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Interactions between Nova and brPTB Proteins in Alternative Splicing and Nuclear Localization

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Γιτσα, Νικο και Χριστινα, σας αγαπω πολυ πολυ.

Table of Contents

List of Tables and Figures

Abbreviations

aa: amino acid

ALS: Amyotrophic Lateral Sclerosis

ARE: AU-Rich Element

ASF/SF2: Associated Splicing Factor/Splicing Factor 2

BBP: Branch point-Binding Protein

BGH: Bovine Growth Hormone

brPTB: brain-enriched Polypyrimidine Tract-Binding protein

CAMKIIα: Calcium calmodulin-dependent protein Kinase type II, alpha subunit

CBC: Cap Binding Complex

CELF: CUG-BP and ETR-3-Like Factors

CGRP: Calcitonin Gene-Related Peptide

CMV: Cytomegalovirus

CNS: Central Nervous System

CTD: C-Terminal Domain

cTNT: cardiac Troponin-T

CUG-BP: CUG-Binding Protein

DCS: Downstream Control Sequence

DICE: Differentiation Control Element

DM: Myotonic Dystrophy

E (n): embryonic day

EAAT2: Excitatory Amino Acid Trasporter 2

EJC: Exon-exon Junction Complex

Elav: Embryonic Lethal Abnormal Visual system

ER: Endoplasmic Reticulum

ERK: Extracellular signal-Regulated Kinase

ESE: Exonic Splicing Enhancer sequence

ESS: Exonic Splicing Silencer sequence

ETR: Elav-Type RNA-binding protein

FGFR2: fibroblast growth factor receptor 2

FTDP-17: Frontotemporal Dementia and Parkinsonism linked to chromosome 17

FMR: Fragile X/Mental Retardation

FBP: Fuse-Binding Protein

$GABA_AR\gamma2$: $GABA_A$ receptor $\gamma2$ subunit

GAD: Gal4 Activation Domain

GFP: Green Fluorescent Protein

GlyR$\alpha2$: glycine receptor $\alpha2$ subunit

GST: Glutathione S-transferase

HIV: Human Immunodefficiency Virus

HNS: HuR Nuclear Shuttling Domain

hnRNP: heterogeneous nuclear ribonucleoprotein

IRES: Internal Ribosomal Entry Site

ISE: Intronic Splicing Enhancer sequence

ISS: Intronic Splicing Silencer sequence

kDa: kilo Dalton

KH: hnRNP K Homology

KNS: hnRNP K Nuclear Shuttling domain

KSRP: KH-type Splicing Regulatory Protein

LOX: erythroid 15-lipoxygenase

MAP2: Microtubule-Associated Protein 2

MBP: Myelin Basic Protein

mRNA: messenger RNA

MSE: Muscle-specific Splicing Enhancer

NES: Nuclear Export Signal

NLS: Nuclear Localization Signal

NMDA: *N*-methyl-D-aspartate

NPC: Nuclear Pore Complex

PABP: Poly(A)-Binding Protein

PCR: Polymerase Chain Reaction

PML: Promyelocytic Leukaemia

PNC: PeriNucleolar Compartment

PND: Paraneoplastic Neurologic Disease

POMA: Paraneoplastic Opsoclonus Myoclonus Ataxia

PSF: PTB-associated Splicing Factor

PSI: P-element Somatic Inhibitor

PTB: Polypyrimidine Tract-Binding protein

RBD: RNA-Binding Domain

RBP: RNA-Binding Protein

RNP: RiboNucleoProtein (consensus sequence or motif)

RRE: Rev Response Element

RRM: RNA-Recognition Motif

rRNA: ribosomal RNA

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction assay

SF1: Splicing Factor 1

SIP: SMN-Interacting Protein

SLE: Systemic Lupus Erythematosus

SMA: Spinal Muscular Atrophy

SMN: Survival of Motor Neurons

snRNP: small nuclear RiboNucleoProtein

snRNA: small nuclear RNA

SR: Serine-Arginine

Sxl: Sex lethal protein

TRF: Telomeric Repeat Factor

UTR: Untranslated Region

VP: Vasopressin Precursor protein

ZBP: Zipcode-Binding Protein

Abstract

The Nova paraneoplastic antigens are neuron-specific RNA-binding proteins that are essential for neuronal viability and participate in the control of alternative splicing. In this study, the yeast two-hybrid system is used to isolate Nova-interacting proteins. A novel RNA-binding protein named brPTB is identified, that is closely related to the poly-pyrimidine tract-binding protein (PTB) and is enriched in the brain at the mRNA and protein levels. brPTB interacts with Nova proteins *in vitro* and *in vivo*. Splicing assays in kidney epithelial cell lines show that brPTB inhibits the effect of Nova1 in the inclusion of alternatively spliced exons in two target pre-mRNAs: glycine receptor α2 subunit (GlyRα2) and $GABA_A$ receptor γ2 subunit ($GABA_AR\gamma2$). Furthermore, in the case of GlyRα2, brPTB binds to a site adjacent to Nova1 in a 90 nucleotide fragment of intronic RNA upstream of the alternatively spliced exon, but with an affinity more than 10-fold lower than Nova1. When the brPTB site is mutated, binding is abolished and the inhibitory effect on Nova1-dependent exon inclusion disappears. In addition, it is shown that the inhibitory effect of brPTB on Nova1 splicing does not occur in neuronal cells. These results suggest that brPTB is a tissue-restricted RNA-binding protein that specifically interacts with Nova and inhibits its ability to activate exon selection. Nova proteins localize in distinct nuclear foci, but fail to co-localize with any of the known proteins that occupy sub-nuclear structural domains. Both endogenous and transfected brPTB and Nova proteins co-localize in these foci in neuroblastoma cell lines but not in non-neuronal cells. A model is proposed whereby alternative splicing and nuclear localization mediated by Nova and brPTB are linked as the consequence of their protein interaction in specific cell types.

Chapter 1 – General Introduction

RNA-binding proteins and RNA-binding domains

In eukaryotic cells, the path of RNA molecules, from DNA transcribed in the nucleus to protein synthesized in the cytoplasm, encompasses a multitude of steps, including capping, polyadenylation, splicing, editing, nuclear export, localization, translation, stability and, finally, turnover. The existence of these separate biochemical stages in the metabolism of RNA provides the distinct opportunity for regulation at different levels and with broad outcomes. The effectors of these processes are RNA-binding proteins (RBPs) consisting of diverse families of proteins that mediate their actions through direct binding to RNA targets.

Amino acid sequence analysis and phylogenetic data on a plethora of existing RBPs has resulted in the recognition of numerous sub-families, each with distinct features most notable of which are the various RNA-binding motifs (Burd and Dreyfuss, 1994). More recently, structural information has provided concrete evidence for the mechanism of RNA binding and its implications in the proposed functional role of these proteins. A review of RBP families and their most prominent members is therefore warranted before any detailed examination of their cellular function. An informative way to categorize these RBPs is based on the amino acid sequence similarities and predicted functional significance of their RNA-binding motifs (Burd and Dreyfuss, 1994; Varani and Nagai, 1998).

RRM-type RNA-binding proteins

Newly transcribed RNAs in the nucleus, called heterogeneous nuclear RNAs (hnRNAs), exist in complexes with proteins, forming heterogeneous nuclear ribonucleoprotein particles (hnRNPs; Dreyfuss et al., 1993; Matunis et al., 1993). Early studies have identified a number of proteins involved in the assembly of these particles and have determined, albeit crudely, the sequence requirements for their RNA binding by ribohomopolymer binding assays (Dreyfuss et al., 1988; Swanson and Dreyfuss, 1988). Comparisons of the sequence and overall structural organization of different hnRNP proteins established common motifs that were hypothesized to bind RNA, and led to the identification of the RNA Recognition Motif (RRM) that, since then, has been recognized as the most prevalent RNA-binding domain (Swanson et al., 1987; Siomi and Dreyfuss, 1997).

The RRM motif was first identified as a repeated domain in the sequence of the hnRNP protein A1 and the polyadenylate binding protein (PABP; Adam et al., 1986). Each RRM (also called RNP, for ribonucleoprotein motif) consists of 80-100 amino acids that comprises of a secondary structure with two α helices and four β sheets separated by loops of variable length (Nagai et al., 1990). The amino acid sequences of the first and third β sheets constitute the most conserved elements of the RRM and have been named RNP2 and RNP1, respectively (Burd and Dreyfuss, 1994; see also Figure 2). The remainder of the RRM domain contains many conserved hydrophobic residues that are thought to mediate proper folding but play a small role in actual RNA-binding specificity (Birney et al., 1993). The sequence specificity of RNA-binding and, ultimately, the function of these RRM-containing proteins have been shown to differ substantially (see

below). Nevertheless, the evolutionary conservation of several residues and their similar secondary structure suggested that these proteins share common folding properties and similar protein-RNA interface features (Kenan et al., 1991).

When the crystal structure of the first RRM domain was solved (from the splicing factor U1A snRNP), it was found that the highly conserved RNP2 and RNP1 segments form β sheets that lie juxtaposed and exposed to solvent, available for contact with RNA molecules (Nagai et al., 1990). Co-crystal structures of the RRM domains from this and other proteins bound to RNAs (with or without intramolecular base-pairing) have determined the specific characteristics of RNA-protein recognition and have provided insight into the mechanism guiding sequence-specific RNA binding (Oubridge et al., 1994; Handa et al., 1999).

From these and other studies, a general model for RNA binding by RRM domains has emerged that involves a general recognition of the RNA molecule by the charged and aromatic amino acid residues of RNP2 and RNP1, and a more sequence-specific interaction with the variable loop element before RNP1 (Burd and Dreyfuss, 1994). Added complexity and specificity can be achieved when proteins that contain more than one RRM domains (such as the translation regulator PABP, the splicing factor $U2AF^{65}$ and the hnRNP protein A1) require their contiguous RNA-binding motifs for proper recognition of the same RNA molecule (Nietfeld et al. 1990; Zamore et al., 1992). Interestingly, other proteins that contain multiple RRMs may bind distinct RNA targets with each RNA-binding domain and perform different functions. For instance, the U1A snRNP protein has been shown through UV cross-linking and immunoprecipitation assays to bind to the U1 snRNA through the first RRM and to a polyadenylation signal

via its second RRM, suggesting that it plays a role in both splicing and polyadenylation and perhaps providing a link between the two processes (Lutz and Alwine, 1994).

Members of the RRM-type of RBPs include a variety of proteins with different RNA-binding specificities and diverse functions in RNA metabolism. For example, hnRNP A1 contains two RRM domains and has been shown to influence 5' splice site selection *in vitro* (Ge and Manley, 1990; Mayeda and Krainer, 1992). Furthermore, it has been suggested to compete with another RRM-containing protein (ASF/SF2) that favors more proximal 5' splice sites. These results have also been verified by *in vivo* overexpression/RT-PCR splicing assays (Caceres et al., 1994). Sex lethal is a Drosophila RRM-containing protein that activates a female-specific splicing switch by binding to a polypyrimidine tract upstream of a repressed exon. This is hypothesized to block the binding of another RRM protein, $U2AF^{65}$, an essential splicing activator that is now free to activate the lower-affinity female-specific site (Valcarcel et al., 1993).

The hnRNP I protein, also known as the polypyrimidine tract binding protein (PTB), was first identified as a protein that binds the polypyrimidine tract that typically precedes 3' splice sites and was later recognized as an hnRNP protein (Garcia-Blanco et al., 1989; Ghetti et al., 1992). It contains four RRM domains that have been suggested to play different roles in RNA-binding, dimerization and splicing repression (Gil et al., 1991; Patton et al., 1991; reviewed in Wagner and Garcia-Blanco, 2001). *In vitro* splicing assays and immunodepletion experiments have implicated PTB in the control of alternative splicing through a mechanism that possibly involves repression of 3' splice sites via competition with $U2AF^{65}$ for binding, similar to the one proposed for Sex lethal (Singh et al., 1995; reviewed in Valcarcel and Gebauer, 1997; see below).

Thus, the interplay between various RRM proteins on specific RNA targets and, more importantly, the competition for binding of these RNAs has been shown to regulate various processes such as alternative splicing and polyadenylation. This underscores the biological significance of the RRM domain in regulating RNA-binding and mediating control of RNA processing and calls for more studies that would clearly identify the specific sequence requirements and understand the pathophysiology behind disorders involving mutations in RRM proteins that result in aberrant RNA-binding.

KH-type RNA-binding proteins

The first member of this sub-family of RNA-binding proteins was the hnRNP K protein, whose amino acid sequence revealed three similar domains that were named KH motifs (for hnRNP K Homology; Siomi et al., 1993a). Sequence comparisons identified other KH-type RBPs including the product of the fragile X/mental retardation gene (FMRP; Siomi et al., 1993b), the paraneoplastic neurologic disease antigens Nova1 and Nova2 (Buckanovich et al., 1993; Yang et al., 1998) and the yeast splicing factor Mer-1 (Nandabalan et al., 1993). Most of these proteins have one to three KH motifs containing approximately 70 amino acids each, with a core Gly-X-X-Gly sequence, conserved hydrophobic residues and a variable loop (Burd and Dreyfuss, 1994). Until recently, data for the function of KH-containing proteins in the binding of RNA had been limited and consisted mostly of *in vitro* assays (Siomi et al., 1993b; Ashley et al., 1993; Siomi et al., 1994; Buckanovich et al., 1996; Yang et al., 1998). Mutations of highly conserved residues in the KH domain impaired RNA binding ability *in vitro* (Siomi et al., 1994) and have been associated with human disease (De Boulle et al., 1993).

Structural data on the KH motif came first from NMR spectroscopy studies on the vigilin protein and showed the globular KH domain to contain three antiparallel β sheets on one face backed by three α helices on the other (Musco et al., 1996). Further analysis of the structures of single KH domains from FMRP, Nova, and hnRNP K, supported the conclusion that the RNA-protein interface is most likely located in the loop between the first two helices that contains the strongly conserved tetrapeptide GXXG (Musco et al., 1997; Baber et al., 1999; Lewis et al., 1999). Definitive evidence for this interaction was provided by the co-crystal structure of the third Nova2 KH domain in association with a single stem loop RNA molecule (Lewis et al., 2000). These studies showed that, unlike the β sheet of the RRM motif, it is an α/β platform flanked by the invariant GXXG motif of the KH domain that recognizes and binds RNA. Furthermore, the residue mutated in a severe form of fragile X/mental retardation disease is predicted by this analysis to change the hydrophobicity of the α/β platform and alter the RNA-binding properties of FMRP.

Even though they are not as numerous as the RRM-type RBPs, KH-containing proteins have often been implicated in human disease. Whether their function is ablated by mutations (FMRP) or autoantibodies (Nova), there has been evidence linking the specific defect in RNA-binding of these proteins to the underlying disease (De Boulle et al., 1993; Siomi et al., 1994; Buckanovich et al., 1996; Jensen et al., 2000a; Lewis et al., 2000). KH-type proteins must therefore play significant roles in RNA biology that, when perturbed in some human disorders, can cause distinct phenotypes that are likely to provide more clues for the biological function of these proteins *in vivo*.

Sequence similarities between the KH-type RBP Sam68 (see below), a female germ-cell specific tumor-suppressor gene from Caenorhabditis elegans named GLD-1

and the shrimp GRP33 protein prompted the identification of the KH motif-containing GSG (for GRP33/Sam68/GLD-1) super-domain (Jones and Schedl, 1995). Mutations in a conserved residue of the KH domain in GSG eliminate the tumor-supressor function of GLD-1 and implicate RNA-binding in this function as well as in oogenesis for which GLD-1 is essential (Jonas and Schedl, 1995). The cloning of the cDNA for the splicing factor SF1 identified it as another member of this sub-family of KH-type RBPs (Arning et al., 1996).

Other RNA-binding domains

Several other motifs have been described that can bind RNA and characterize additional sub-families of RBPs. The arginine-rich motif (ARM) is, as the name suggests, a short domain (10-20 amino acids) of mostly arginine residues (Burd and Dreyfuss, 1994). Described mostly in viral proteins, this domain is thought to recognize RNA as an α helix (Lazinski et al., 1989; Tan et al., 1993). The role of arginine residues in these protein-RNA interactions is thought to be two-fold: increasing the non-specific binding of RNA through their positive charges while forming specific hydrogen bonds. The HIV Rev and Tat proteins which contain this domain, have been implicated in diverse functions concerning RNA metabolism such as trans-activation of transcription and export of unspliced viral RNA (Malim et al., 1989). Surprisingly, the same motif can assume two different conformations during RNA-binding: the Rev ARM binds as an α helix while the Tat peptide is a β sheet and yet they both penetrate the major groove of their respective RNA targets (Puglisi et al., 1995).

