser, D. N., Dibbens, L. M., Singh, R., Phillips, F., Wallace, R. H., Richards, M. C., Williams, D. A., Mulley, J. C., Berkovic, S. F., *et al.* (2002). Truncation of the GABA(A)-receptor gamma2 subunit in a family with generalized epilepsy with febrile seizures plus. Am J Hum Genet *70*, 530-536.

Hentze, M. W., and Kuhn, L. C. (1996). Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci U S A *93*, 8175-8182.

Hentze, M. W., and Kulozik, A. E. (1999). A perfect message: RNA surveillance and nonsense-mediated decay. Cell *96*, 307-310.

Herrera, F., Triana, L., and Bosch, I. (1988). Importance of polysomal mRNA-associated polypeptides for protein synthesis initiation in yeast. Eur J Biochem *175*, 87-92.

Hertel, K. J., and Maniatis, T. (1998). The function of multisite splicing enhancers. Mol Cell *1*, 449-455.

Hirose, Y., and Manley, J. L. (2000). RNA polymerase II and the integration of nuclear events. Genes Dev *14*, 1415-1429.

Hirose, Y., Tacke, R., and Manley, J. L. (1999). Phosphorylated RNA polymerase II stimulates pre-mRNA splicing. Genes Dev *13*, 1234-1239.

Holmberg, L., Melander, Y., and Nygard, O. (1994a). Probing the conformational changes in 5.8S, 18S and 28S rRNA upon association of derived subunits into complete 80S ribosomes. Nucleic Acids Res *22*, 2776-2783.

Holmberg, L., Melander, Y., and Nygard, O. (1994b). Probing the structure of mouse Ehrlich ascites cell 5.8S, 18S and 28S ribosomal RNA in situ. Nucleic Acids Res *22*, 1374-1382.

Homanics, G. E., Harrison, N. L., Quinlan, J. J., Krasowski, M. D., Rick, C. E., de Blas, A. L., Mehta, A. K., Kist, F., Mihalek, R. M., Aul, J. J., and Firestone, L. L. (1999). Normal electrophysiological and behavioral responses to ethanol in mice lacking the long splice variant of the gamma2 subunit of the gamma-aminobutyrate type A receptor. Neuropharmacology *38*, 253-265.

Hou, V. C., Lersch, R., Gee, S. L., Ponthier, J. L., Lo, A. J., Wu, M., Turck, C. W., Koury, M., Krainer, A. R., Mayeda, A., and Conboy, J. G. (2002). Decrease in hnRNP A/B expression during erythropoiesis mediates a pre-mRNA splicing switch. Embo J *21*, 6195-6204.

Howe, K. J. (2002). RNA polymerase II conducts a symphony of pre-mRNA processing activities. Biochim Biophys Acta *1577*, 308-324.

Huang, Y., and Steitz, J. A. (2001). Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. Mol Cell *7*, 899-905.

Huang, Y. S., Carson, J. H., Barbarese, E., and Richter, J. D. (2003). Facilitation of dendritic mRNA transport by CPEB. Genes Dev *17*, 638-653.

Huang, Y. S., Jung, M. Y., Sarkissian, M., and Richter, J. D. (2002). N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. Embo J *21*, 2139-2148.

Huber, K. M., Kayser, M. S., and Bear, M. F. (2000). Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. Science *288*, 1254-1257.

Iborra, F. J., Jackson, D. A., and Cook, P. R. (2001). Coupled transcription and translation within nuclei of mammalian cells. Science *293*, 1139-1142.

Iborra, F. J., Pombo, A., Jackson, D. A., and Cook, P. R. (1996). Active RNA polymerases are localized within discrete transcription "factories' in human nuclei. J Cell Sci *109 (Pt 6)*, 1427-1436.

Ishigaki, Y., Li, X., Serin, G., and Maquat, L. E. (2001). Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell *106*, 607-617.

Izaurralde, E., Jarmolowski, A., Beisel, C., Mattaj, I. W., Dreyfuss, G., and Fischer, U. (1997). A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. J Cell Biol *137*, 27-35.

Jensen, K. B., Dredge, B. K., Stefani, G., Zhong, R., Buckanovich, R. J., Okano, H. J., Yang, Y. Y., and Darnell, R. B. (2000a). Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. Neuron *25*, 359-371.

Jensen, K. B., Musunuru, K., Lewis, H. A., Burley, S. K., and Darnell, R. B. (2000b). The tetranucleotide UCAY directs the specific recognition of RNA by the Nova K-homology 3 domain. Proc Natl Acad Sci U S A *97*, 5740-5745.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet *1*, 20-29.

Ji, X., Kong, J., and Liebhaber, S. A. (2003). In vivo association of the stability control protein alphaCP with actively translating mRNAs. Mol Cell Biol *23*, 899-907.

Jin, Y., Suzuki, H., Maegawa, S., Endo, H., Sugano, S., Hashimoto, K., Yasuda, K., and Inoue, K. (2003). A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing via the pentanucleotide GCAUG. Embo J *22*, 905-912.

Johannes, G., Carter, M. S., Eisen, M. B., Brown, P. O., and Sarnow, P. (1999).

Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. Proc Natl Acad Sci U S A *96*, 13118-13123.

Johnstone, O., and Lasko, P. (2001). Translational regulation and RNA localization in Drosophila oocytes and embryos. Annu Rev Genet *35*, 365-406.

Jumaa, H., and Nielsen, P. J. (1997). The splicing factor SRp20 modifies splicing of its own mRNA and ASF/SF2 antagonizes this regulation. Embo J *16*, 5077-5085.

Kadener, S., Cramer, P., Nogues, G., Cazalla, D., de la Mata, M., Fededa, J. P., Werbajh, S. E., Srebrow, A., and Kornblihtt, A. R. (2001). Antagonistic effects of T-Ag and VP16 reveal a role for RNA pol II elongation on alternative splicing. Embo J *20*, 5759-5768.

Kadener, S., Fededa, J. P., Rosbash, M., and Kornblihtt, A. R. (2002). Regulation of alternative splicing by a transcriptional enhancer through RNA pol II elongation. Proc Natl Acad Sci U S A *99*, 8185-8190.

Kaminski, A., Hunt, S. L., Patton, J. G., and Jackson, R. J. (1995). Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. Rna *1*, 924-938.

Kaminski, A., and Jackson, R. J. (1998). The polypyrimidine tract binding protein (PTB) requirement for internal initiation of translation of cardiovirus RNAs is conditional rather than absolute. Rna *4*, 626-638.

Kang, H., and Schuman, E. M. (1996). A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science *273*, 1402-1406.

Kataoka, N., Yong, J., Kim, V. N., Velazquez, F., Perkinson, R. A., Wang, F., and Dreyfuss, G. (2000). Pre-mRNA splicing imprints mRNA in the nucleus with a novel

RNA-binding protein that persists in the cytoplasm. Mol Cell *6*, 673-682.

Keegan, L. P., Gallo, A., and O'Connell, M. A. (2001). The many roles of an RNA editor. Nat Rev Genet *2*, 869-878.

Keene, J. D. (2001). Ribonucleoprotein infrastructure regulating the flow of genetic information between the genome and the proteome. Proc Natl Acad Sci U S A *98*, 7018-7024.

Keene, J. D., and Tenenbaum, S. A. (2002). Eukaryotic mRNPs may represent posttranscriptional operons. Mol Cell *9*, 1161-1167.

Kelleher, D. J., and Gilmore, R. (1997). DAD1, the defender against apoptotic cell death, is a subunit of the mammalian oligosaccharyltransferase. Proc Natl Acad Sci U S A *94*, 4994-4999.

Kemp, B. E., and Pearson, R. B. (1990). Protein kinase recognition sequence motifs. Trends Biochem Sci *15*, 342-346.

Kennedy, M. B. (1998). Signal transduction molecules at the glutamatergic postsynaptic membrane. Brain Res Brain Res Rev *26*, 243-257.

Kennedy, M. B. (2000). Signal-processing machines at the postsynaptic density. Science *290*, 750-754.

Khandjian, E. W., Corbin, F., Woerly, S., and Rousseau, F. (1996). The fragile X mental retardation protein is associated with ribosomes. Nat Genet *12*, 91-93.

Kiledjian, M., Wang, X., and Liebhaber, S. A. (1995). Identification of two KH domain proteins in the alpha-globin mRNP stability complex. Embo J *14*, 4357-4364.

Kim, E., Naisbitt, S., Hsueh, Y. P., Rao, A., Rothschild, A., Craig, A. M., and Sheng, M. (1997). GKAP, a novel synaptic protein that interacts with the guanylate kinase-like

domain of the PSD-95/SAP90 family of channel clustering molecules. J Cell Biol *136*, 669-678.

Kim, V. N., Kataoka, N., and Dreyfuss, G. (2001). Role of the nonsense-mediated decay factor hUpf3 in the splicing-dependent exon-exon junction complex. Science *293*, 1832-1836.

Kim, Y. K., Hahm, B., and Jang, S. K. (2000). Polypyrimidine tract-binding protein inhibits translation of bip mRNA. J Mol Biol *304*, 119-133.

Koushika, S. P., Lisbin, M. J., and White, K. (1996). ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Curr Biol *6*, 1634-1641.

Koushika, S. P., Soller, M., and White, K. (2000). The neuron-enriched splicing pattern of Drosophila erect wing is dependent on the presence of ELAV protein. Mol Cell Biol *20*, 1836-1845.

Krainer, A. R., Conway, G. C., and Kozak, D. (1990). The essential pre-mRNA splicing factor SF2 influences 5' splice site selection by activating proximal sites. Cell *62*, 35-42.

Krainer, A. R., Maniatis, T., Ruskin, B., and Green, M. R. (1984). Normal and mutant human beta-globin pre-mRNAs are faithfully and efficiently spliced in vitro. Cell *36*, 993-1005.

Krichevsky, A. M., and Kosik, K. S. (2001). Neuronal RNA granules: a link between RNA localization and stimulation-dependent translation. Neuron *32*, 683-696.