The RGG box is an RNA-binding domain that was first described in hnRNP U (Kiledjian and Dreyfuss, 1992; Puglisi et al., 1992). It contains 20 to 25 amino acids with closely spaced arginine-glycine-glycine (RGG) repeats that assume a β spiral conformation (Ghisolfi et al., 1992; Burd and Dreyfuss, 1994). Curiously, most proteins that contain an RGG box (such as hnRNP A1, FMRP, and Nucleolin) also contain other RNA-binding domains, most often an RRM motif, suggesting that perhaps the RGG box is not involved in sequence specific RNA binding, but rather facilitates binding by other domains.

Other RNA-binding domains that have been described, but not yet adequately characterized, include the double stranded RNA-binding motif (DSRM), the zinc finger/knuckle motif and the cold shock domain (Burd and Dreyfuss, 1994; Siomi and Dreyfuss, 1997). Recently, it has also been proposed that the homeodomain of the Drosophila protein bicoid, usually involved in DNA binding and transcription activation, can also bind the 3' UTR of RNA and act as a translational repressor (Dubnau and Struhl, 1996).

Functions of RNA-binding proteins

As mentioned above, the processing of RNA molecules, from heterogeneous nuclear RNAs (hnRNAs or pre-mRNAs) to mature transcripts ready to be translated in the ribosome, involves many steps that are regulated by RBPs. The effect of RBPs on the metabolism of RNA is a consequence of direct binding which can alter the RNA's structural conformation, affect its localization pattern, facilitate or impede its accessibility to regulatory molecules (including other proteins), and control its stability.

Consequently, RBPs play a crucial role in the post-transcriptional control of gene expression and thus of cellular processes in general (Siomi and Dreyfuss, 1997). Many of the varied functions of RBPs are coupled to preceding or subsequent events in the lifecycle of the RNA molecule hence providing continuity and an added opportunity for control and regulation. The functions of RBPs will be reviewed here in a sequential manner, as the transcript moves from the nucleus to the cytoplasm.

Transcription and mRNA processing

Even though the general transcription factors are DNA-binding proteins, the role played by RNA-binding proteins in the control of transcription (and especially its termination) is significant and has been mostly recognized through the study of retroviral RBPs and their function in trans-activation and anti-termination (e.g. the HIV protein Tat and the phage N protein, respectively; Rees et al., 1996; Su et al., 1997; Legault et al., 1998). An analysis of the function of these RBPs is not in the scope of this thesis. Instead, a review of RBP functions in transcription will be limited to recent intriguing findings that provide evidence for the spatial, temporal and functional linkage of transcription to mRNA processing (collectively defined as the biochemical reactions that transform newly synthesized pre-mRNA to mature mRNA, including 5' capping, splicing of introns and 3' cleavage and polyadenylation).

Reports have shown that mRNA processing *in vivo* occurs co-transcriptionally (Beyer and Osheim, 1988; Bauren et al., 1998). The carboxy-terminal domain (CTD) of RNA polymerase II (RNA pol II) has emerged as a leading candidate in mediating the coupling of all three major mRNA processing reactions to transcription, but the precise

mechanism for this remains unknown (McCracken et al., 1997). In agreement with this hypothesis, the CTD domain contains tandem peptide repeats that have been proposed to mediate protein-protein interactions and are the regulatory target of phosphorylation by kinases (Komarnitsky et al., 2000; Schroeder et al., 2000).

The activation of the c-Src kinase during mitosis has been shown to result in the interaction with and phosphorylation of a KH-type RBP named Sam68 (Fumagalli et al., 1994; Taylor and Shalloway, 1994). Sam68 is a functional homologue of the HIV protein Rev since dominant negative mutants of Sam68 block the transactivation of RRE-containing transcripts (Rev Response Element; Reddy et al., 1999). Phosphorylation of Sam68 abolishes its RNA-binding activity and its ability to act as a Rev homologue (Derry et al., 2000). This suggests that the regulation of transcription and pre-mRNA processing is linked to signal transduction pathways, highlighting the fact that RNA-binding proteins may play crucial roles in the response of the cell to external stimuli.

The mRNA processing reactions (capping, splicing and polyadenylation) are thought to be interdependent, but all three can be separately stimulated by the RNA pol II CTD (Fong and Bentley, 2001). Furthermore, different domains of the CTD and specific interactions with other RBPs or protein complexes (e.g. the cleavage stimulation factor; CstF) have been proposed to be responsible for this effect (Fong and Bentley, 2001). As a domain of RNA pol II, the CTD is uniquely positioned during RNA transcription to mediate and couple subsequent processing reactions (Roberts et al., 1998). Apart from its physical location in the protein complex on transcribed RNA, its ability to interact with other proteins enables the CTD to attract other RBPs, such as members of the SR family

of splicing factors, to transcription sites in the nucleus where splicing can occur concurrently (Yuryev et al., 1996; Misteli and Spector, 1999; see below).

The enzymatic complexes regulating and affecting 5' capping and 3' cleavage and polyadenylation of the pre-mRNA are quite large and involve many factors including many RNA-binding proteins that are not going to be discussed here (for reviews see Colgan and Manley, 1997; Bentley, 1998; Shatkin and Manley, 2000). It is of interest to note however, that accumulating data on these processes point to the interpretation that they do not occur independently of each other and are instead part of an intricate regulation pattern that ensures the correct processing of the mRNA on all fronts. For example, splicing factors have also been shown to regulate polyadenylation by binding to intronic enhancer sequences (Lou et al., 1996; Lou et al., 1998). Furthermore, RBPs involved in processing seem to be involved in the regulation of other, downstream events in the metabolism of mRNA. The proteins mediating 5' capping are members of the cap binding complex (CBC) and have recently been shown to interact with the nuclear pool of the translation initiation factor eIF4G and possibly facilitate the transfer of mRNA to the cytoplasm for efficient translation (Fortes et al., 2000; McKendrick et al., 2001).

Splicing

Given that splicing concerns the physical joining of discontinuous exons in eukaryotic pre-mRNAs, it is expected that RNA-binding proteins will play a crucial role in this process. The basal splicing machinery of the cell consists of small nuclear RNAs (snRNAs) and a number of associated RBPs resulting in the formation of five complexes (U1, U2, U4, U5 and U6 snRNPs, for small nuclear ribonucleoprotein particles) that

associate with newly synthesized pre-mRNA in an orderly way (reviewed in Staley and Guthrie, 1998; Smith and Valcarcel, 2000; Hastings and Krainer, 2001). In addition, the spliceosome, as it is assembled on the new transcript, will eventually contain a multitude of non-snRNP RBPs such as members of the SR family (named for their common motif rich in serine and arginine residues), the U2 auxiliary factor (U2AF), splicing factor 1 (SF1; also known as branch point binding protein, BBP) and others.

Despite the fact that introns can vary substantially in size and sequence, they maintain several conserved motifs, most prominently dinucleotides in their 5′ and 3′ ends (splice donor and acceptor site, respectively), a polypyrimidine tract, and a branch point adenosine. These motifs are recognized by components of the splicing machinery: U1 snRNP binds to the 5′ splice site; U2AF is composed of two subunits, $U2AF^{65}$ and $U2AF^{35}$, which bind to the polypyrimidine tract and the 3' splice site, respectively; SF1 binds to the branch point adenosine. This forms the commitment complex which, with the addition of U2 snRNP and ATP, becomes the pre-spliceosome. In turn, the recognition of the pre-spliceosome by the U5-U4-U6 tri-snRNP results, after rearrangements, in the fully-competent spliceosome. Furthermore, the interaction of U1 snRNP with the 5' splice site enhances the recognition and splicing of the upstream 3' splice site, a result that helped establish the idea behind the exon definition model (Robberson et al., 1990; Kuo et al., 1991). This model predicts that protein-protein interactions across the exon facilitate the recognition of the upstream 3' and downstream 5' splice sites and help "define" the exon and mark it for splicing.

Alternative (as opposed to constitutive) splicing concerns exons that for various reasons are not always included in the mature mRNA. These include cassette exons,

mutually exclusive exons and the existence of alternative splice sites (5' or 3'). Particular intronic or exonic sequences besides the splice sites (which in these cases usually have weak consensus) are the cis-acting elements responsible for the regulation of alternative splicing and can function positively (enhancers) or negatively (repressors or silencers). These sequences have been therefore defined as intronic or exonic splicing enhancers or silencers (ISE, ESE, ISS and ESS). Successful splicing events in this case are characterized by the interplay of general splicing factors (e.g. snRNPs) with specific regulatory factors (trans-acting enhancer or repressor factors of splicing).

The SR proteins are a family of RBPs that have been grouped together by their similarity in structural organization and function in pre-mRNA splicing (Zahler et al., 1992, Fu, 1995). They contain at least one RRM-type RNA-binding domain in their N-terminus and a C-terminal domain characterized by multiple arginine-serine dipeptide repeats (RS domain) which can mediate protein-protein interactions. The role of the SR proteins is proposed to be in forming bridging interactions between essential splicing factors recognizing different splice sites. For example, the SR proteins ASF/SF2 and SC35 interact with U1 snRNP and the heterodimer U2AF through their RS domains, thereby forming a complex across the intron and facilitating the necessary transesterification reactions that will join the two exons (Wu and Maniatis, 1993; Kohtz et al., 1994). In concert with this, SR protein-bound enhancer elements can activate weak 3' splice sites supporting a role for SR proteins in stabilizing U2AF binding (Buvoli et al., 1997). Alternatively, the interactions between SR proteins (perhaps recruited by specific exonic elements) and splice site-bound general factors could favor exon definition (Smith and Valcarcel, 2000; see above).

Thus, SR proteins can function both as general splicing factors (in bridging splice sites; see above) and as specific splicing enhancers (dependent on the binding of cis-elements). For example, ASF/SF2 has been described to function as a sequence-specific splicing enhancer in alternative splicing (see below), but also as a general factor in constitutive splicing as the interaction with U1 snRNP and U2AF would imply. The latter is supported by experiments in a tet-inducible system that have shown ASF/SF2 to be an essential gene for cell viability and required for alternative splicing, that cannot be rescued by other SR proteins (Wang et al., 1996). Importantly, the RS domain is responsible for mediating the enhancer sequence-dependent splicing function of SR proteins and it can be uncoupled from its function in general splicing (Graveley and Maniatis, 1998). In conclusion, SR proteins can play important roles in alternative splicing as they are recruited to regulated exons directly by intronic or exonic cis-elements (Lavigueur et al., 1993; Sun et al., 1993) or indirectly through protein-protein interactions with other trans-acting splicing factors (Lynch and Maniatis, 1996).

In this way, they can also function in the coupling of splicing to previous biochemical processes in RNA metabolism (e.g. transcription). The SR proteins ASF/SF2 and 9G8 stimulate splicing of an exon in the fibronectin pre-mRNA *in vivo*, through an exonic splicing enhancer sequence (ESE). Significantly, promoter swapping experiments and mutational analysis of cis-acting elements have shown that successful splicing regulation by SR proteins in this transcript depends on promoter structure, suggesting that interactions between RNA pol II and the promoter could regulate alternative splicing by influencing SR proteins (Cramer et al., 1999). Recent experiments have shed light on the molecular mechanism behind this coupling by showing that a

novel transcriptional activator termed p52 co-localizes with ASF/SF2 and their interaction modulates alternative splicing *in vitro* and *in vivo* (Ge et al., 1998).

Another large family of RBPs that has been implicated in the regulation of alternative splicing are the hnRNP proteins. This hypothesis is consistent with their specific localization in the nucleus and their early binding of hnRNA. The hnRNP A1 protein was found to have the opposite effect from ASF/SF2 in 5' splice site selection *in vivo* (Caceres et al., 1994). However, this was the result of overexpression experiments with minigene constructs in HeLa cells and the two proteins were not tested in the same experiment to prove antagonism. Nevertheless, they are in agreement with previous *in vitro* mRNA splicing data where addition of hnRNP A1 counteracts the specific stimulatory effect of ASF/SF2 (Mayeda and Krainer, 1992; Sun et al., 1993). The positive effect of ASF/SF2 is thought to be mediated by stimulating the binding of U1 snRNP to the 5' splice site (Eperon et al., 1993). While the molecular mechanism of splicing repression by hnRNP A1 has not been elucidated, it has been shown to depend on splicing silencer sequences (Blanchette and Chabot, 1999; Caputi et al., 1999; Del Gatto-Konczak et al., 1999). In these studies, *in vivo* splicing assays using minigene constructs from a variety of alternatively spliced transcripts were performed in several cell lines and the effect of co-transfected hnRNP A1 and/or ASF/SF2 was measured by RT-PCR. Recent data support a similar interaction between hnRNP A1 and ASF/SF2 in the control of 3' splice site choice as well (Bai et al., 1999). The effect on both 5' and 3' splice site choice by these proteins lends support to the exon definition model and suggests a role for constitutive splicing factors in the regulation of tissue-specific

alternative splicing, perhaps through slight variations in their levels or modifications (see below).

The hnRNP I/PTB protein binds CU-rich sequences in the polypyrimidine tract of introns and was for that reason hypothesized to be involved in constitutive splicing (Garcia-Blanco et al., 1989; Patton et al., 1991). However, since then, PTB has been found to mediate the repression of exon inclusion through the binding of intronic splicing silencer (ISS) elements in a number of alternatively spliced mRNAs including c-src (Chan and Black, 1997), αactinin (Southby et al, 1999), fibroblast growth factor receptor-2 (Carstens et al., 2000), α-tropomyosin (Gooding et al., 1998), β-tropomyosin (Mulligan et al., 1992), and $GABA_A$ receptor γ2 subunit (Ashiya and Grabowski, 1997). Mutation of the PTB sites within the different ISSs results in reduced binding *in vitro* and reverses exon repression *in vivo* (Gooding et al., 1994; Perez et al., 1997a; Chou et al., 2000). Furthermore, PTB-mediated exon repression can be abolished *in vitro* by competition with exogenous RNAs (Ashiya and Grabowski, 1997; Chan and Black, 1997) or immunodepletion of PTB (Southby et al, 1999; Chou et al., 2000) and can be reconstituted by adding back recombinant PTB protein (Ashiya and Grabowski, 1997; Southby et al, 1999; Chou et al., 2000).

These results, together with its presumed ubiquitous protein distribution, have prompted the suggestion that PTB is a universal splicing repressor of weak exons (Wagner and Garcia-Blanco, 2001). However, PTB is unlikely to act independently of other factors in exon repression for a number of reasons. First, PTB is expressed in tissues where some of these exons are included in the mature mRNA. The first event in which a role for PTB in splicing repression was described involved an exon in the β-

tropomyosin pre-mRNA (Mulligan et al., 1992) that was not included in non-muscle cells (Guo et al., 1991). Second, the presence of exogenous PTB binding sites is not sufficient for the repression of heterologous exons (Lin and Patton, 1995; Perez et al., 1997a; Gooding et al., 1998). Third, additional silencer sequences and presumably the corresponding trans-acting RBP factors are required for the repression of some exons (Del Gatto-Konczak et al., 1999; Carstens et al., 2000). In addition, PTB has been isolated as part of a multi-protein complex associated with some of these regulatory elements (Chan and Black, 1997; Grossman et al., 1998; Chou et al., 2000). Finally, little data exists for the requirement of PTB as a splicing repressor *in vivo*. Studies of knock-out and transgenic mice (if viable) will likely settle the questions of where PTB is actually expressed, whether it is an essential splicing factor, and what its exact role is in the splicing of a number of target pre-mRNAs.

The observation that the intronic binding sites for PTB sometimes overlap those for U2AF (Lin et al., 1995; Singh et al., 1995) has resulted in the hypothesis that the mechanism of splicing repression by PTB involves the competition for binding sites with general splicing factors, similar to the mechanism described for the action of the Drosophila RBP Sex lethal (Valcarcel et al., 1993; see below). However, in most of the regulated exons mentioned above there is no such overlap. Another proposed model for the function of PTB takes advantage of the existence of multiple PTB sites in most of these transcripts, often located in both introns flanking the repressed exon (Perez et al., 1997a; Southby et al., 1999; Carstens et al., 2000; Chou et al., 2000). Together with evidence that PTB can multimerize (Perez et al., 1997b; Oh et al., 1998), it is plausible that RNA-binding on specific ISSs and protein-protein interactions of multiple PTB

monomers across the exon results in its repression, possibly by inhibiting the accessibility of ESEs (Chou et al., 2000; Wagner and Garcia-Blanco, 2001). A similar model has been proposed for the action of hnRNP A1 on the regulation of an alternative exon in its own transcript (Blanchette and Chabot, 1999). In this case however, binding of multiple ISSs in the flanking introns by hnRNP A1 results in the inclusion of the downstream exon.

A novel family of RBPs that have recently been described to regulate cell-specific alternative splicing are the CELF protein (CUG-BP and ETR-3-Like Factors; Ladd et al., 2001). CUG-binding protein (CUG-BP), the first homologue of this family, was identified due to its ability to bind CUG-triplet repeats and was recognized to have a similar structure to hnRNP proteins (Timchenko et al., 1996). CUG-BP has been implicated in the pathogenesis of myotonic dystrophy (DM), possibly through its regulation of the alternative splicing of the cardiac troponin T (cTNT) pre-mRNA (Philips et al., 1998). Elav-Type RNA binding protein 3 (ETR-3) is similar in sequence to CUG-BP and has also been shown to bind CUG-triplet repeats (Lu et al., 1999). Members of the CELF family have been shown to be differentially expressed in a developmentally-regulated way and to bind *in vitro* to muscle-specific splicing enhancer sequences (MSEs) present in introns flanking an alternatively spliced exon in the cTNT pre-mRNA (Ladd et al., 2001). Furthermore, it was shown that members of this family activate MSE-dependent exon inclusion in a cTNT minigene *in vivo*, and that ETR-3 protein expression differences during development correlate with exon usage in cTNT splicing. This suggests that the regulation of alternative splicing could be the result of competition and/or synergism among many RBPs that function as a complex (see below).

Overall, SR and hnRNP proteins have been known to act as enhancers or repressors in the alternative splicing of numerous transcripts, including their own in some autoregulatory cases (Smith and Valcarcel, 2000). The exact mechanism for this function is not known but has been proposed to be mediated by the binding of specific cis-acting RNA elements (exonic or intronic splicing enhancers and silencers). Future studies including biochemical and structural analyses of the RNA-protein interactions, determination of the domains involved in protein-protein interactions and genetic studies (such as knock-out and transgenic animals) will help elucidate the molecular mechanism, the likely RNA targets and the biological significance of the function of RBPs in splicing.

One of the most significant consequences of alternative splicing in the biology of a complex organism is the spatial and temporal differential processing of mRNAs and the generation thereby of diverse proteins from the same transcript. This would suggest that the regulation of alternative splicing can be modulated in a tissue- or developmental stage-specific manner. Several mechanisms have been proposed to this effect including variations in the relative concentrations of general splicing factors, post-translational modifications, and the existence of tissue-, cell- or developmentally-restricted splicing factors (Lopez, 1998). One of the best studied examples of this is the hnRNP-like protein Sex lethal (Sxl) of Drosophila melanogaster that is exclusively present in female flies and sets up a sex-specific splicing pattern of downstream effector genes in the sexual determination pathway (Boggs et al., 1987; Bell et al., 1988). In the case of the transformer mRNA, this is thought to occur by the binding of Sxl to the polypyrimidine tract thus forcing the essential splicing factor U2AF to activate a lower-affinity female

specific splice site (Valcarcel et al., 1993). A similar mechanism has been proposed for the splicing inhibition by Sxl of another RNA target, msl-2 (Merendino et al., 1999).

This result, together with the proposed competition between $U2AF^{65}$ and PTB on the polypyrimidine tract, would suggest that a fundamental characteristic of alternative splicing is the existence of splice sites with different affinities and that the regulation of exon inclusion or exclusion depends on binding competition by splicing factors. The default splicing pattern would then be one that includes exons with the strongest splice sites (i.e. closest to the consensus for binding by the general splicing factors). On the other hand, the inclusion of regulated exons (which contain weaker splice sites) will depend on the balance between enhancer and silencer elements and the actions of the RBPs that recognize them. This interplay between multiple RBP factors would require the formation of large complexes on the pre-mRNA in a regulated and orderly way.

Examples of this can be found in the study of alternative splicing in Drosophila development where the P-element transposase is only produced in germline cells (Laski et al., 1986). In somatic cells, the retention of intron 3 in the P element transcript results in the production of a transposase inhibitor and requires a regulatory sequence in exon 3 (an exonic splicing silencer, ESS). This ESS is bound by numerous trans-acting factors including U1 snRNP, the Drosophila hnRNP protein hrp48, and the P-element somatic inhibitor protein (PSI), a germ-cell specific KH-type RBP that functions in splicing repression (Siebel et al., 1992; Siebel et al., 1994). The mechanism of splicing inhibition in this complex is not clear but is known to involve an interaction between PSI and U1 snRNP (Labourier et al., 2001). Furthermore, transcriptional repression of the P-element promoter results in reduced splicing of intron 3 in a negative autoregulatory feedback

loop, and provides additional evidence for the coupling of splicing to transcription (Roche et al., 1995).

Another example is provided by the Drosophila gene doublesex where female-specific processing of exon 4 requires a purine-rich exonic splicing enhancer (ESE). This element is bound by an RBP complex including the RS domain-containing Tra and Tra2 proteins, and the Drosophila homologues of the SR proteins ASF/SF2 and 9G8 (Heinrichs and Baker, 1995; Lynch and Maniatis, 1996). The assembly of this complex is cooperative, depends on Tra and is thought to activate splicing by stabilizing U2AF binding. Two human homologues of Tra-2 have been identified as the first mammalian RBPs that are sequence-specific splicing activators without being essential splicing factors since HeLa cell extracts that are lacking Tra2 proteins can still function in constitutive splicing (Tacke et al., 1998).

It is likely though that the paradigms established in Drosophila concerning alternative splicing are not entirely accurate in mammals where the existence of all-or-nothing splicing patterns is improbable due to the complexity, number and density of cells. Instead, models that have been proposed to explain the mechanism of splicing regulation in mammalian cells suggest that an interplay is at work between positive and negative trans-acting factors and between constitutive splicing factors and sequence- or cell- specific proteins. This results in promoting exon selection (positive regulation) or repressing exon inclusion (negative regulation) according to the relative levels of the factors involved which interact with the pre-mRNA as a complex (Grabowski, 1998). These complexes are a testament to the intricate balance of power between competing signals that will eventually lead to the inclusion or not of an exon.

The reasons for the existence of such a complex system of regulation in mammalian cells are multifaceted (Smith and Valcarcel, 2000). First, there are more genes and splicing events (compared to lower organisms) that would require many single, dedicated, transcript-specific splicing factors. In the model described above, the same proteins in different combinations can achieve regulation of multiple transcripts. Second, since there are various RBPs involved in splicing regulation, there will have to be numerous binding sites for them on the pre-RNA. In some cases, these cis-acting signals are not the best consensus sequences for binding and only provide for weak protein-RNA interactions. Consequently, recognition of the RNA and successful splicing will depend on the interaction of multiple elements and allows for an additional layer of control. Finally, multiple splicing components provide for sensitive responses to varying levels of any of these proteins as well as to the signals that regulate them and produce differences in splicing patterns that are better attuned to environmental cues or developmental requirements.

However, even in this model, the requirement for some tissue- or cell-specific factors remains, whether it is in the actual splicing regulator that exists in different levels among various cell types or in other regulatory proteins (such as kinases) that in turn affect the post-translational state of splicing factors. A lot of work on alternative splicing regulation in mammalian systems has concentrated in the nervous system where the functional diversification of proteins resulting from differential mRNA processing can be advantageous in allowing neurons to transmit and respond to specific electrical or chemical signals and mediate information processing through plasticity, learning and

memory. The regulation of alternative splicing by RBPs in the nervous system will be discussed below.

Nuclear export and shuttling

In addition to altering its structural characteristics, RBPs can provide the RNA with localization and targeting signals. Many RBPs have been shown to move between the nucleus and the cytoplasm through the nuclear pore complex (NPC) and it is reasonable to assume that this transport affects the localization pattern of bound RNAs (reviewed in Nigg, 1997; Nakielny and Dreyfuss, 1999). The fact that different classes of RNA molecules use distinct and non-overlapping mechanisms of nuclear export (Jarmolowski et al., 1994), together with the existence of several components of the NPC that seem to recognize assorted signals on the transported proteins (Yang et al., 2001), suggest that there are multiple layers of control and varying degrees of regulation in the nuclear transport of protein and mRNA. For both nuclear import and export, many peptide signal sequences and their corresponding receptors have been identified, and the molecular mechanisms for the trafficking of proteins (many of them RBPs) through the NPC have begun to be elucidated.

The best characterized peptide sequence guiding nuclear import (called a nuclear localization signal, NLS), is the mono- or bi-partite NLS that contains one or two groups of positively charged residues, respectively (exemplified by the SV40 T antigen and nucleoplasmin sequences). NLS sequences are bound by the importin β receptor that mediates nuclear entry (Gorlich et al., 1995). Other co-factors (such as RanGDP; see

below) are required for this process and many more signal-receptor pairs have been identified (Nakielny and Dreyfuss, 1999).

Since mRNA is synthesized in the nucleus and must be transported out to the cytoplasm, a more in-depth review of some relevant facts about the nuclear export of mRNA ribonucleoprotein particles (mRNPs) and the shuttling of some RBPs is appropriate. Various elements on the mRNA seem to influence its nuclear export. The 5' cap and the associated cap-binding complex (CBC) proteins stimulate nuclear export (Izaurralde et al., 1992; Visa et al., 1996). Also, the existence of unspliced introns in the mRNA prevents its export into the cytoplasm and provides a mechanism for the prevention of improper translation of unprocessed messages (see below). The most important mediator of mRNA export, however, is the binding by RBPs and the specific peptide signals that they contain.

Insight into the function of RBPs in the export of RNA came from the study of retroviruses and their regulated export of intron-containing RNAs. The HIV protein Rev was the first RNA-binding protein identified to contain a nuclear export signal (NES) consisting of a short leucine-rich peptide (Fischer et al., 1995; Wen et al., 1995). Rev was found to interact with and use the cellular receptor protein Crm1 for nuclear export (Fornerod et al., 1997; Stade et al., 1997). Crm1 associates with RanGTP (a small, GTP-bound GTPase providing energy for the transport) and the NES sequence in a manner that is inhibited by leptomycin B *in vivo*, an observation that has allowed the identification of many Rev-like RNA export pathways (reviewed in Izaurralde and Mattaj, 1995; Stutz and Rosbash, 1998).

The discovery that some hnRNP proteins are able to shuttle between the nucleus and the cytoplasm has led to the identification of shuttling or bi-directional signals that provide information for both the import and export of the protein (Pinol-Roma and Dreyfuss, 1992; Michael et al., 1995; Lee et al., 1996b). Specifically, the hnRNP protein A1 has been shown to shuttle due to the M9 sequence, a domain of approximately 40 amino acids rich in glycine and aromatic residues (Michael et al., 1995). The receptor for the M9 signal has been identified as the transportin protein, which differs from the importin family of receptors and acts independently of the import pathway of proteins containing classical NLS sequences (Pollard et al., 1996).

The hnRNP K protein also contains a bi-directional shuttling domain that has been named KNS (for hnRNP K Nuclear Shuttling domain) and differs from both the classical NLS domain and the M9 sequence of hnRNP A1 (Michael et al., 1997). This domain has been shown, in competition assays with saturating levels of other signals, to mediate the import of hnRNP K protein independently of importin or transportin proteins and suggests that the KNS domain utilizes a novel import pathway, one that possibly does not require soluble factors.

Surprisingly, the hnRNP K protein also contains a classic bi-partite NLS sequence, whose deletion does not impair nuclear localization since the KNS is able to mediate nuclear import (Michael et al., 1997). In contrast to hnRNP A1, the nuclear localization of full length hnRNP K protein does not depend on RNA pol II function. However, when a construct of the hnRNP K protein lacking the classic NLS domain was tested in the presence of actinomycin D (a transcription inhibitor), it was found that nuclear import became transcription dependent (Michael et al., 1997). This would

suggest a role for hnRNP proteins A1 and K in the nuclear export of mRNAs and the existence of a waste-preventing mechanism blocking their re-import into the nucleus in the absence of active transcription. In the latter case, the function of hnRNP proteins in the nucleus mediating RNA export would not be required, but their contribution to cytoplasmic processes could be useful (see below).

These results, together with previous observations on the shuttling of other hnRNPs suggest different functions for these proteins according to their localization and shuttling patterns (Pinol-Roma and Dreyfuss, 1991). hnRNPs C and U do not shuttle and localize to the nucleus by virtue of their classic NLS in a transcription-independent manner. hnRNPs A1 and I lack a classic NLS, shuttle, and their nuclear localization is transcription–dependent. Finally, hnRNP K contains both a classic NLS and a shuttling domain and its nuclear localization becomes transcription-dependent only in the absence of the classic NLS. This organization hints at the existence of at least two distinct pathways for the nuclear import of these proteins that are under differential regulation depending on whether the proteins function in the export of mRNA. The prediction would be that hnRNPs A1 and I mediate RNA export while hnRNP K does so only when its classical NLS domain is masked, perhaps through protein-protein interactions or other associations that result in conformational changes in the protein.

It is becoming increasingly clear, therefore, that the export of mRNA from the nucleus, where it was synthesized and processed, to the cytoplasm, where it will be translated, is mediated by RBPs and occurs in ribonucleoprotein (RNP) complexes. The fact that these RBPs have other functions in the metabolism of RNA, strongly suggests that nuclear transport does not occur independently of preceding events or without

influencing subsequent ones. Earlier evidence had shown that the nucleocytoplasmic transport of some RBPs depends on transcription, as described above (Pinol-Roma and Dreyfuss, 1991; Pinol-Roma and Dreyfuss, 1992; Lee et al., 1996b).

More recent reports have linked mRNA nuclear export to pre-mRNA processing (Huang and Carmichael, 1996; Luo and Reed, 1999; Brodsky and Silver, 2000; Daneholt, 2001) and splicing (Kataoka et al, 2000; Le Hir et al, 2000a; Zhou et al., 2000; Huang and Steitz, 2001; Le Hir et al., 2001; Luo et al., 2001). In the latter case, RBPs provide a means by which to imprint newly spliced transcripts with information that will follow them to the cytoplasm and perhaps regulate downstream events (Matsumoto et al. 1998; Le Hir et al., 2000b). These RBPs that associate with mRNAs only as a consequence of splicing events have been referred to as the exon-exon junction complex (EJC) and include the splicing factors SRm160, DEK and RNPS1, the shuttling protein Y14 and the mRNA export factor REF/Aly.

Since some of the hnRNP proteins that play a role in splicing are also able to shuttle between the nucleus and the cytoplasm (e.g. hnRNP A1), it has been suggested that they may associate with RNA transcripts during earlier biochemical steps and follow or guide it through subsequent events (Kataoka et al., 2000; Mili et al., 2001; Zenklusen et al., 2001). While the two modes of RNA export (via an hnRNP particle or through splicing-dependent mRNP complexes with EJC proteins) may seem to be competing, they can provide additional regulatory steps for the cell or allow it to identify, for example, intron-containing transcripts and retain them in the nucleus (reviewed in Reed and Magni, 2001). In support of this model, protein components of the EJC have been

found to belong to or interact with proteins of the mRNA surveillance machinery (Kim et al., 2001b; Lykke-Andersen et al., 2001; see below).

Other proteins that have been shown to shuttle and could mediate the export of bound mRNAs include the HuR protein (also known as HuA), a member of the Hu family of paraneoplastic antigens (Fan and Steitz, 1998a). The shuttling domain of HuR, which lacks a classical NLS, was named HNS (for HuR Nuclear Shuttling sequence) and exhibits similarity to the M9 domain of hnRNP A1 in its sequence composition as well as in regulating nuclear localization in a transcription-dependent fashion. However, the function of HuR and the proposed consequence of its shuttling most likely concerns its role in the stability of bound mRNA and will be discussed later. Another RBP that has been shown to shuttle between the nucleus and the cytoplasm and has been proposed to be involved in the regulation of RNA stability is the hnRNP D protein (Loflin et al., 1999). In addition, members of the SR family of proteins have been shown to shuttle and could play a role in the export of RNA (Caceres et al., 1998). Indeed, two shuttling SR proteins have been shown recently to promote the nuclear export of intronless RNA and could provide the necessary adaptor function for molecules not exposed to EJC proteins or bound by hnRNP protein during splicing (Huang and Steitz, 2001).