Krichevsky, A. M., and Kosik, K. S. (2002). RNAi functions in cultured mammalian neurons. Proc Natl Acad Sci U S A *99*, 11926-11929.

Kuhse, J., Kuryatov, A., Maulet, Y., Malosio, M. L., Schmieden, V., and Betz, H. (1991).

Alternative splicing generates two isoforms of the alpha 2 subunit of the inhibitory glycine receptor. FEBS Lett *283*, 73-77.

Kullmann, M., Gopfert, U., Siewe, B., and Hengst, L. (2002). ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. Genes Dev *16*, 3087-3099.

Kwek, K. Y., Murphy, S., Furger, A., Thomas, B., O'Gorman, W., Kimura, H., Proudfoot, N. J., and Akoulitchev, A. (2002). U1 snRNA associates with TFIIH and regulates transcriptional initiation. Nat Struct Biol *9*, 800-805.

Kwon, S., Barbarese, E., and Carson, J. H. (1999). The cis-acting RNA trafficking signal from myelin basic protein mRNA and its cognate trans-acting ligand hnRNP A2 enhance cap-dependent translation. J Cell Biol *147*, 247-256.

Labourier, E., Blanchette, M., Feiger, J. W., Adams, M. D., and Rio, D. C. (2002). The KH-type RNA-binding protein PSI is required for Drosophila viability, male fertility, and cellular mRNA processing. Genes Dev *16*, 72-84.

Ladd, A. N., Charlet, N., and Cooper, T. A. (2001). The CELF family of RNA binding proteins is implicated in cell-specific and developmentally regulated alternative splicing. Mol Cell Biol *21*, 1285-1296.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., *et al.* (2001). Initial sequencing and analysis of the human genome. Nature *409*, 860-921.

Lang, B. D., Li, A., Black-Brewster, H. D., and Fridovich-Keil, J. L. (2001). The brefeldin A resistance protein Bfr1p is a component of polyribosome-associated mRNP complexes in yeast. Nucleic Acids Res *29*, 2567-2574.

Le Hir, H., Izaurralde, E., Maquat, L. E., and Moore, M. J. (2000a). The spliceosome

deposits multiple proteins 20-24 nucleotides upstream of mRNA exon-exon junctions. Embo J *19*, 6860-6869.

Le Hir, H., Moore, M. J., and Maquat, L. E. (2000b). Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon-exon junctions. Genes Dev *14*, 1098-1108.

Lee, M. S., Henry, M., and Silver, P. A. (1996). A protein that shuttles between the nucleus and the cytoplasm is an important mediator of RNA export. Genes Dev *10*, 1233-1246.

Lerner, E. A., Lerner, M. R., Janeway, C. A., Jr., and Steitz, J. A. (1981). Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc Natl Acad Sci U S A *78*, 2737-2741.

Leski, M. L., and Steward, O. (1996). Protein synthesis within dendrites: ionic and neurotransmitter modulation of synthesis of particular polypeptides characterized by gel electrophoresis. Neurochem Res *21*, 681-690.

Lewis, H. A., Chen, H., Edo, C., Buckanovich, R. J., Yang, Y. Y., Musunuru, K., Zhong, R., Darnell, R. B., and Burley, S. K. (1999). Crystal structures of Nova-1 and Nova-2 K-homology RNA-binding domains. Structure Fold Des *7*, 191-203.

Lewis, H. A., Musunuru, K., Jensen, K. B., Edo, C., Chen, H., Darnell, R. B., and Burley, S. K. (2000). Sequence-specific RNA binding by a Nova KH domain: implications for paraneoplastic disease and the fragile X syndrome. Cell *100*, 323-332.

Lim, L. P., and Sharp, P. A. (1998). Alternative splicing of the fibronectin EIIIB exon depends on specific TGCATG repeats. Mol Cell Biol *18*, 3900-3906.

Lisbin, M. J., Qiu, J., and White, K. (2001). The neuron-specific RNA-binding protein

ELAV regulates neuroglian alternative splicing in neurons and binds directly to its pre-mRNA. Genes Dev *15*, 2546-2561.

Luo, M. J., and Reed, R. (1999). Splicing is required for rapid and efficient mRNA export in metazoans. Proc Natl Acad Sci U S A *96*, 14937-14942.

Luo, M. L., Zhou, Z., Magni, K., Christoforides, C., Rappsilber, J., Mann, M., and Reed, R. (2001). Pre-mRNA splicing and mRNA export linked by direct interactions between UAP56 and Aly. Nature *413*, 644-647.

Lykke-Andersen, J., Shu, M. D., and Steitz, J. A. (2000). Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. Cell *103*, 1121-1131.

Lykke-Andersen, J., Shu, M. D., and Steitz, J. A. (2001). Communication of the position of exon-exon junctions to the mRNA surveillance machinery by the protein RNPS1. Science *293*, 1836-1839.

Lynch, K. W., and Maniatis, T. (1996). Assembly of specific SR protein complexes on distinct regulatory elements of the Drosophila doublesex splicing enhancer. Genes Dev *10*, 2089-2101.

Makeyev, A. V., and Liebhaber, S. A. (2002). The poly(C)-binding proteins: a multiplicity of functions and a search for mechanisms. Rna *8*, 265-278.