Cytoplasmic localization

The specific localization of mRNAs in the cytoplasm is an important means by which to achieve spatially regulated protein synthesis and its aftermaths during development and beyond (Bandziulis et al., 1989; Curtis et al, 1995; Mohr and Richter, 2001). Most signals on the mRNA controlling cytoplasmic localization have been identified in the 3'

untranslated region (UTR) and are bound by RBPs that mediate this process (Jansen, 2001). Significantly, many of these RBPs have been found to belong to the hnRNP family and support a general model for the function of these proteins in the cytoplasm after binding their mRNA targets in the nucleus and mediating their export (Shyu and Wilkinson, 2000).

Several mechanisms for the localization of mRNA in the cytoplasm have been described. Active transport of bound mRNA by RBPs, presumably through interactions with motor proteins and the cytoskeleton, occurs with some hnRNP proteins suggesting that the cytoplasmic localization of transcripts depends on previous, nuclear events. These processes have been visualized in Drosophila embryos and Xenopus oocytes, and the proteins involved show homology to mammalian hnRNPs (Cote et al., 1999; Lall et al., 1999). Other mechanisms for the selective cytoplasmic localization of mRNA involve localized stabilization of transcripts (as observed for a Drosophila heat shock protein; Bashirullah et al., 1999) or local trapping of the RNA (as is the case with the Staufen RBP anchoring bicoid message in Drosophila; St. Johnston et al., 1991).

The zipcode-binding protein (ZBP-1) is an interesting RBP that contains RNA-binding domains of both the KH- and RRM-type (Ross et al., 1997). It was identified as a protein that binds the 3'UTR "zipcode" element of β-actin mRNA that had been previously shown to be necessary for the correct cytoplasmic localization of that transcript in fibroblasts (Kislauskis et al., 1994). ZBP-1 is highly homologous to another protein named Vera (or Vg1RBP), which has been shown to mediate the specific cytoplasmic localization of a Xenopus mRNA (Deshler et al., 1997; Havin et al., 1998). While Vera is thought to mediate the association of its bound mRNA with microtubules

(Elisha et al., 1995), the localization of β-actin mRNA by ZBP-1 requires the presence of microfilaments (Sundell and Singer, 1991; Ross et al., 1997). Furthermore, the sequence elements recognized by these two homologous proteins on the 3' UTR of their respective mRNA targets are radically different. These results suggest that proteins of similar sequence and structure can not only have different binding specificities but also utilize different cellular machineries to achieve cytoplasmic localization. A related function of RBPs concerns the facilitation of local actin polymerization, possibly in order to better anchor mRNA molecules in the cytoplasm (Zhao et al., 2001).

Functional associations between the endoplasmic reticulum (ER) and RBPs responsible for the cytoplasmic localization of mRNA have also been described. The Xenopus protein Vera co-fractionates with ER membranes (Deshler et al., 1997), while the mammalian homologue of the Drosophila RBP Staufen co-localizes with the rough ER and co-sediments with polysomes (Marion et al., 1999). Furthermore, specific signals on RNAs are responsible for their localization to specific ER subdomains (Choi et al., 2000). These results suggest that cytoplasmic localization of mRNA and association with particular sub-domains of the ER is mediated by RBPs and specifies an important mechanism for the eventual localization of proteins to different cellular compartments.

In contrast to nuclear transport, little is know about the exact molecular mechanism and the peptide signals responsible for the movement and the correct cytoplasmic localization of RBPs and, consequently, of bound mRNAs. Recently, functional interactions between RBPs and motor proteins that could mediate movement across the cytoskeleton have been reported. Deletion of the microtubule motor protein kinesin I in Drosophila germ cells results in the specific absence of Staufen protein and

oskar mRNA (whose cytoplasmic localization is dependent on intact Staufen function) from the posterior pole (Brendza et al., 2000). Importantly, this mislocalization was corrected by the addition of a wild type kinesin I transgene.

The RBP Swallow is required for the correct localization of the bicoid mRNA to the anterior pole of the Drosophila oocyte (Nusslein-Volhard et al., 1987). In a recent study, the anterior localization of Swallow protein was found to require an intact microtubule network with correct polarity (Schnorrer et al., 2000). Using yeast-two-hybrid screens, Swallow was found to interact with the motor protein dynein with which it co-immunoprecipitates in ovarian extracts. Furthermore, in transgenic flies carrying a deletion of the Swallow peptide domain that interacts with dynein, Swallow protein did not localize to the anterior pole (Schnorrer et al., 2000). Even though the localization of bicoid mRNA in these mutants was not tested, these results suggest an important functional interaction between RBPs that are responsible for mRNA localization and motor proteins of the cytoskeleton. As mentioned above, specific requirements for microtubules and microfilaments have also been established for the cytoplasmic localization of Vg-1 and β-actin mRNAs, respectively.

Additionally, in budding yeast, the localization of the cell fate determinant Ash1 to the presumptive daughter nucleus occurs at the level of the mRNA and depends on the presence of its 3' UTR and an intact actin cytoskeleton (Takizawa et al., 1997). Protein-protein interactions between a novel family of RBPs (the She proteins) have been shown in this case to be important for the tethering of Ash1 mRNA to the Myo4p myosin motor protein and could explain the requirement for both the 3'UTR and the actin cytoskeleton for proper localization of this message (Bohl et al., 2000; Long et al., 2000). Another

novel RBP named Loc1 that binds double stranded RNA, has been shown to also be required for efficient localization of Ash1 mRNA (Long et al., 2001). Surprisingly, the localization of this protein is exclusively nuclear, suggesting again that previous (nuclear) events are necessary for the cytoplasmic localization of RNA and highlighting the importance of cross-talk between nucleus and cytoplasm and the crucial role that RBPs play in mediating this communication.

Translation control, stability and turnover

The mRNP complexes involved in translation initiation, elongation and termination are well characterized and will not be considered here. However, a couple of interesting instances involving the regulation of translation and its coupling to other events in mRNA metabolism (especially cytoplasmic localization and nuclear transport) have been reported to involve several RBPs, particularly members of the hnRNP family.

The hnRNP protein A2 has been implicated in the translational regulation of the mRNA for myelin basic protein (MBP) *in vivo* (Kwon et al., 1999). Specifically, hnRNP A2 enhances the translation of a heterologous transcript that includes a particular sequences from the 3' UTR of MBP mRNA. Coupled with the described function of hnRNP A2 in shuttling and the cytoplasmic localization of this transcript (Munro et al., 1999; see below) these results place hnRNP A2 in the forefront of an integrated role in the metabolism of target mRNAs. The function of other shuttling hnRNP proteins in the regulation of translation has also been described. The hnRNP proteins A1 and I/PTB have been shown to mediate cap-dependent translation (Svitkin et al., 1996), and hnRNP I/PTB has also been implicated in the regulation of cap-independent translation in viral

transcripts (Kaminski et al., 1995). Also, hnRNP C, even though considered to be a mostly nuclear protein, has been associated with the binding of an internal ribosomal entry site (IRES; Sella, 1999).

Another example of the involvement of hnRNP proteins in translation control concerns hnRNPs K and E. These proteins have been affinity purified from rabbit reticulocytes using a particular sequence in the 3'UTR of the erythroid 15-lipoxygenase gene (LOX) called the differentiation control element (DICE; Ostareck et al., 1997). This element had previously been shown to be responsible for the translational silencing of LOX mRNA until the final steps of reticulocyte maturation (Ostareck-Lederer et al., 1994). More recent evidence has suggested that the mechanism of silencing by DICE and hnRNPs K and E entails the inhibition of joining by the 60S ribosomal subunit in the formation of the 80S subunit at the AUG start codon (Ostareck et al., 20001). Moreover, the regulation of this translational silencing is mediated through the phosphorylation of hnRNP K by the extracellular-signal-regulated kinase (ERK). This phosphorylation is necessary both for the cytoplasmic localization of hnRNP K and its ability to silence translation of DICE-containing mRNAs (Habelhah et al., 2001).

Several lines of evidence suggest that the regulation of mRNA translation is coupled to the stability and turnover of the transcript. For example, interactions between translation initiation factors and decapping enzymes have been proposed to arbitrate the decision on whether the transcript will undergo translation or degradation (Schwartz and Parker, 2000; Vilela et al., 2000). Additionally, the poly(A)-binding protein (PABP) has been hypothesized to provide a link between translation and mRNA decay by being part of a complex bound to an RNA instability element (Grosset et al., 2000). Another protein

identified in this complex is hnRNP D which had been previously implicated in mRNA stability and highlights again the centrality of hnRNP proteins in mRNA metabolism (Loflin et al., 1999).

The Hu proteins are a family of proteins that have been identified as target antigens in paraneoplastic encephalomyelitis/sensory neuropathy (Szabo et al., 1991). They consist of at least four homologous members each with three RRM-type RNA-binding motifs (HuA through D; Okano and Darnell, 1997). They are similar to the Drosophila proteins Sex lethal and Elav that have been implicated in the regulation of sex determination and neuronal development, respectively, possibly through the control of alternative splicing (Robinow et al., 1988; Valcarcel, 1993; Koushika et al., 2000). Hu proteins have been shown to bind AU-rich elements (AREs) in the 3'UTR of mRNAs and have been hypothesized to play a role in the stability and degradation of ARE-containing messages (Fan et al., 1997; Jain et al., 1997; Myer et al., 1997; Fan and Steitz, 1998b; Ford et al., 1999). Moreover, transfection of HuB results in increased steady-state levels of the mRNA encoding for neurofilament M protein and induces the formation of neurites in cell culture (Antic et al., 1999). However, it is not known whether the increased level of message was due to increased stability or translation initiation. Furthermore, the specific details of this binding and the exact mechanism by which Hu proteins affect mRNA translation and/or stability have not yet been discerned.

A novel function of RBPs emerging from recent reports has been the prevention of unspliced transcripts from exiting the nucleus and the regulation of nonsense-mediated decay of such mRNAs that do cross the nuclear membrane into the cytoplasm. This is particularly interesting since one of the prototype RBPs with a role in RNA transport is

the HIV protein Rev which actually mediates nuclear export of unspliced, viral mRNAs. After the initial observation that splicing mutants in the yeast allowed pre-mRNA to be mistakenly transported to the cytoplasm (Legrain and Rosbach, 1989), current work has focused on the role of specific RBPs in nonsense-mediated mRNA decay and the functional coupling of this surveillance process to previous events (i.e. splicing). The successful result of splicing (witnessed by the occurrence of exon-exon junctions and the formation of the EJC) serves as both a mediator of mRNA nuclear export (see above) and a marker for the mRNA surveillance machinery of the cell in order to avoid nonsense-mediated decay of the mature transcript in the cytoplasm. RBPs in the EJC (such as Y14 and RNPS1) were found to interact with the mRNA surveillance machinery (hUpf complex) and mediate its function (Kim et al., 2001a; Lykke-Andersen et al., 2001).

Taken together, the evidence described in the preceding sections point out the multiple functions of many RBPs and especially the hnRNP proteins. This suggests that individual hnRNP proteins could be trans-acting factors that bind their mRNA targets shortly after or concurrently with transcription, participate in their splicing and processing reactions, guide them through nuclear export and mediate their cytoplasmic localization and selective translation. A striking example is provided by the hnRNP I/PTB protein that has been implicated in the control of mRNA splicing, poly(A) cleavage, nuclear export, cytoplasmic localization and translation (Valcarcel and Gebauer, 1997; Moreira et al., 1998; Cote et al., 1999; Gosert et al., 2000; see above). Thus, hnRNP proteins provide a strong model for the integrated function of RBPs and a distinct opportunity for the regulation of RNA metabolism in multiple, linked steps (Shyu and Wilkinson, 2000).

RNA-binding proteins in the nervous system

The particular functions of RBPs in the nervous system will be considered in the context of two processes on the elucidation of which significant progress has been made recently and which constitute the focus of this thesis, namely the regulation of alternative splicing and the trafficking and localization of RBPs and mRNA, particularly at the synapse. Neuronal functions of RBPs will also be considered as those emerge from the study of neurologic diseases, especially neurodegeneration.

Neuron-specific alternative splicing

The regulation of the inclusion of alternatively spliced (cassette) exons in the nervous system has furthered our understanding of the general aspects of alternative splicing as well as the particular tissue- or cell-specific factors and processes that are required. The tyrosine kinase c-src gene contains a small alternative exon (called N1) that is only included in neurons (Pyper and Bolen, 1989). A cis-acting element promoting the neuron-specific inclusion of this exon has been identified in the downstream intron (an Intronic Splicing Enhancer, ISE) and has been shown to activate inclusion of a heterologous exon in the human β-globin gene (Min et al., 1995; Modafferi and Black, 1997). UV-cross-linking experiments identified a complex of proteins that bind to this element only in neuronal cell nuclear extracts (Min et al., 1995).

This complex included the hnRNP F and H proteins and a novel KH-containing RBP named KSRP (for KH-type Splicing Regulatory Protein) that is homologous to the Drosophila PSI protein and the human transcription factor Fuse-Binding Protein (FBP; Min et al., 1995; Min et al., 1997; Chou et al., 1999). Immunodepletion experiments

followed by the addition of recombinant proteins established a role for these proteins in the activation of the N1 exon *in vitro*. The fact that none of the identified trans-acting RBPs is neuron-specific has precluded any conclusions that their action on N1 splicing is sufficient for its neuron-specific inclusion. Cross-linking of KSRP to the downstream ISE (also called the downstream control sequence, DCS) was enhanced in nuclear extracts from neuronal cells compared to non-neuronal cells and the expression of KSRP protein (but not mRNA) was slightly increased in neuronal cell lines (Min et al., 1997).

Additional sequences were found to regulate exon N1 inclusion including a splicing enhancer sequence in exon N1 (an ESE) that cooperates with the ISE and a splicing silencer sequence in the upstream intron (an ISS) that represses the action of the downstream ISE (Chan and Black, 1995; Modafferi and Black, 1999). Mutagenesis analysis and *in vitro* splicing assays with nuclear extracts have identified a conserved CUCUCU element in the upstream intron to be required for splicing repression (Chan and Black, 1995). RNA competition experiments have suggested that this repression is mediated by RBPs and UV-cross-linking identified PTB as one of the proteins bound to the upstream ISS (Chan and Black, 1995; Chan and Black, 1997). Addition of purified PTB to RNA competition experiments with this upstream ISS restores repression of the N1 exon (Chan and Black, 1997), while immunodepletion experiments in HeLa nuclear extracts suggest that PTB is necessary for N1 exon skipping (Chou et al., 2000).

PTB can be cross-linked to CUCUCU sequence elements in both the upstream and downstream ISS and mutations in the downstream binding sequence can cause the dissociation of PTB from the upstream ISS (Chou et al., 2000). In neuronal cells, where N1 is included, PTB dissociates from the upstream ISS in the presence of ATP. These

results suggest that an ATP-dependent event in neuronal cells is responsible for the removal of PTB from the upstream ISS, which in turn destabilizes PTB binding to the downstream ISS and allows splicing of the N1 exon. A neuronally-enriched homologue of PTB (called nPTB) was isolated from neuronal cell lines with affinity chromatography using DCS RNA and was found to bind the CUCUCU sequence more stably than PTB but to be a weaker splicing repressor *in vitro* (Markovtsov et al., 2000). This result lead to the hypothesis that nPTB mediates N1 exon inclusion through a permissive mechanism by binding the repressor element and preventing binding by PTB. However, it is not certain that PTB functions in the repression of N1 splicing *in vivo* or that nPTB is a splicing activator (or, more accurately, a de-repressor). No *in vivo* splicing assays have been done on c-src and the presumed protein-protein interactions in the recruitment of a regulatory splicing complex have not been verified. Furthermore, there is no *in vivo* data on the correlation of PTB levels and splicing patterns of c-src or, for that matter, of any other presumed PTB splicing target.