Malim, M. H., Bohnlein, S., Hauber, J., and Cullen, B. R. (1989). Functional dissection of the HIV-1 Rev trans-activator--derivation of a trans-dominant repressor of Rev function. Cell *58*, 205-214.

Malim, M. H., and Cullen, B. R. (1991). HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. Cell

65, 241-248.

Maniatis, T., and Reed, R. (2002). An extensive network of coupling among gene expression machines. Nature *416*, 499-506.

Maniatis, T., and Tasic, B. (2002). Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature *418*, 236-243.

Manley, G. T., Smitt, P. S., Dalmau, J., and Posner, J. B. (1995). Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites. Ann Neurol *38*, 102-110.

Maquat, L. E. (2002). NASty effects on fibrillin pre-mRNA splicing: another case of ESE does it, but proposals for translation-dependent splice site choice live on. Genes Dev *16*, 1743-1753.

Maquat, L. E., and Carmichael, G. G. (2001). Quality control of mRNA function. Cell *104*, 173-176.

Marchand, V., Mereau, A., Jacquenet, S., Thomas, D., Mougin, A., Gattoni, R., Stevenin, J., and Branlant, C. (2002). A Janus splicing regulatory element modulates HIV-1 tat and rev mRNA production by coordination of hnRNP A1 cooperative binding. J Mol Biol *323*, 629-652.

Marini, C., Harkin, L. A., Wallace, R. H., Mulley, J. C., Scheffer, I. E., and Berkovic, S. F. (2003). Childhood absence epilepsy and febrile seizures: a family with a GABA(A) receptor mutation. Brain *126*, 230-240.

Markovtsov, V., Nikolic, J. M., Goldman, J. A., Turck, C. W., Chou, M. Y., and Black, D. L. (2000). Cooperative assembly of an hnRNP complex induced by a tissue-specific homolog of polypyrimidine tract binding protein. Mol Cell Biol *20*, 7463-7479.

Martin, K. C., Casadio, A., Zhu, H., Yaping, E., Rose, J. C., Chen, M., Bailey, C. H., and Kandel, E. R. (1997). Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. Cell *91*, 927-938.

Matsumoto, K., Wassarman, K. M., and Wolffe, A. P. (1998). Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA. Embo J *17*, 2107-2121.

Mayeda, A., and Krainer, A. R. (1992). Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. Cell *68*, 365-375.

Mayeda, A., Munroe, S. H., Caceres, J. F., and Krainer, A. R. (1994). Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. Embo J *13*, 5483-5495.

McCracken, S., Fong, N., Rosonina, E., Yankulov, K., Brothers, G., Siderovski, D., Hessel, A., Foster, S., Shuman, S., and Bentley, D. L. (1997a). 5'-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II. Genes Dev *11*, 3306-3318.

McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. (1997b). The C-terminal domain of RNA polymerase II couples mRNA processing to transcription. Nature *385*, 357-361.

McFarlane, S. (2003). Metalloproteases: carving out a role in axon guidance. Neuron *37*, 559-562.

McPherson, J. D., Marra, M., Hillier, L., Waterston, R. H., Chinwalla, A., Wallis, J., Sekhon, M., Wylie, K., Mardis, E. R., Wilson, R. K., *et al.* (2001). A physical map of the human genome. Nature *409*, 934-941.

Mendez, R., and Richter, J. D. (2001). Translational control by CPEB: a means to the

end. Nat Rev Mol Cell Biol *2*, 521-529.

Michael, W. M., Choi, M., and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. Cell *83*, 415-422.

Miller, S., Yasuda, M., Coats, J. K., Jones, Y., Martone, M. E., and Mayford, M. (2002). Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. Neuron *36*, 507-519.

Min, H., Turck, C. W., Nikolic, J. M., and Black, D. L. (1997). A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. Genes Dev *11*, 1023-1036.

Missler, M., Fernandez-Chacon, R., and Sudhof, T. C. (1998). The making of neurexins. J Neurochem *71*, 1339-1347.

Missler, M., and Sudhof, T. C. (1998). Neurexins: three genes and 1001 products. Trends Genet *14*, 20-26.

Misteli, T., and Spector, D. L. (1999). RNA polymerase II targets pre-mRNA splicing factors to transcription sites in vivo. Mol Cell *3*, 697-705.

Modrek, B., and Lee, C. (2002). A genomic view of alternative splicing. Nat Genet *30*, 13-19.

Modrek, B., Resch, A., Grasso, C., and Lee, C. (2001). Genome-wide detection of alternative splicing in expressed sequences of human genes. Nucleic Acids Res *29*, 2850-2859.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., Edmondson, J., and Axel, R. (1996). Visualizing an olfactory sensory map. Cell *87*, 675-

686.

Mortillaro, M. J., Blencowe, B. J., Wei, X., Nakayasu, H., Du, L., Warren, S. L., Sharp, P. A., and Berezney, R. (1996). A hyperphosphorylated form of the large subunit of RNA polymerase II is associated with splicing complexes and the nuclear matrix. Proc Natl Acad Sci U S A *93*, 8253-8257.

Musunuru, K., and Darnell, R. B. (2001). Paraneoplastic neurologic disease antigens: RNA-binding proteins and signaling proteins in neuronal degeneration. Annu Rev Neurosci *24*, 239-262.

Nakielny, S., and Dreyfuss, G. (1999). Transport of proteins and RNAs in and out of the nucleus. Cell *99*, 677-690.

Niepmann, M. (1996). Porcine polypyrimidine tract-binding protein stimulates translation initiation at the internal ribosome entry site of foot-and-mouth-disease virus. FEBS Lett *388*, 39-42.

Niessing, D., Dostatni, N., Jackle, H., and Rivera-Pomar, R. (1999). Sequence interval within the PEST motif of Bicoid is important for translational repression of caudal mRNA in the anterior region of the Drosophila embryo. Embo J *18*, 1966-1973.

Nogues, G., Kadener, S., Cramer, P., Bentley, D., and Kornblihtt, A. R. (2002). Transcriptional activators differ in their abilities to control alternative splicing. J Biol Chem *277*, 43110-43114.

Okano, H. J., and Darnell, R. B. (1997). A hierarchy of Hu RNA binding proteins in developing and adult neurons. J Neurosci *17*, 3024-3037.

Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., Nikaido, I., Osato, N., Saito, R., Suzuki, H., *et al.* (2002). Analysis of the mouse transcriptome based

on functional annotation of 60,770 full-length cDNAs. Nature *420*, 563-573.

Osheim, Y. N., and Beyer, A. L. (1991). EM analysis of Drosophila chorion genes: amplification, transcription termination and RNA splicing. Electron Microsc Rev *4*, 111-128.

Ostareck, D. H., Ostareck-Lederer, A., Shatsky, I. N., and Hentze, M. W. (2001). Lipoxygenase mRNA silencing in erythroid differentiation: The 3'UTR regulatory complex controls 60S ribosomal subunit joining. Cell *104*, 281-290.

Ostareck, D. H., Ostareck-Lederer, A., Wilm, M., Thiele, B. J., Mann, M., and Hentze, M. W. (1997). mRNA silencing in erythroid differentiation: hnRNP K and hnRNP E1 regulate 15-lipoxygenase translation from the 3' end. Cell *89*, 597-606.

Ostareck-Lederer, A., Ostareck, D. H., and Hentze, M. W. (1998). Cytoplasmic regulatory functions of the KH-domain proteins hnRNPs K and E1/E2. Trends Biochem Sci *23*, 409-411.

Ouyang, Y., Rosenstein, A., Kreiman, G., Schuman, E. M., and Kennedy, M. B. (1999). Tetanic stimulation leads to increased accumulation of Ca(2+)/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons. J Neurosci *19*, 7823-7833.

Palmiter, R. D., Sandgren, E. P., Avarbock, M. R., Allen, D. D., and Brinster, R. L. (1991). Heterologous introns can enhance expression of transgenes in mice. Proc Natl Acad Sci U S A *88*, 478-482.

Pickering, B. M., Mitchell, S. A., Evans, J. R., and Willis, A. E. (2003). Polypyrimidine tract binding protein and poly r(C) binding protein 1 interact with the BAG-1 IRES and stimulate its activity in vitro and in vivo. Nucleic Acids Res *31*, 639-646.

Pinol-Roma, S., and Dreyfuss, G. (1991). Transcription-dependent and transcription-independent nuclear transport of hnRNP proteins. Science *253*, 312-314.

Pinol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. Nature *355*, 730-732.

Pollard, A. J., Krainer, A. R., Robson, S. C., and Europe-Finner, G. N. (2002). Alternative splicing of the adenylyl cyclase stimulatory G-protein G alpha(s) is regulated by SF2/ASF and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and involves the use of an unusual TG 3'-splice Site. J Biol Chem *277*, 15241-15251.

Polydorides, A. D., Okano, H. J., Yang, Y. Y., Stefani, G., and Darnell, R. B. (2000). A brain-enriched polypyrimidine tract-binding protein antagonizes the ability of Nova to regulate neuron-specific alternative splicing. Proc Natl Acad Sci U S A *97*, 6350-6355.

Posner, J. B. (1992). Pathogenesis of central nervous system paraneoplastic syndromes. Rev Neurol (Paris) *148*, 502-512.

Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Kettenmann, H., Schofield, P. R., and Seeburg, P. H. (1989). Importance of a novel GABAA receptor subunit for benzodiazepine pharmacology. Nature *338*, 582-585.

Proudfoot, N. J., Furger, A., and Dye, M. J. (2002). Integrating mRNA processing with transcription. Cell *108*, 501-512.

Quinlan, J. J., Firestone, L. L., and Homanics, G. E. (2000). Mice lacking the long splice variant of the gamma 2 subunit of the GABA(A) receptor are more sensitive to benzodiazepines. Pharmacol Biochem Behav *66*, 371-374.

Ramos, A., Hollingworth, D., Major, S. A., Adinolfi, S., Kelly, G., Muskett, F. W., and Pastore, A. (2002). Role of dimerization in KH/RNA complexes: the example of Nova

KH3. Biochemistry *41*, 4193-4201.