Another transcript that contains a neuron-specific cassette exon is the $GABA_A$ receptor γ2 subunit ($GABA_AR\gamma2$) mRNA. Since the initial discovery that transcripts of $GABA_AR\gamma2$ in brain tissues can contain a cassette exon of 24 nucleotides (Whiting et al., 1990), mutagenesis analysis on minigene constructs and transfection/RT-PCR splicing assays in neuronal cell lines have identified an intronic repressor element near the 3' splice site upstream of the alternatively spliced exon 9 (Zhang et al., 1996). UV-cross-linking and RNA competition assays with HeLa cell nuclear extracts suggested that this element is bound by PTB (Ashiya and Grabowski, 1997). Furthermore, the addition of recombinant PTB protein in the RNA-competed *in vitro* splicing assays results in exon 9

repression. A sequence alignment from intronic splicing repressor elements from this and other transcripts with a demonstrated affinity for PTB *in vitro*, identified a UUCUCU consensus (Patton et al., 1991; Mulligan et al., 1992; Chan and Black, 1995; Singh et al., 1995; Ashiya and Grabowski, 1997).

When UV-cross-linking/RNA competition assays were repeated in rat brain nuclear extracts, the levels of PTB protein were reduced and a protein (tentatively called p59) with different electrophoretic mobility from PTB (59 kDa vs. a 60 kDa doublet) but, nevertheless, immunoreactive to an hnRNP I-specific monoclonal antibody was observed (Ashiya and Grabowski, 1997). Together with the results on the differential regulation of c-src splicing in neuronal cells (Chan and Black, 1997; see above), this suggested a model whereby exon repression by PTB is alleviated in the brain through the differential expression of PTB protein and its p59 homologue (Grabowski, 1998).

In addition to $GABA_AR\gamma2$, the repression of the neuron-specific exon in two additional transcripts, clathrin light chain B and *N*-methyl-D-aspartate (NMDA) receptor NR1 subunit, was shown, in splicing switch assays with RNA competitors, to depend on PTB-binding sites (Zhang et al., 1999). Again, the existence of a neural form of PTB correlated with exon inclusion in neuronal extracts, and the addition of recombinant PTB protein switched splicing to the non-neuronal pathway. Besides being mostly correlative, these studies do not present concrete evidence on the factors responsible for the regulation of neuron-specific splicing in these pre-mRNAs. The significance of the cis-acting sequence elements identified has not been substantiated *in vivo*, and the trans-acting factors involved in splicing regulation are only hinted at. The cloning of the

presumably neuron-specific PTB homologue would be a step in the right direction, as would the characterization of its function in splicing regulation *in vivo*.

A well-studied example of the coupling between alternative splicing and other events in mRNA processing (e.g. polyadenylation), concerns the calcitonin/calcitonin gene-related peptide (CGRP) transcript. Downstream of alternative exon 4 exists an ISE that resembles a pseudo exon, mutations on which reduce *in vitro* binding by PTB (Lou et al., 1996; Lou et al. 1998). Exon 4 contains a polyadenylation site and is included in thyroid C cells, thereby generating the shorter calcitonin mRNA. In neurons, exon 4 is repressed and a second polyadenylation site at the end of exon 6 generates CGRP mRNA.

In vivo transfection/RT-PCR splicing assays in non-neuronal cell lines using a CGRP minigene resulted in the unexpected finding that PTB binding of the ISE enhances exon 4 inclusion (Lou et al., 1999). Mutations in the ISE that favored binding by $U2AF^{65}$ resulted in exon repression *in vivo* and inhibited polyadenylation *in vitro*. However, the apparent enhancement of exon inclusion by PTB can be explained if one considers that the PTB-mediated repression of the 3' splice site of the pseudo exon results in increased usage of the upstream 3' splice site of exon 4. Moreover, an additional binding site for PTB (a pyrimidine tract) was identified close to the poly(A) signal sequence in exon 4 (Lou et al., 1999) similar to the PTB biding site identified near the poly(A) signal of the mouse C2 complement gene (Moreira et al., 1992). These results, while preliminary and unconfirmed *in vivo*, suggest that an added function of PTB is in the regulation of polyadenylation and possibly in its functional coupling to splicing.

The Nova family of RBPs have been isolated through their involvement in paraneoplastic opsoclonus myoclonus ataxia (POMA) and consist of two identified

members, Nova1 and Nova2 (Buckanovich et al., 1993; Yang et al., 1998). They have been shown to bind RNA through ribohomopolymer and filter-binding assays and their *in vitro* sequence preferences for RNA targets have been studied through RNA selection strategies (Buckanovich and Darnell, 1997; Yang et al., 1998). Finally, crystal structures have demonstrated the mode of RNA binding (Lewis et al., 2000). A Nova1 knock-out mouse was recently described that established Nova as the first mammalian neuron-specific splicing regulator (Jensen et al., 2000a).

A combination of RT-PCR, RNAse protection and co-transfection assays identified two transcripts whose alternative exons were positively regulated by Nova: the glycine receptor α2 subunit and the $GABA_A$ receptor γ2 subunit (Jensen et al., 2000a). In Nova1-null mice the inclusion of alternatively spliced exons in these transcripts, but not others tested, was reduced compared to their wild type litter mates. This effect was similar to the one seen in co-transfection/splicing assays with minigene constructs of the same mRNAs (Jensen et al., 2000a; K. Dredge and R. Darnell, unpublished observations). Furthermore, recent evidence suggests that the action of Nova in regulating alternative splicing is part of an interplay with other tissue-restricted factors (Polydorides et al., 2000; see Chapter 4). Even though the difference, if any, in the physiological function of these splicing isoforms is not known, the fact that they are both receptors for inhibitory neurotransmitters is in agreement with a lack of inhibitory control phenotype, observed in patients with POMA (Darnell, 1996).

Recently, the Hu proteins have also been implicated in the control of alternative splicing in the nervous system. The Drosophila HuB homologue Elav (Embryonic lethal abnormal vision) is a neuron-specific RBP that has been shown to regulate the alternative

splicing of neuroglian, armadillo and erect wing transcripts (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001). The authors used quantitative RT-PCR in Elav-null eye imaginal disc clones and ectopic Elav-expressing wing imaginal discs (the equivalent of knock-out and transgenic mice) to show that the neuron-specific splice forms of armadillo and erect wing transcripts, but not of several ubiquitously-expressed genes, depend on and correspond to Elav levels (Koushika et al., 2000). Transgenic flies carrying reporter minigene constructs of neuroglian were also used to identify the sequences responsible for Elav-mediated alternative splicing (Lisbin et al., 2001). Furthermore, as mentioned above, the Elav-like ETR proteins play a role in the regulation of cTNT splicing (Ladd et al., 2001). With the exception of HuA/R, the expression of Hu proteins and their Drosophila homologue Elav is restricted to the nervous system making them important factors in tissue-specific splicing regulation (Okano and Darnell, 1997).

Post-synaptic localization of mRNA

The first example of an mRNA being selectively localized in the neuronal dendrite was reported by in situ hybridization assays for the transcript of the dendritic-specific microtobule-associated protein MAP2 (Garner et al., 1988). Since then, a number of messages have been found to be localized in dendritic processes including the alpha subunit of type II calcium calmodulin-dependent protein kinase (CAMKIIα), the mRNA encoding for the vasopressin precursor protein (VP) and the immediate-early gene Arc (Benson et al., 1992; Miyashiro et al., 1994; Lyford et al., 1995; Crino and Eberwine, 1996; Mayford et al., 1996b; Mohr et al., 2001). As well, the localization of β-actin mRNA has been observed in neurites and growth cones (Bassell et al., 1998).

This localization has been proposed to provide a way for neurons to achieve localized protein synthesis with all the advantages this might confer, i.e. polarity, synaptic plasticity, memory, etc. Consistent with this hypothesis, the existence of selectively localized polyribosomes in postsynaptic dendritic sites in CNS neurons has been long known (Steward and Levy, 1982). Significantly, the localization of some of those mRNAs in dendrites is stimulated by synaptic activity, a regulatory mechanism which is present exclusively in neurons, suggesting that selective localization of messages post-synaptically can indeed constitute a mechanism mediating plasticity. For example, the localization of Arc mRNA in post-synaptic hippocampal dendrites and the resulting selective protein synthesis was enhanced by high frequency activation and also resulted in the selective accumulation of newly synthesized Arc protein in the same area (Steward et al., 1998). Furthermore, this activity-dependent mRNA localization requires the activation of NMDA receptors (Steward and Worley, 2001). The expression of an activated CaMKII transgene that is independent of calcium regulation resulted in a loss of hippocampal long-term potentiation and a deficit in spatial memory formation, implying that the selective localization of transcripts in post-synaptic dendrites, is crucial for the physiology of neurons (Mayford et al., 1996a).

While the cellular mechanisms responsible for the localization of these mRNAs are not yet understood, some of the cis-acting elements on the 3' UTR that are necessary for dendritic targeting have been identified and seem to share common features (Mori et al., 2000). Therefore, it is reasonable to assume that trans-acting RBPs are responsible for the dendritic localization of mRNA. Recently, the *in vitro* interaction of PABP with the 3' UTR sequence responsible for the dendritic localization of the VP mRNA has been

reported (Mohr et al., 2001), but these results are preliminary and their *in vivo* significance unclear since binding of the 3' UTR by PABP is required for stabilization and translation initiation.

Another example of an RBP controlling cytoplasmic localization in the central nervous system (CNS), involves MBP mRNA in oligodendrocyte peripheral processes (Ainger et al., 1997). While the signals in the 3' UTR of the transcript responsible for this effect have been characterized, the actual trans-acting protein regulating this effect remains elusive. Contradictory reports have implicated one of the quaking KH-type proteins and the hnRNP protein A2 in the localization of MBP mRNA (Hoek et al., 1998; Munro et al., 1999; Li et al., 2000). However, in both cases the evidence is circumstantial and indirect as no co-localization or direct binding *in vivo* was shown. The possibility that quaking is the RBP responsible for the localization of MBP mRNA in oligodendrocytes is intriguing since mutations in the quaking gene have been correlated with demyelination phenotypes (Ebersole, 1996).

RNA-binding proteins and neurologic disease

The importance of the role played by RBPs in normal cell function is underscored when such processes are perturbed by genetic defects or other diseases targeting specific gene products (e.g. autoimmune disorders). Furthermore, the study of various diseases has allowed for the identification of many RBPs and has hinted at their function *in vivo*. For example, defects in spermatogenesis have been attributed to specific deletions of a gene encoding for a protein with an RNA-binding domain (Reijo et al, 1995; Eberhart et al., 1996) but specific *in vivo* RNA targets for this protein and its homologues have not yet

been identified. Autoantibodies from patients with systemic lupus erythematosus (SLE) have been used to clone many of the proteins that participate in the formation of snRNPs, the essential splicing factors (Hinterberger et al., 1983; Wieben et al., 1985).

Many RBPs have been implicated in the pathogenesis of neurologic disease. Quaking is a KH-type RBP that also contains signal transduction motifs and has been implicated in the localization of target mRNAs in oligodendrocytes (see above). Mutations in the quaking gene-product result in severe defects in myelination and embryogenesis (Ebersole et al., 1996). Another function of quaking has been described in the induction of apoptosis (Chen and Richard, 1998). Curiously, other KH-type RBPs have been implicated in the induction of apoptosis as well, including the Drosophila homologues of Sam68, KEP1 and Sam50 (Di Fruscio et al., 1998), the paraneoplastic antigen Nova (Jensen et al., 2000a), the cellular p53 target MCG10 (Zhu and Chen, 2000), and the Drosophila homologue of FMRP (Wan et al., 2000). In the case of Nova and FMRP proteins, apoptosis has been observed in neuronal cells suggesting that the particular function of these proteins in mRNA metabolism is related to programmed cell death, and providing clues about the mechanism of dysfunction in neurologic disease.

Other neurologic disorders have been identified where the protein involved is thought to play a role in splicing regulation either as a regulator of the general splicing machinery, such as the survival of motor neurons (SMN) protein in spinal muscular atrophy (SMA; Pellizzoni et al., 1998; see below) or by controlling the neuron-specific splicing of an alternatively regulated exon, such as the Nova protein in POMA (see above). SMA is a relatively common motor neuron degenerative disease that results from mutations in the SMN gene (Lefebvre et al., 1995). SMN was found to interact and co-

localize with SIP1 (SMN-Interacting Protein 1), and this interaction is essential for the biogenesis of snRNPs (Fischer et al., 1997; Liu et al., 1997). Dominant negative SMN mutants cause the redistribution of snRNP complexes and inhibit pre-mRNA splicing *in vitro* (Pellizzoni et al., 1998). However, apart from a role as a protein whose function is critical in mRNA biogenesis, these studies do not specifically explain the pathogenesis of SMA, or the actual cause of the commonly associated fatality in this disorder.

Neurologic disorders where the biochemical cause for the erratic mRNA processing is not completely understood include inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) and amyotrophic lateral sclerosis (ALS). In both cases, however, the involvement of RBPs and alternative splicing mechanisms is highly suspected. In FTDP-17, some mutations identified in patients with the disease map to a 5' splice site and result in the increased usage of an alternatively spliced exon in the mRNA for tau, a microtubule-associated protein (Hutton et al., 1998). The inclusion of this exon increases the number of microtubule-binding repeats in the tau protein and this has been proposed to explain the increased tau deposits in the neurons and glial cells of these patients (Spillantini et al., 1997). In the case of ALS, the mRNA of the glutamate transporter EAAT2 (Excitatory Amino Acid Trasporter 2) was found to be abnormally spliced, but only in neuro-pathologically affected areas in patients with the disease (Lin et al., 1998). *In vitro* expression of these aberrant transcripts resulted in proteins that were either rapidly degraded or predicted to function as dominant negatives (Lin et al., 1998), thus providing a link to the glutamate-mediated excitotoxicity that has been postulated to cause ALS (Rothstein, 1996). However, the cellular event responsible for this abnormal splicing pattern has not been identified yet.

The paraneoplastic neurologic disorders (PNDs) are a rare group of syndromes that involve autoimmune responses to antigens ectopically expressed by systemic tumors (reviewed in Darnell, 1996; Musunuru and Darnell, 2001). These antigens are normally expressed in the nervous system where they are suspected to have important functions in the physiology and development of neurons. A successful anti-tumor immune response results in high-titer antibodies that, by crossing into the central nervous system (CNS), can cause specific neurological symptoms. The clinical presentation of PND patients allows for insight into the various functions of these onconeural antigens that have been cloned using patient sera (Buckanovich et al., 1993; Yang et al., 1998). Importantly, among the categories of onconeural antigens so far identified is a group of RNA-binding proteins that include the Nova and Hu proteins (see above). Furthermore, since these proteins are present exclusively in neurons (except for HuA/R; Okano and Darnell, 1997), the study of their interactions with RNA targets or other proteins provides a means to examine neuron-specific cellular functions.

The abundance of genetic or immunologically-mediated defects in neuronal physiology attributable to improper function of RBPs together with specific examples of RBP functions in the nervous system as described above, attests to the magnitude of the role played by RBPs in the regulation of RNA metabolism in neurons. Furthermore, it underscores the fact that this role is likely to be important in the development and general maintenance of the nervous system, including information processing in the brain. In any case, neurodegenerative disorders have been associated with abnormal mRNA processing either as a primary defect (SMA) or as a specific dysfunction in a particular transcript or pathway (FTDP-17, ALS, and POMA). A better knowledge of the interactions between

the proteins involved and their RNA targets (and not only in neurons) will clearly open the way for the understanding of physiological processes as well as the reasons why these processes go wrong in disease and the consequences thereof. Insight from molecular, genetic, biochemical and structural studies will be crucial in elucidating the mechanism of RNA binding, the important cis- and trans-acting elements involved, the relative sequence requirements and the functional significance of protein-RNA and protein-protein interactions.

Summary

The study of RNA-binding proteins has been an area of tremendous growth in recent years and it is highlighted by the existence of many human disorders where the function of a specific RBP is affected. These disorders, as well as data on the structure and localization of these proteins, provide the framework for examining their role in the cellular metabolism of mRNA. A central theme in this introduction has been that sequential biochemical steps in the metabolic evolution of mRNA within the cell are tightly linked spatially and functionally and that this coupling can be mediated by RBPs. Since they are unlikely to act completely independently of other factors, investigating interacting partners of RBPs is useful in elucidating the mechanism of this coupling and ultimately providing clues for their biological role. This thesis concerns the study of the RBP Nova, the search for its interacting protein partners and the ways these interactions may affect its function in alternative splicing and nuclear localization.

Chapter 2 – Materials and Methods

Yeast two-hybrid library screens

Full-length Nova1, full-length Nova2 and Nova2 spacer (amino acids 230-407) bait constructs were cloned into the LexA vector pBTM116 (trp+; kindly provided by Drs. Susan Smith and Titia de Lange), and transformed with LiOAc into the LD40 yeast strain (also provided by Drs. Susan Smith and Titia de Lange). LD40 yeast are leu-/trp- and contain the lacZ and His+ genes under the control of LexA promoters. The resulting transformants were control-tested to make sure that there was no induction of lacZ expression independently of prey constructs (colony lift/X-gal assays without a co-transformed Gal4 activation domain construct). Screens of adult mouse brain or E 11.5 whole mouse Matchmaker cDNA libraries (in the pGAD10 vector, leu+; Clontech) were performed following the Matchmaker System protocol (Clontech). The total number of successful transformants in each screen (colonies that were trp+/leu+) was divided by the number of independent clones in each library to give the percentage screened (% scr. in Table 1). Large scale LiOAc transformations of each amplified library into LD40 yeast already carrying a bait construct were plated onto triple dropout media (trp-, leu-, his-). The resulting colonies that were able to grow in these plates were his+ and were also assayed for β-galactosidase production (blue staining) in a colony lift/X-gal assay (following a modified protocol from the Matchmaker System, Clontech; Yang, 1997, PhD Thesis, Rockefeller University). These colonies were deemed true positives when their β-galactosidase staining turned white upon removal of the bait plasmid (by growing in trp+/leu- single dropout media). The prey plasmid was then extracted from the white colonies and sequencing of the clones was performed with vector-specific primers (Operon). Sequences were analyzed with MacVector Software and compared to the NCBI database for homology. Screens with full length Nova2 baits were performed by Yolanda Yang and have been previously described (Yang, 1997). All DNA sequencing throughout this work was performed by the Rockefeller University Protein/DNA Technology Center.

Yeast two-hybrid protein interaction assays

Deletion constructs of Nova2 (in pBTM116) and brPTB (in pGAD424; Clontech) were made by PCR with specifically designed primers (Operon, see list below), gel-extracted (Qiaex II, Qiagen), subcloned into pGEMT vectors (Promega), verified by sequencing, digested with SacI and SalI restriction endonucleases and cloned into the SacI/SalI site of pGAD424 vectors. For the experiments mapping yeast-two-hybrid interactions between deletion constructs (Table 2, Figure 8), plasmids were sequentially transformed (first pBTM116 bait constructs, then pGAD424 prey constructs) into LD40 yeast with LiOAc and plated in the appropriate media (first trp-, then trp-/leu-). Colony lift/X-gal assays were used to determine the strength of the interaction, judged by the intensity of blue staining. Interactions between Nova full length and partial deletion constructs were also tested as a control for the intensity of the blue staining.

Cloning of mouse brPTB cDNA

A gel-extracted (Qiaex II, Qiagen) 1.3 kb EcoRI fragment of coding sequence from the brPTB clone isolated in the yeast-two-hybrid screen was used to make a radioactive DNA probe (^{32}P-dATP, Amersham; Prime-it II Kit, Stratagene) and to screen an adult mouse brain cDNA phage library (Uni-ZAP XR, Stratagene). A total of 5.4×10^5 phage clones were screened (following the protocol in Molecular Cloning, by Sambrook, Fritch and Maniatis, Cold Spring Harbor Laboratory Press, 1989), and after three rounds of initial screening and two rounds of secondary isolation and hybridization, four single clones were identified, three of which were identical by restriction digest analysis. Sequencing revealed that the two unique clones were overlapping and contained the 1.6 kb predicted open reading frame and a 1.4 kb 3'UTR as shown in Figure 1.

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).

RNA preparation and Northern blot analysis

Total RNA was extracted from wild type mice using a modified guanidine-acid phenol protocol as previously described (Yang et al., 1998), separated on a 1 % agarose/2.2 M formaldehyde gel and transferred to a nylon membrane (NEN) by capillary transfer in 10X SSC (Molecular Cloning, by Sambrook, Fritch and Maniatis, Cold Spring Harbor Laboratory Press, 1989). The integrity and size of the RNA samples was determined by ethidium bromide staining of a duplicate gel. The membranes were UV cross-linked, and hybridized using the QuikHyb system and following the manufacturer's protocol (Stratagene). The radioactively labeled probes used to screen the membrane included the one used to clone brPTB cDNA (see above) and a 1.2 kb EcoRI fragment from the coding sequence of mouse PTB, also isolated in the yeast two-hybrid screen, gel extracted (Qiaex II, Qiagen) and labeled in the same way (^{32}P-dATP, Amersham; Prime-it II Kit, Stratagene). Exposure of the washed, wrapped membranes was carried out overnight (-80°C) on Kodak X-OMAT AR film.

Protein preparation and Western blot analysis

Fresh tissue specimens were obtained from adult wild type mice, homogenized in 1X RIPA buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 1 % NP-40, 0.5 % DOC, 0.1 % SDS) and left on ice for 20 minutes. Alternatively, the medium from cultured cells was aspirated and the cells were resuspended in 1X lysis buffer (1% Triton X-100, 10 mM Tris-Cl pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, complete protease inhibitors-Boehringer Mannheim) and vortexed. Cell debris were precipitated by centrifuging at 14,000 rpm for 15 minutes and the supernatant was passed through a 20G needle to shear chromosomal DNA. Proteins were separated by 10 % SDS-PAGE (following the protocol in Current Protocols in Protein Science, John Wiley & Sons, Inc, 1995) and transferred to PVDF membranes (Millipore). Equal

loading of total protein from each tissue (50 μg/lane) was determined by Bradford assay and verified by Coomassie blue staining of a duplicate gel. 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 primary antibodies in 5 % milk in R buffer at 4°C overnight and washed five 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 incubation for an hour at room temperature with secondary, HRP-conjugated antibodies (Jackson Immunoresearch) in 5 % milk in R buffer, the membranes were again washed five times with alternating high salt/high detergent buffers and the signal was detected by chemiluminescence (NEN). The membranes were wrapped (SaranWrap) and exposed on Biomax MR film (Kodak).

Generation of polyclonal anti-brPTB antibody

After an unsuccessful attempt to generate an antibody against a peptide unique to brPTB, a polyclonal anti-brPTB antibody was produced by injection of two rabbits with full-length recombinant brPTB fusion protein (see Fusion protein synthesis below) and Freund's adjuvant (Pocono Rabbit Farm & Laboratory). Pre- and post-immune test sera were assayed by Western Blot against purified recombinant PTB and brPTB fusion proteins (50 ng/lane) to determine antibody reactivity, specificity and appropriate dilution (see Figure 4). Of the two animals immunized with the full-length brPTB protein, one developed immunity against brPTB and its serum was used in all subsequent experiments at a 1:5000 dilution. The other animal exhibited background reactivity to brPTB protein even in the pre-immune state.

Antibodies

The following primary antibodies were used throughout this work:

POMA patients' Ri serum (Buckanovich et al., 1993)

Nova2-specific anti-peptide antibody (Yang, 1997)

polyclonal anti-HuA (generously provided by Dr. Joan Steitz)

polyclonal anti-PTB (generously provided by Dr. Doug Black)

monoclonal anti-myc (9E10, generously provided by Dr. Jan Karlseder)

monoclonal anti-T7 (Novagen)

monoclonal M5 anti-flag (Sigma)

polyclonal anti-GST (Sigma)

monoclonal anti-GFP (Clontech)

monoclonal anti-MAP2 (Sigma)

monoclonal anti-GFAP (DAKO)

polyclonal anti-matrin3 (generously provided by Dr. Somanathan)

polyclonal anti-coilin p80 (R288, generously provided by Dr.Gall)

polyclonal anti-Nopp140 (RE10, generously provided by Dr. Meier)

monoclonal anti-SIP-1 (generously provided by Dr. Dreyfuss)

monoclonal anti-Sm (Y12, generously provided by Dr. Gall)

monoclonal anti-SC35 (generously provided by Dr. Gall)

monoclonal anti-SR (16H3, Covance)

monoclonal anti-SR (1H4, Covance)

monoclonal anti-CTD domain of RNA polII (Covance).

A variety of HRP-, Cy2-, Cy3- and Cy5-conjugated species-specific secondary antibodies were used as appropriate, following dilutions suggested by the manufacturer (Jackson Immunoresearch). DAPI stain (Sigma) was used at 1000 μg/mL to visualize cell nuclei.

Fusion protein synthesis

Full-length Nova1 and full-length Nova2 recombinant GST-fusion proteins were produced and purified as previously described (Yang, 1997). Full-length T7/His tagged brPTB and PTB proteins were produced by subcloning EcoRI fragments from the yeast-two-hybrid prey vectors into pET21 (Novagen) and transforming BL21 competent cells, followed by standard IPTG-induction and purification by nickel-chelation chromatography.

GST pull-down assays

In vitro GST pull-down assays were performed as previously described (Yang, 1997). Briefly, the indicated amounts of GST and T7-tagged purified recombinant fusion proteins were incubated in a low stringency buffer and GST proteins were isolated using Glutathione-Sepharose 4B beads (Pharmacia) following the manufacturer's protocol. Any T7-tagged proteins pulled down with the GST proteins were eluted in SDS loading buffer, analyzed in SDS/PAGE and detected by Western blot. *In vivo* GST pull-down assays were performed by transfecting (Fugene6, Boehringer Mannheim) N2A cells with Nova1 and Nova2 eukaryotic GST fusion protein expression constructs (cloned in the pEBG vector, kindly provided by Dr. T Shishido). Cells were harvested two days later, lysed on ice for 45 minutes (1 % Triton X-100, 10 mM Tris-Cl pH 7.6, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, complete protease inhibitors-Boehringer Mannheim) and the DNA pellet was removed by centrifugation. Nuclei were disrupted by sonication and GST pull-downs were performed with Glutathione-Sepharose 4B beads (Pharmacia) following the manufacturer's protocol.

Cell cultures

All cell lines were maintained in 10% FBS/DMEM with penicillin/streptomycin, grown to 60% confluence in 6 well plates or chamber slides and transfected using Fugene6 according to the manufacturer's protocol (Boehringer-Mannheim). 293T cells (ATCC) are a primary human embryonal kidney cell line transformed with the SV40 T antigen. N2A cells (ATCC) are a mouse neuroblastoma cell line.

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, fixed (10 minutes in 2 % paraformaldehyde in PBS at room temperature), washed, permeabilized (10 minutes in 0.5 % NP-40 in PBS at room temperature), washed again, blocked (1 hour 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 hour at room temperature. 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 (Revlon). The slides were observed under multiple wavelength fluorescence from a mercury lamp (AttoArc), in an upright microscope (Zeiss Axiolpan). Pictures were taken with a digital camera (Hamamatsu Orca), using Openlab software (Improvision) and presented with Photoshop software (Adobe).

Immunofluorescence of tissue sections

For immunofluorescent staining of rat and mouse brains, tissue sections were fixed in formalin/PBS and paraffin embedded as described above. After permeabilization in 0.5 % NP-40, the sections were blocked in 0.2 % gelatin and 0.5 % bovine serum albumin in PBS. Primary anti-brPTB polyclonal antiserum (1:5000 dilution) and anti-MAP2 monoclonal antibody (SIGMA, 1:500), anti-GFAP monoclonal antibody (DAKO Corporation, 1:25) or POMA patient sera (1:500) were incubated with tissue sections at 4°C overnight. Proteins were visualized using Cy2 anti-mouse IgG and Cy3 anti-rabbit IgG or Cy5 anti-human IgG and Cy2 anti-rabbit IgG (all from Jackson ImmunoResearch) by confocal microscopy (Zeiss). We confirmed that sections stained with brPTB/Cy2 gave no signal when detected at the wavelength of Cy5 in the absence of POMA/Cy5.

Immunohistochemistry

Adult rats (male Sprague-Dawley), E 16 Rat embryo and P 5 mouse (male CD1) were used. Brains were perfused with formalin/PBS, paraffin embedded, and sectioned at 14 μm. After deparaffinization, sections were boiled in 0.01 M citric acid (pH 6.0) in a microwave for 10 minutes. All immune reactions were preceded by a blocking step (PBS, 0.05 % triton X-100, 2 % normal horse serum), and were carried out at 4°C overnight. All sections were washed (PBS, 0.05 % triton X-100), incubated with biotinylated secondary antibodies (Vector Laboratories) and washed again. Signals were enhanced by addition of HRP-conjugated avidin (Vector Laboratories), developed with

diaminobenzidene (DAB) in the presence of H_2O_2, and visualized by light microscopy using a Zeiss Axioplan microscope.

GFP-fusion constructs and live immunofluorescence

All GFP-Nova constructs were prepared by PCR of Nova fragments with specifically designed primers (Operon, see list below), gel-extracted (Qiaex II, Qiagen), sub-cloned into the pGEMT shuttle vector (Promega), verified by sequencing, digested with SalI and BglII restriction endonucleases and cloned into SalI/BglII sites of the pEGFP-C1 vector (Clontech). Plated N2A cells were transfected with Cesium Chloride preparations of the plasmid DNA and observed two days later live, under a mercury lamp (AttoArc) with an inverted microscope (Zeiss Axiovert S100). Pictures were taken as before. Specific amino acid mutations on all GFP constructs were made with the QuikChange Site-directed Mutagenesis Kit (Stratagene) and site specific primers (see list below) and verified by sequencing. The constructs presented in Figure 29 were prepared as follows:

GFP vector: empty GFP vector (pEGFP-C1, Clontech)

GFP-Nova1: full length Nova1 sequence (amino acids 1-506) inserted into pEGFP-C1

GFP-Nova2: full length Nova2 sequence (amino acids 1-489) inserted into pEGFP-C1

GFP-NLS: amino acids 25-41 of Nova1 protein (putative NLS) inserted into pEGFP-C1

GFP-NLS K27E: as in GFP-NLS with a mutation substituting glutamate for lysine 27

GFP-NLS K40E: as in GFP-NLS with a mutation substituting glutamate for lysine 40

GFP-NLS K27,40E: as in GFP-NLS with glutamate substituting for lysines 27 and 40

GFP-Nova1 K27E: as in GFP-Nova1 with a mutation substituting glutamate for lysine 27

GFP-Nova1 K40E: as in GFP-Nova1 with a mutation substituting glutamate for lysine 40

GFP-Nova1 K27,40E: as in GFP-Nova1 with glutamate substituting for lysines 27 and 40

GFP-Nova1 ΔNLS: as in GFP-Nova1 with a mutation deleting amino acids 24-41

GFP-Nova1 KH1/2: amino acids 1-242 of Nova1 protein inserted into pEGFP-C1

GFP-Nova1 sp/KH3: amino acids 242-503 of Nova1 protein inserted into pEGFP-C1

GFP-Nova1 spacer: amino acids 242-423 of Nova1 protein inserted into pEGFP-C1

GFP-Nova1 t.spacer: amino acids 242-360 of Nova1 protein inserted into pEGFP-C1

In vivo *splicing assays*

Cells (30% confluent in 6-well plates, Corning) were transfected with 0.25 μg of the GlyRα2 minigene (described in Jensen et al., 2000a), 0.125 μg of pCMV β-galactosidase (Gibco-BRL) and variable amounts of pcXHookNova1, pflagbrPTB and pcXHook empty vector. The total amount of DNA transfected in each experiment was kept constant by the addition of corresponding amounts of the appropiate empty vectors. Construction of pcXHookNova1 has been described (Jensen et al., 2000a); pflagbrPTB and pmycPTB were made by sub-cloning EcoRI fragments of the yeast-two-hybrid prey isolates of brPTB and PTB into pcDNAflag and pcDNAmyc respectively (both parent vectors were kindly provided by Drs. Jan Karlseder and Titia de Lange). RNA was collected 40 hours after transfection and RT-PCR was performed with primers (one radioactively labeled) specific for exons 2 and 4 of GlyRα2 as previously described (Jensen et al., 2000a). Exons 3A and 3B were distinguished by SspI digest of Exon 3A-containing PCR product and their ratio was determined by non-denaturing SDS-PAGE, followed by quantitation with a Molecular Dynamics Storm Phosphorimager and ImageQuant software. The $GABA_AR\gamma2$ minigene construct was made by Kate Dredge. Mutations on both minigene constructs were made with the QuikChange Site-directed Mutagenesis Kit (Stratagene) and specifically designed primers (Operon) and verified by sequencing.