Raught, B., Gingras, A. C., and Sonenberg, N. (2001). The target of rapamycin (TOR) proteins. Proc Natl Acad Sci U S A *98*, 7037-7044.

Rechsteiner, M., and Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem Sci *21*, 267-271.

Reed, R., and Hurt, E. (2002). A conserved mRNA export machinery coupled to pre-mRNA splicing. Cell *108*, 523-531.

Reed, R., and Magni, K. (2001). A new view of mRNA export: separating the wheat from the chaff. Nat Cell Biol *3*, E201-204.

Roberts, G. C., Gooding, C., Mak, H. Y., Proudfoot, N. J., and Smith, C. W. (1998). Co-transcriptional commitment to alternative splice site selection. Nucleic Acids Res *26*, 5568-5572.

Ruskin, B., and Green, M. R. (1985). An RNA processing activity that debranches RNA lariats. Science *229*, 135-140.

Ruskin, B., and Green, M. R. (1990). RNA lariat debranching enzyme as tool for analyzing RNA structure. Methods Enzymol *181*, 180-188.

Ruskin, B., Krainer, A. R., Maniatis, T., and Green, M. R. (1984). Excision of an intact intron as a novel lariat structure during pre-mRNA splicing in vitro. Cell *38*, 317-331.

Satoh, K., Yanai, H., Senda, T., Kohu, K., Nakamura, T., Okumura, N., Matsumine, A., Kobayashi, S., Toyoshima, K., and Akiyama, T. (1997). DAP-1, a novel protein that interacts with the guanylate kinase-like domains of hDLG and PSD-95. Genes Cells *2*, 415-424.

Scheetz, A. J., Nairn, A. C., and Constantine-Paton, M. (2000). NMDA receptor-

mediated control of protein synthesis at developing synapses. Nat Neurosci *3*, 211-216.

Schell, T., Kulozik, A. E., and Hentze, M. W. (2002). Integration of splicing, transport and translation to achieve mRNA quality control by the nonsense-mediated decay pathway. Genome Biol *3*, REVIEWS1006.

Schmucker, D., Clemens, J. C., Shu, H., Worby, C. A., Xiao, J., Muda, M., Dixon, J. E., and Zipursky, S. L. (2000). Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell *101*, 671-684.

Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J., Luhrmann, R., and Hurt, E. (1997). Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores. Embo J *16*, 3256-3271.

Shyu, A. B., and Wilkinson, M. F. (2000). The double lives of shuttling mRNA binding proteins. Cell *102*, 135-138.

Silberstein, S., Kelleher, D. J., and Gilmore, R. (1992). The 48-kDa subunit of the mammalian oligosaccharyltransferase complex is homologous to the essential yeast protein WBP1. J Biol Chem *267*, 23658-23663.

Singer, J. H., Talley, E. M., Bayliss, D. A., and Berger, A. J. (1998). Development of glycinergic synaptic transmission to rat brain stem motoneurons. J Neurophysiol *80*, 2608-2620.

Siomi, H., Matunis, M. J., Michael, W. M., and Dreyfuss, G. (1993). The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. Nucleic Acids Res *21*. 1193-1198.

Sisodia, S. S., Sollner-Webb, B., and Cleveland, D. W. (1987). Specificity of RNA maturation pathways: RNAs transcribed by RNA polymerase III are not substrates for

splicing or polyadenylation. Mol Cell Biol *7*, 3602-3612.

Smith, P. J., Spurrell, E. L., Coakley, J., Hinds, C. J., Ross, R. J., Krainer, A. R., and Chew, S. L. (2002). An exonic splicing enhancer in human IGF-I pre-mRNA mediates recognition of alternative exon 5 by the serine-arginine protein splicing factor-2/alternative splicing factor. Endocrinology *143*, 146-154.

Spector, D. L. (2001). Nuclear domains. J Cell Sci *114*, 2891-2893.

Spingola, M., and Ares, M., Jr. (2000). A yeast intronic splicing enhancer and Nam8p are required for Mer1p-activated splicing. Mol Cell *6*, 329-338.

Stamm, S. (2002). Signals and their transduction pathways regulating alternative splicing: a new dimension of the human genome. Hum Mol Genet *11*, 2409-2416.

Stamm, S., Zhang, M. Q., Marr, T. G., and Helfman, D. M. (1994). A sequence compilation and comparison of exons that are alternatively spliced in neurons. Nucleic Acids Res *22*, 1515-1526.

Stamm, S., Zhu, J., Nakai, K., Stoilov, P., Stoss, O., and Zhang, M. Q. (2000). An alternative-exon database and its statistical analysis. DNA Cell Biol *19*, 739-756.

Steward, O., and Levy, W. B. (1982). Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. J Neurosci *2*, 284-291.

Steward, O., and Schuman, E. M. (2001). Protein synthesis at synaptic sites on dendrites. Annu Rev Neurosci *24*, 299-325.

Steward, O., Wallace, C. S., Lyford, G. L., and Worley, P. F. (1998). Synaptic activation causes the mRNA for the IEG Arc to localize selectively near activated postsynaptic sites on dendrites. Neuron *21*, 741-751.

Steward, O., and Worley, P. F. (2001). Selective targeting of newly synthesized Arc

mRNA to active synapses requires NMDA receptor activation. Neuron *30*, 227-240.

Strasser, K., and Hurt, E. (2001). Splicing factor Sub2p is required for nuclear mRNA export through its interaction with Yra1p. Nature *413*, 648-652.

Strasser, K., Masuda, S., Mason, P., Pfannstiel, J., Oppizzi, M., Rodriguez-Navarro, S., Rondon, A. G., Aguilera, A., Struhl, K., Reed, R., and Hurt, E. (2002). TREX is a conserved complex coupling transcription with messenger RNA export. Nature *417*, 304-308.

Sun, X., Perlick, H. A., Dietz, H. C., and Maquat, L. E. (1998). A mutated human homologue to yeast Upf1 protein has a dominant-negative effect on the decay of nonsense-containing mRNAs in mammalian cells. Proc Natl Acad Sci U S A *95*, 10009-10014.

Suzuki, H., Jin, Y., Otani, H., Yasuda, K., and Inoue, K. (2002). Regulation of alternative splicing of alpha-actinin transcript by Bruno-like proteins. Genes Cells *7*, 133-141.

Tabuchi, K., and Sudhof, T. C. (2002). Structure and evolution of neurexin genes: insight into the mechanism of alternative splicing. Genomics *79*, 849-859.

Tang, S. J., Reis, G., Kang, H., Gingras, A. C., Sonenberg, N., and Schuman, E. M. (2002). A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. Proc Natl Acad Sci U S A *99*, 467-472.

Tasic, B., Nabholz, C. E., Baldwin, K. K., Kim, Y., Rueckert, E. H., Ribich, S. A., Cramer, P., Wu, Q., Axel, R., and Maniatis, T. (2002). Promoter choice determines splice site selection in protocadherin alpha and gamma pre-mRNA splicing. Mol Cell *10*, 21-33.

Tenenbaum, S. A., Carson, C. C., Lager, P. J., and Keene, J. D. (2000). Identifying mRNA subsets in messenger ribonucleoprotein complexes by using cDNA arrays. Proc

Natl Acad Sci U S A *97*, 14085-14090.

Therrien, M., Wong, A. M., Kwan, E., and Rubin, G. M. (1999). Functional analysis of CNK in RAS signaling. Proc Natl Acad Sci U S A *96*, 13259-13263.

Therrien, M., Wong, A. M., and Rubin, G. M. (1998). CNK, a RAF-binding multidomain protein required for RAS signaling. Cell *95*, 343-353.

Toba, G., Qui, J., Koushika, S. P., and White, K. (2002). Ectopic expression of Drosophila ELAV and human HuD in Drosophila wing disc cells reveals functional distinctions and similarities. J Cell Sci *115*, 2413-2421.

Valcarcel, J., and Gebauer, F. (1997). Post-transcriptional regulation: the dawn of PTB. Curr Biol *7*, R705-708.

Valcarcel, J., Singh, R., Zamore, P. D., and Green, M. R. (1993). The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. Nature *362*, 171-175.

Van Buskirk, C., and Schupbach, T. (2002). Half pint regulates alternative splice site selection in Drosophila. Dev Cell *2*, 343-353.

van der Flier, A., and Sonnenberg, A. (2001). Structural and functional aspects of filamins. Biochim Biophys Acta *1538*, 99-117.

van der Houven van Oordt, W., Diaz-Meco, M. T., Lozano, J., Krainer, A. R., Moscat, J., and Caceres, J. F. (2000). The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. J Cell Biol *149*, 307-316.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., *et al.* (2001). The sequence of the human

genome. Science *291*, 1304-1351.

Visa, N., Alzhanova-Ericsson, A. T., Sun, X., Kiseleva, E., Bjorkroth, B., Wurtz, T., and Daneholt, B. (1996). A pre-mRNA-binding protein accompanies the RNA from the gene through the nuclear pores and into polysomes. Cell *84*, 253-264.

Wallace, R. H., Marini, C., Petrou, S., Harkin, L. A., Bowser, D. N., Panchal, R. G., Williams, D. A., Sutherland, G. R., Mulley, J. C., Scheffer, I. E., and Berkovic, S. F. (2001). Mutant GABA(A) receptor gamma2-subunit in childhood absence epilepsy and febrile seizures. Nat Genet *28*, 49-52.

Wang, X., Kiledjian, M., Weiss, I. M., and Liebhaber, S. A. (1995). Detection and characterization of a 3' untranslated region ribonucleoprotein complex associated with human alpha-globin mRNA stability. Mol Cell Biol *15*, 1769-1777.

Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P., Agarwala, R., Ainscough, R., Alexandersson, M., An, P., *et al.* (2002). Initial sequencing and comparative analysis of the mouse genome. Nature *420*, 520-562.

Wells, D. G., Dong, X., Quinlan, E. M., Huang, Y. S., Bear, M. F., Richter, J. D., and Fallon, J. R. (2001). A role for the cytoplasmic polyadenylation element in NMDA receptor-regulated mRNA translation in neurons. J Neurosci *21*, 9541-9548.