Filter binding assays

Radioactively labeled 90 nt fragments of wild type or mutant GlyRα2 intron2 and an unrelated rRNA fragment were synthesized as described (Jensen et al., 2000b). $GABA_AR\gamma2$ 85 nt fragments of intronic RNA were made by PCR with specific primers, the 5' of which contained the T7 RNA polymerase sequence (Operon, see list below). Filter binding assays using Nova1 and brPTB fusion proteins were performed as described (Buckanovich and Darnell, 1997; Yang, et al., 1998) and the Kd was determined by graph analysis on KaleidaGraph software.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays were performed by combining Nova1 and brPTB fusion proteins, at the concentrations indicated, in gel shift buffer (250 mM KOAc, 50 mM Tris-OAc pH 7.7, 10 mM DTT, 5 mM $Mg(OAC)_2$, 1 mg/mL tRNA), adding radioactively labeled wild type or mutant GlyRα2 RNA fragments (final concentration 0.6 nM) and Ficoll loading buffer (final concentration 2.5% Ficoll) and incubating for 30 minutes at 4°C. The samples were separated by non-denaturing 8% PAGE/0.5 X TBE at 200V, 4°C, and exposed on Kodak Biomax MR film overnight (-80 °C).

Primers

For intronic GlyRα2 and $GABA_AR\gamma2$ RNA fragments:

#GlyRFr905'T7:

AGTAATACGACTCACTATAGGGATCATGCAGTTCTGGTTTAAT

#GlyRfr903': AGCTCCATCAACATCTGTGG

#T7GABA100:

GAAATTAATACGACTCACTATAGGGAGTAATTTGTCTTATTTTGTTTC

For mutations in PTB sites in GlyRα2 and $GABA_AR\gamma2$ minigenes and RNA fragments:

#Gly90PTBC/T5': GCAGTTCTGGTTTAATTTTTTTTTTTTGCAGTCTCATCATC

#Gly90PTBC/T3': GATGATGAGACTGCAAAAAAAAAAAAATTAAACCAGAACTC

#GABA-GA2.5': GTCTTATTTTGTTTCGATTTCTCGATTTTTTTTCCTTTTCC

#GABA-GA2.3':

GGAAAAGGAAAAAAAATCGAGAAATCGAAACAAAATAAGAC

#GABA-GA1.5': GCAATTCGATTTTCTGTCTACAAATCCAAAGCTTCTTCGG

#GABA-GA1.3': CCGAAGAAGCTTTGGATTTGTAGACAGAAAATCGAATTGC

For mutations in amino acids of the Nova1 NLS in pEGFP constructs:

#N1K27E5': CCGGACTCGCGGGAAAGGCCGCTTGAAGC

#N1K27E3': GCTTCAAGCGGCCTTTCCCGCGAGTCCGG

#N1K40E5': GCCGGCAGCACCGAGAGGACCAACACG

#N1K40E3': CGTGTTGGTCCTCTCGGTGCTGCCGGC

For brPTB deletion mutants in yeast-two-hybrid prey constructs:

#brPTB30: CTCGAGTGGACGGAATTGTCACTGAGG

#brPTB31: CTCGAGAAGATAAAATGGATGGGGC

#brPTB32: CTCGAGACAAAGAACTAAAGACAGATAATAC

#brPTB33: CTCGAGAGAGTGCGGTGACTCCAGC

#brPTB34: CTCGAGTTGTGAATTTGAATGTAAAATAC

#brPTB35: CTCGAGTGCCTGGAGTCTCAGC

#brPTB36rc: CGGCCGGATTGGAGTACTGGATGTAAA

#brPTB37rc: CGGCCGGTTTGGAAAAATCAATCC

#brPTB38rc: CGGCCGCCACTCGGCCAGCGG

#brPTB39rc: CGGCCGCGATTGTTGACTTGGAGAAAGAC

#brPTB40: GTCGACTGGACGGAATTGTCACTGAGG

#brPTB41: GTCGACAAATGGATGGGGCTCCCT

#brPTB42: GTCGACACAAAGAACTAAAGACAGATAA

#brPTB43: GTCGACAGAGTGCGGTGACTCCAGC

#brPTB44: GTCGACTTGTGAATTTGAATGTAAAATAC

Chapter 3 – A novel, brain-enriched homologue of PTB interacts with Nova

Introduction

The Nova proteins constitute a family of neuron-specific, RNA-binding proteins that have been implicated as the antigens in paraneoplastic opsoclonus-myoclonus ataxia (POMA). They have high homology with the hnRNP K protein with which they share their organization of three RNA-binding domains (RBD) of the KH (hnRNP K Homology) type. Nova1 was one of the very first human, neuron-specific, RNA-binding proteins (RBP) to be identified, but its role remained unclear at first. It was cloned by screening a human cerebellar cDNA expression library with a high-titer serum from a POMA patient (Buckanovich et al., 1993).

Using the same serum to screen a small cell lung cancer expression library, yielded a second family member named Nova2 (Yang et al., 1998). Interestingly, the expression patterns of the two proteins in neurons are almost complementary with Nova1 restricted to the hypothalamus and ventral midbrain, hindbrain and spinal cord and Nova2 present at highest levels in the cortex, thalamus, inferior colliculus, inferior olive and the external granule cell layers of the cerebellum (Buckanovich et al., 1993; Buckanovich et al., 1996; Yang et al., 1998).

The fact that Nova1 expression in the developing nervous system is restricted to neurons of the ventral brain stem, ventral spinal cord and cerebellum suggested initially that it might be important in the development and patterning of these structures

(Buckanovich et al., 1993). Indeed, later work showed that Nova1 is essential for neuronal viability since null mice exhibit apoptotic cell death in hindbrain and ventral spinal cord neurons (Jensen et al., 2000a).

Nova proteins are similar in their amino acid sequence and structural features to two other KH-type RBPs, hnRNP K and the fragile X mental retardation protein (FMRP) They all harbor two adjacent KH domains, a spacer and a third RNA-binding domain (KH domain in Nova and hnRNP K, RGG box in FMRP). Both FMRP and hnRNP K contain nuclear export sequences and have been shown to shuttle between the nucleus and the cytoplasm (Michael et al., 1997; Feng et al., 1997). Many nuclear KH-type RBPs have been shown to regulate pre-mRNA splicing in different organisms, such as MER-1 in yeast (Nandabalan and Roeder, 1995), PSI in Drosophila (Siebel et al., 1995), and SF1 and KSRP in mammals (Arning et al., 1996; Min et al., 1997). However, the best characterized role for hnRNP K and FMRP involves translation control in the cytoplasm (DeBoulle et al., 1993; Ostareck et al, 1997).

Because of the sequence homology of Nova with hnRNP K and the presence of consensus KH-type RNA-binding motifs, it was hypothesized that it too is an RNA-binding protein. A variety of *in vitro* and *in vivo* experiments demonstrated that Nova1 is an RNA-binding protein with a consensus sequence for binding and identified putative target RNA transcripts in the metabolism of which Nova1 could play a role (Buckanovich et al., 1996; Buckanovich et al., 1997). Similarly, Nova2 was found to bind RNA targets *in vitro* but with a sequence specificity that differed from Nova1 (Yang, 1997; Yang et al., 1998). However, the disparity in the RNA sequences is subtle and Nova proteins are able to bind each other's targets, albeit with slightly smaller affinity.

Besides clues obtained from sequence and structure similarities to hnRNP K and FMRP, insight into the function of Nova1 came from studies of its binding to specific physiologic RNA targets, such as the glycine receptor α2 subunit (GlyRα2) pre-mRNA (Buckanovich et al., 1997). Structural and biochemical studies of the Nova1 protein-RNA interaction demonstrated it is of high affinity, specificity and functional importance and determined the particular RNA sequence characteristics and peptide domain requirements (Buckanovich and Darnell, 1997; Lewis et al., 2000; Jensen et al., 2000b).

As well as making contacts with RNA, many RNA-binding proteins have been known to engage in protein-protein interactions that are likely to be important in mediating their biological function. For example, in the eukaryotic nucleus, many ribonucleoprotein (RNP) complexes are known to exist (e.g. the spliceosome) whose function is both dependent on and a consequence of specific protein contacts within these complexes. Thus, the search for specific protein interactions of RBPs is expected to yield important information on their function within the cell. Various domains of RBPs have been hypothesized to be responsible for such protein-protein interactions including the RNA-binding motifs themselves (RRM- or KH-type; Chen et al., 1997; Kielkopf et al., 2001), the RS domains of SR proteins (Valcarcel and Green, 1996; Xiao and Manley, 1997), and the glycine-rich domain of hnRNP A1 and Sxl (Cartegni et al., 1996; Wang et al., 1997).

Several findings support the idea that Nova proteins may undergo protein-protein interactions in addition to RNA binding. First, the co-crystal structure of a Nova-RNA complex reveals that most of the KH domain is accessible for protein-protein interactions, even when bound to RNA (Lewis et al., 2000). Dynamic light scattering

experiments showed that the KH3 of Nova1 is capable of forming dimers in solution and tetramers in the presence of RNA. Second, 32% of Nova1 (164/510 aa) and 39% of Nova2 (190/492 aa) peptide sequences consist of an unstructured spacer domain linking the second and third KH domains (Buckanovich et al., 1993; Yang et al., 1998). This spacer domain is the region of greatest sequence divergence between Nova1 and Nova2 family members (56% identity and 66% homology at the amino acid level; the KH domains are 89% identical, 96% homologous) and between Nova and several closely related KH-domain containing proteins (hnRNP E1/2, hnRNP K; Lewis et al., 1999). The spacer region contains several proline-rich and alanine/glycine-rich stretches that may represent protein interaction motifs. Third, there are several reports on the existence of homotypic and heterotypic interactions between KH-containing RBPs such as the Fragile X Mental Retardation Protein (FMRP) family (Siomi et al., 1996), the quaking protein (Zorn and Krieg, 1997), and the Sam68 protein (Chen et al., 1997). Furthermore, preliminary experiments from our lab have shown that Nova proteins are able to form homodimers and heterodimers *in vitro* and interact in the yeast-two-hybrid system (Yang, 1997; Yang et al., 1998).

To examine whether Nova1 and Nova2 interact specifically with other proteins and to identify what those may be, yeast two-hybrid screens were performed using Nova proteins as bait. The results shed new light to our understanding of Nova biology and made possible the exploration of the effect of specific protein interactions on the *in vivo* function of Nova.

Results

Identification of proteins that interact with Nova1 and Nova2

Yeast two-hybrid screens were performed in order to identify proteins that might interact with Nova1 and Nova2. Adult mouse brain and embryonic day 11.5 (E 11.5) whole mouse cDNA libraries were screened with various Nova bait constructs including full length Nova1, full length Nova2, and the Nova2 spacer (amino acids 230-407). The latter domain of Nova2 does not contain any RNA binding motifs and has been previously shown to mediate at least part of the homotypic and heterotypic interactions between Nova proteins (Yang, 1997).

As summarized in Table 1, a number of different RNA-binding proteins were isolated in the yeast-two-hybrid screens as possible Nova interactors, including two novel sequences. One of these novel sequences (novel RRM) contained RRM-type RNA-binding motifs and exhibited a brain-specific mRNA expression pattern (data not shown). The other, was named brain-enriched polypyrimidine tract-binding protein (brPTB) because it displayed a strong sequence similarity to PTB (also known as hnRNP I) and was characterized further (see below).

Strikingly, several of the proteins pulled out of the libraries belong to distinct, well-defined families such as the paraneoplastic antigen Hu protein family (HuA, HuB and HuD) and the PTB protein family (matrin3, hnRNP I/PTB, hnRNP L, and brPTB). Additionally, other proteins that play a role in RNA metabolism were found to interact with Nova2. SRp20 is a member of the SR family of splicing factors with a role in pre-mRNA processing and alternative splicing regulation (Zahler et al., 1992; Heinrichs and Baker, 1997; Neugebauer and Roth, 1997). YL2/p32, a protein known to co-purify with

ASF/SF2 and interact with the HIV protein Rev, is thought to mediate nuclear export of unspliced messages (Luo et al. 1994, Tange et al. 1996).

Cloning of brain-enriched Polypyrimidine Tract-Binding Protein (brPTB)

Since the sequence of brPTB identified in the yeast-two-hybrid screen lacked a portion of the 5' end (as determined by the absence of alignment with PTB in that region), an EcoRI fragment was used to make a radioactively-labeled probe and screen an adult mouse brain cDNA phage library. A 3.0 kb brPTB cDNA containing the predicted full length coding sequence was isolated after three rounds of screening (see Materials and Methods).

The 1.6 kb open reading frame encodes for a predicted 532 amino acids and is followed by a 1.4 kb 3' UTR (Figure 1). Sequence analysis shows that brPTB protein shares 73% identity and 84% similarity with PTB at the amino acid level. These numbers jump to 80% and 91%, respectively, over the four RRM domains (Figure 2). The brPTB cDNA also contains a putative nuclear localization signal (NLS) and a consensus polyadenylation signal.

A sub-family of RRM-type RNA-binding proteins interact with Nova

Interestingly, some of the proteins that were identified in the yeast two-hybrid screen with Nova baits (hnRNP I/PTB, hnRNP L, brPTB and matrin 3) form a distinct sub-group of the RRM-type family of RNA-binding proteins based on amino acid homologies over their RRM domains (Figure 2; Burd and Dreyfuss, 1994). While the sequence of these proteins matches the RRM domain consensus overall, they differ significantly over their ribonucleoprotein motifs (RNPs), which are thought to mediate the actual binding to

RNA targets (Nagai et al., 1990). Specifically, the PTB/brPTB family lacks the glycine/phenylalanine doublets (GF) that are prominent in the RNP1 motif of most RRMs and instead contain hydrophobic and polar amino acids. In the case of RNP2, the consensus among RRM-containing proteins calls for the presence of glycine and hydrophobic residues whereas the PTB/brPTB family in addition to the hydrophobic residues, contains a histidine and polar amino acids.

The interaction of these proteins with the Nova family members was equally robust (Table 2). In assays measuring the interaction between the PTB/brPTB family members with Nova1 and Nova2 in the yeast-two-hybrid system, their behavior was indistinguishable. All four proteins were capable, when fused to the Gal4 activation domain (GAD), to interact with Nova1 or Nova2 fused to the LexA binding domain and activate the LexA promoter thus conferring the ability to grow in his- media. In colony lift/X-gal assays, the same interactions resulted in strong blue staining confirming their strong interaction with Nova1 and Nova2.

brPTB expression in the nervous system

To examine the expression pattern of brPTB, Northern blot analysis was performed on several mouse tissues (Figure 3). brPTB mRNA was detected preferentially in the brain, including the cortex, cerebellum and brainstem, with very reduced levels evident in some organs outside the nervous system, most notably the heart. brPTB is expressed at similar levels across different developmental stages, ranging from E18 to adult. In contrast, a PTB probe detected mRNA in all tissues, although the possibility that this probe might cross-react with brPTB cannot be ruled out.

In order to evaluate the tissue expression of brPTB protein, we generated a specific antibody by immunizing rabbits with a purified, recombinant full-length brPTB fusion protein. While the pre-immune serum was non-reactive, the anti-brPTB polyclonal serum (α-brPTB) recognizes a protein of approximately 57-59 kDa (Figure 4A). Furthermore, this antibody is brPTB-specific at this dilution (1:5000) since it reacts with brPTB fusion protein but not with the same amount of PTB (Figure 4B).

Western blot analysis using this brPTB antibody revealed that brPTB protein, like its mRNA, is enriched in neural tissues, including the cortex, cerebellum, brainstem and spinal cord (Figure 5). brPTB is also expressed at high levels in the testis and at considerably lower levels in the liver, heart, lung, skeletal muscle and thymus with no expression evident at all in the spleen and kidneys. This pattern contrasts with the strict brain-specific expression of Nova proteins.