Wickens, M., Elizabeth B. Goodwin, Judith Kimble, Sidney Strickland, and Matthias Hentze. Translational Control in Developmental Decisions. In Translational Control Second Edition. (Michael Mathews, ed.) Cold Spring Harbor Press, New York.

Wilkinson, M. F., and Shyu, A. B. (2002). RNA surveillance by nuclear scanning? Nat Cell Biol *4*, E144-147.

Will, C. L., and Luhrmann, R. (2001). Spliceosomal UsnRNP biogenesis, structure and

function. Curr Opin Cell Biol *13*, 290-301.

Wilson, J. E., Connell, J. E., and Macdonald, P. M. (1996). aubergine enhances oskar translation in the Drosophila ovary. Development *122*, 1631-1639.

Wolff, B., Sanglier, J. J., and Wang, Y. (1997). Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem Biol *4*, 139-147.

Wu, L., Wells, D., Tay, J., Mendis, D., Abbott, M. A., Barnitt, A., Quinlan, E., Heynen, A., Fallon, J. R., and Richter, J. D. (1998). CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. Neuron *21*, 1129-1139.

Wu, Q., and Maniatis, T. (2000). Large exons encoding multiple ectodomains are a characteristic feature of protocadherin genes. Proc Natl Acad Sci U S A *97*, 3124-3129.

Wu, Q., Zhang, T., Cheng, J. F., Kim, Y., Grimwood, J., Schmutz, J., Dickson, M., Noonan, J. P., Zhang, M. Q., Myers, R. M., and Maniatis, T. (2001). Comparative DNA sequence analysis of mouse and human protocadherin gene clusters. Genome Res *11*, 389-404.

Xie, J., and Black, D. L. (2001). A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. Nature *410*, 936-939.

Xu, Q., Modrek, B., and Lee, C. (2002). Genome-wide detection of tissue-specific alternative splicing in the human transcriptome. Nucleic Acids Res *30*, 3754-3766.

Xu, R., Teng, J., and Cooper, T. A. (1993). The cardiac troponin T alternative exon contains a novel purine-rich positive splicing element. Mol Cell Biol *13*, 3660-3674.

Yamamoto, Y. Y., Tsuji, H., and Obokata, J. (1995). 5'-leader of a photosystem I gene in

Nicotiana sylvestris, psaDb, contains a translational enhancer. J Biol Chem *270*, 12466-12470.

Yang, Y. Y., Yin, G. L., and Darnell, R. B. (1998). The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. Proc Natl Acad Sci U S A *95*, 13254-13259.

Yao, I., Hata, Y., Ide, N., Hirao, K., Deguchi, M., Nishioka, H., Mizoguchi, A., and Takai, Y. (1999). MAGUIN, a novel neuronal membrane-associated guanylate kinase-interacting protein. J Biol Chem *274*, 11889-11896.

Yong, V. W., Power, C., Forsyth, P., and Edwards, D. R. (2001). Metalloproteinases in biology and pathology of the nervous system. Nat Rev Neurosci *2*, 502-511.

Yuryev, A., Patturajan, M., Litingtung, Y., Joshi, R. V., Gentile, C., Gebara, M., and Corden, J. L. (1996). The C-terminal domain of the largest subunit of RNA polymerase II interacts with a novel set of serine/arginine-rich proteins. Proc Natl Acad Sci U S A *93*, 6975-6980.

Zhang, G., Taneja, K. L., Singer, R. H., and Green, M. R. (1994). Localization of pre-mRNA splicing in mammalian nuclei. Nature *372*, 809-812.

Zhang, L., Ashiya, M., Sherman, T. G., and Grabowski, P. J. (1996). Essential nucleotides direct neuron-specific splicing of gamma 2 pre-mRNA. Rna *2*, 682-698.

Zhang, M., and Green, M. R. (2001). Identification and characterization of yUAP/Sub2p, a yeast homolog of the essential human pre-mRNA splicing factor hUAP56. Genes Dev *15*, 30-35.

Zhang, W., Liu, H., Han, K., and Grabowski, P. J. (2002). Region-specific alternative splicing in the nervous system: implications for regulation by the RNA-binding protein

NAPOR. Rna *8*, 671-685.

Zhou, Z., Luo, M. J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000). The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans. Nature *407*, 401-405.

Zhu, D., Xu, G., Ghandhi, S., and Hubbard, K. (2002). Modulation of the expression of p16INK4a and p14ARF by hnRNP A1 and A2 RNA binding proteins: implications for cellular senescence. J Cell Physiol *193*, 19-25.

Zhu, J., Mayeda, A., and Krainer, A. R. (2001). Exon identity established through differential antagonism between exonic splicing silencer-bound hnRNP A1 and enhancer-bound SR proteins. Mol Cell *8*, 1351-1361.

Zong, Q., Schummer, M., Hood, L., and Morris, D. R. (1999). Messenger RNA translation state: the second dimension of high-throughput expression screening. Proc Natl Acad Sci U S A *96*, 10632-10636.