Immunohistochemical studies were performed in order to further define the expression pattern of brPTB protein in rat and mouse tissues (Figure 6). Strong reactivity to the brPTB-specific antibody was present in most brain tissues including cerebellum, brainstem, spinal cord and hypothalamus (Figure 6A, B, C and D). brPTB was also expressed in the peripheral nervous system and neural crest derivatives, including the dorsal root and trigeminal ganglia (Figure 6C and D), the cochlear spiral and intestinal ganglion cells (Figure 6E and F) and the adrenal medulla (Figure 6C). Non-neural tissues that expressed brPTB included the heart and, at low levels, the liver and adrenal glands (Figure 6C and data not shown).

To examine the subcellular distribution of brPTB, we used immunofluorescence microscopy on rat sections. brPTB protein is predominantly nuclear and stains both

neurons and astrocytes in confocal microscopy images of cells doubly stained for brPTB and the neuronal marker MAP2 (Figure 6G) or the astrocytic marker GFAP (Figure 6H). While not strictly a neuronal protein like Nova, brPTB is preferentially enriched in the nervous system where it stains neurons as well as other cell types such as astrocytes.

brPTB and Nova proteins interact in vitro *and* in vivo

To confirm the results of the yeast two-hybrid assay, and to demonstrate that the Nova-brPTB interaction is not dependent on additional yeast proteins or RNA, we assayed the interaction between brPTB and the Nova proteins *in vitro* and *in vivo*, using a GST pull-down assay. *In vitro*, purified brPTB specifically interacts with Nova1 and Nova2 fusion proteins, and this interaction is not affected by pre-treating each protein sample with RNAse A (Figure 7A).

In N2A cells, a neuroblastoma cell line that expresses endogenous brPTB (see below), transfected Nova1 and Nova2 GST-fusion expression vectors result in the precipitation of the endogenous brPTB protein (Figure 7B). In contrast, a control transfection with a vector expressing GST protein alone does not pull down brPTB. Taken together, these data demonstrate that brPTB and Nova proteins are capable of interacting directly and independently of an RNA intermediate in both the test tube and cultured cell lines.

We further dissected the interaction between brPTB and the Nova proteins by using truncation constructs in the yeast-two hybrid assay (Figure 8). The spacer domain of Nova2 (aa 230-407) lacks a KH-type RNA-binding motif but is sufficient in producing as robust an interaction with brPTB as were full length Nova1 or Nova2, confirming the

result of the GST pull-down assay that no RNA intermediate is required for the Nova-brPTB interaction.

In contrast, no single brPTB deletion construct was adequate in mediating a strong interaction with Nova. Multiple peptide domains in the brPTB protein seem to be required for the contact with Nova as witnessed by the fact that non-overlapping truncation mutants of brPTB were incapable of interacting with Nova as strongly as the full length protein. From these experiments, at least two important regions in brPTB can be identified that are necessary for the interaction with Nova, one in the N terminus and another in the C terminus. However, these conclusions are preliminary since the question of functionality and proper structural folding of the truncation mutants in the yeast system needs to be addressed. The truncation constructs were indeed capable of expressing proteins of the appropriate size as judged by Western blot assays of yeast cell lysates (data not shown).

Discussion

A novel, brain-enriched, PTB-like protein has been identified that specifically interacts with the neurologic disease antigen Nova. Several earlier studies predicted the existence of a neuronal isoform of PTB. Three distinct isoforms of PTB are known, each with different sizes of a spacer region between the second and third RNA binding domains (Gil et al., 1991; Patton et al., 1991; reviewed in Wagner and Garcia-Blanco, 2001). The existence of a neuronal PTB homologue was predicted from studies of cell extracts that were able to replicate aspects of neuron-specific alternative splicing *in vitro* (reviewed in Grabowski, 1998). These studies documented a PTB-like protein species enriched in nuclear extracts of brain tissue (Ashiya and Grabowski, 1997) and retinoblastoma cell lines (Chan and Black, 1997) that migrated more slowly on SDS-PAGE than PTB (around 59 kDa; named p59 or nPTB). The brPTB cDNA clone identified here predicts a protein that is slightly larger than PTB (57.6 vs. 57.2 kDa; Gil et al., 1991), and is most likely the previously identified p59/nPTB brain-enriched species.

Yeast two-hybrid screens using Nova proteins as bait identified several PTB-related RNA-binding proteins. Four of the most robustly interacting proteins in these screens were PTB, hnRNP L, matrin3 and brPTB. These proteins constitute a subfamily of RRM-type RNA binding proteins, as first suggested by comparisons of PTB and hnRNP L (Ghetti et al., 1992), because their RRM motifs are strongly homologous with each other but only weakly homologous to the canonical RRM consensus sequence. The interactions between Nova and the various members of this subfamily suggest that they may be mediated by a domain shared among these proteins, which seems likely to include the atypical RRM motif itself. However, mapping of the interaction between

Nova and brPTB failed to reveal a single deletion mutant that interacted strongly with Nova, suggesting either that the full-length protein is necessary for the interaction or that several different domains are important for a robust interaction. It is also possible that improper structural folding of the deletion constructs precluded any interaction.

While matrin3 was the only protein that was isolated with both Nova1 and Nova2 baits, it can be further supported from the results of the yeast-two-hybrid screens that the two Nova proteins participate in comparable types of protein-protein interactions and, quite possibly, through the same peptide domains. First, Nova1 and Nova2 have very high amino acid sequence identity and prominent structural similarities (Buckanovich et al, 1996; Yang et al, 1998; Lewis et al, 1999; Lewis et al, 2000). Second, they interact homotypically and heterotypically with each other (Yang, 1997; Yang et al., 1998; Figure 8). Third, they are able to function as baits in yeast-two-hybrid screens and identify proteins belonging to the same family of RNA-binding proteins: Nova1 interacts with HuB, HuD and matrin3 and Nova2 interacts with HuA, hnRNP L, matrin3, PTB and brPTB (Table 1). These interactions of Nova proteins are highly likely to be mediated by similar structural motifs and point to their possible interchangeability within the nervous system. Preliminary evidence from our lab has hinted at a compensatory mechanism for the expression of Nova2 in Nova1-null mice (M. Ruggiu and R. Darnell, unpublished observations).

The region of highest sequence divergence between Nova1 and Nova2 is the spacer region between RNA-binding domains KH2 and KH3. It has not been possible to use the Nova1 spacer as bait in a yeast-two-hybrid screen as it activates the LexA promoter on its own without the need for a GAD-fusion prey. However, given the prey

isolated with the Nova2 spacer and their resemblance to the ones isolated by Nova1 and Nova2 full-length constructs, it is unlikely that drastically different proteins would have been identified by a screen with the Nova1 spacer domain as bait. Furthermore, full-length Nova1 and Nova2 constructs exhibit the same behavior in their interactions with some of the prey isolated in the screens performed here (see Table 2 and Figures 7 and 8). For a complete list of prey isolated with the Nova2 spacer and their interactions with Nova constructs in yeast-two-hybrid see the Appendix.

The experiments presented have identified proteins that interact with Nova1 and Nova2. In the yeast-two-hybrid screens, some proteins were isolated independently in different screens or had sequence and domain similarities with other prey, even belonging to the same sub-family of proteins. Because of this and by virtue of their expression pattern and their functional attributes it seems likely that these interactions are real and biologically significant. In one example, that of the newly-identified, brain-enriched homologue of PTB, the interaction with Nova has been verified *in vitro* and *in vivo*. Thus, attractive hypotheses can be formed about the effect that these interactions may have on the function of Nova proteins and about the roles that Nova proteins may play in RNA metabolism within the cellular environment.

The expression of brPTB is significantly enriched in the brain at the mRNA and protein level (this work; Lillevali et al., 2001). It is also robustly expressed in the testis and at much lower levels in the heart and skeletal muscle. The expression of PTB mRNA is thought to be mostly ubiquitous (Markovtsov et al. 2000; Polydorides et al., 2000; Figure 3) although this is controversial as some reports claim significantly reduced levels in adult brain (Patton et al., 1991; Lillevali et al., 2001) and skeletal muscle (Lillevali et

al., 2001). Reports on the protein expression of PTB are also contradicting in that it is found in several neural cell lines (Markovtsov et al. 2000) but not in nuclear extracts from rat brain (Ashiya and Grabowski, 1997).

Several lines of evidence suggest that the interaction between brPTB and Nova is real and of biological significance. Both proteins are expressed in the same cells, i.e. neurons, and while brPTB is also expressed in other cells of the nervous system, such as glial cells, as well as in other tissues altogether, its brain-enriched expression pattern suggests that it is of functional importance in the nervous system. Nova and brPTB are predominantly nuclear proteins and share homology with hnRNP K and I, respectively, both of which are mostly nuclear proteins that are known to participate in various aspects of RNA metabolism. The two proteins are able to interact *in vitro* without any RNA intermediates and *in vivo* as GST-Nova fusion constructs overexpressed in N2A cells pull down endogenous brPTB protein. An attempt to co-immunoprecipitate endogenous proteins from brain tissues using either the anti-brPTB polyclonal antibody or anti-Nova patient serum was unsuccessful. In the latter case, it is possible that the anti-Nova serum disrupted protein-protein interactions, since it is thought to recognize parts of the spacer and third KH domains of the Nova proteins which have been shown here to mediate, at least partially, the interaction with brPTB (Buckanovich et al., 1996; Dredge and Darnell, personal communication; Figure 8). To verify that brPTB and Nova proteins interact functionally, immunofluorecence studies were performed on their nuclear localization (Chapter 5). The effect of this interaction was tested with *in vivo* splicing assays that have recently been developed for Nova (Chapter 4).

The interactions with known splicing factors and regulators (such as SRp20 and hnRNP I/PTB) would suggest that Nova contributes to the splicing regulation of some of its RNA targets (see Chapter 4). Another function for Nova proteins could be in the nuclear export of mRNA messages due to its interaction with proteins that participate in such transport (e.g. hnRNP L and hnRNP I/PTB). The cellular protein p32/YL2 was originally identified due to its co-purification with the SR protein ASF/SF2 from HeLa cells (Krainer et al., 1991). It has been shown to interact with many viral proteins, including the HIV Rev and Tat proteins (Luo et al., 1994; Yu et al., 1995). The murine homologue of p32, called YL2, increases the nuclear export of unspliced viral messages mediated by Rev (Luo et al., 1994). Furthermore, Rev acts a bridge between its response element (RRE) on the RNA and p32 (Tange et al., 1996). Together with the interaction between p32 and ASF/SF2, this suggests that p32 acts to mediate an interaction between Rev and the splicing machinery, perhaps allowing Rev-bound viral RNAs to bypass the splicing apparatus and other checkpoints and to be exported unspliced. In concert with this theory, p32 has been found to specifically inhibit ASF/SF2 phosphorylation and thus inactivate it as a splicing enhancer or repressor (Petersen-Mahrt et al., 1999). Since Nova contains a Rev-like NES sequence its interaction with p32/YL2, if proven, can have significant functional implications in its action in splicing and nuclear export.

The interaction of Nova with the Hu family of proteins is interesting because these proteins are paraneoplastic antigens as well and may suggest an interplay among proteins whose ectopic expression appears to be beneficial for some tumors. The Hu proteins participate in the control of mRNA stability and translation and a role can be envisioned in these processes for the small but significant portion of Nova that is

cytoplasmic. Finally, many of these proteins (such as SRp20, PTB, matrin3, hnRNP L) form distinct structures in the eukaryotic nucleus and the participation of Nova in the formation of such bodies will be examined (see Chapter 5).

Table 1. Yeast two-hybrid screens with Nova protein constructs.

LD40 yeast host strains were transformed with the indicated bait constructs (Bait, LexA fusions) and mouse libraries (Library, GAD fusions). Positive prey were isolated by growth on His^- media and selected by positive (blue) LacZ staining in a colony lift/X-gal assay (see Materials and Methods). The identities of the interacting proteins were determined by examining prey sequences against the NCBI database (Prey isolated). E 11.5: embryonic day 11.5 whole mouse library. # Clones: total number of independent clones in the library. % scr: percentage of clones in the library that were screened after multiple transformations. aa: amino acid sequence. The first two full-length Nova2 screens presented here were the result of previous work (Yang, 1997).

Bait	Library	# Clones	% scr.	Prey isolated
Nova2 full length	adult brain	1.5×10^6	407	matrin3 HuB brPTB
Nova2 full length	E 11.5	1.2×10^6	758	matrin3 PTB SRp20 hnRNP L
Nova2 (aa 230-407)	E 11.5	1.2×10^6	167	p32/YL2 HuA novel RRM
Nova1 full length	adult brain	1.5×10^6	107	matrin3 HuB HuD
Nova1 full length	E 11.5	1.2×10^6	142	matrin3

Figure 1. Nucleotide and amino acid sequence of mouse brPTB.

The cDNA sequence of mouse brPTB contains a 1.6 kb open reading frame encoding for a predicted 532 amino acids. The four RRM-type RNA-binding domains are boxed. A putative nuclear localization sequence (NLS) is marked with a double underline. The polyadenylation signal located approximately 300 bp upstream of the poly(A) tail is underlined. A bold box indicates an amino acid not present in the cDNA clone from the yeast two-hybrid library. Nucleotide number from the start of the clone is indicated on the right. ***: stop codon.

```
CTGGCTGCGTGGCTCGGTTCTTGGGAGCGAAGCTTTGTCCGGTTCGGCA  ATG GAC GGA ATT GTC ACT GAG GTT GCT GTT GGT GTG AAG AGA GGA TCT GAC GAG   103
                                                    Met Asp Gly Ile Val Thr Glu Val Ala Val Gly Val Lys Arg Gly Ser Asp Glu

CTA CTC TCA GGC AGT GTT CTC AGC AGC CCC AAC TCT AAT ATG AGT GGC ATG GTA GTT ACA GCC AAC GGT AAC GAT AGT AAG AAA TTT AAA GGA   196
Leu Leu Ser Gly Ser Val Leu Ser Ser Pro Asn Ser Asn Met Ser Gly Met Val Val Thr Ala Asn Gly Asn Asp Ser Lys Lys Phe Lys Gly

GAA GAT AAA ATG GAT GGG GCT CCC TCT CGT GTA CTT CAC ATT CGA AAG TTA CCT GGT GAA GTG ACT GAA ACA GAA GTT ATT GCT TTA GGT TTA   289
Glu Asp Lys Met Asp Gly Ala Pro Ser Arg Val Leu His Ile Arg Lys Leu Pro Gly Glu Val Thr Glu Thr Glu Val Ile Ala Leu Gly Leu

CCT TTT GGT AAG GTG ACT AAC ATC CTT ATG CTG AAA GGA AAA AAC CAG GCA TTT TTG GAA CTG GCA ACA GAG GAA GCA GCT ATT ACT ATG GTT   382
Pro Phe Gly Lys Val Thr Asn Ile Leu Met Leu Lys Gly Lys Asn Gln Ala Phe Leu Glu Leu Ala Thr Glu Glu Ala Ala Ile Thr Met Val

AAT TAC TAT TCT GCT GTG ACA CCT CAT CTT CGT AAC CAA CCA ATT TAC ATC CAG TAC TCC AAT CAC AAA GAA CTA AAG ACA GAT AAT ACA TTA   475
Asn Tyr Tyr Ser Ala Val Thr Pro His Leu Arg Asn Gln Pro Ile Tyr Ile Gln Tyr Ser Asn His Lys Glu Leu Lys Thr Asp Asn Thr Leu

AAC CAA CGT GCG CAA GTA GTT CTT CAA GCT GTG ACA GCG GTC CAG ACA GCA AAT ACA CCT CTT AGT GGC ACC ACA GTC AGT GAG AGT GCG GTG   568
Asn Gln Arg Ala Gln Val Val Leu Gln Ala Val Thr Ala Val Gln Thr Ala Asn Thr Pro Leu Ser Gly Thr Thr Val Ser Glu Ser Ala Val

ACT CCA GCC CAG AGT CCA GTA CTT AGA ATA ATT ATT GAC AAT ATG TAC TAC CCT GTA ACA CTT GAT GTC CTT CAC CAA ATA TTT TCT AAG TTT   661
Thr Pro Ala Gln Ser Pro Val Leu Arg Ile Ile Ile Asp Asn Met Tyr Tyr Pro Val Thr Leu Asp Val Leu His Gln Ile Phe Ser Lys Phe

GGT GCT GTA TTG AAG ATA ATC ACA TTT ACA AAA AAC AAC CAG TTT CAG GCT TTG CTC CAG TAT GGT GAT CCG GTA AAC GCT CAA CAA GCC AAG   754
Gly Ala Val Leu Lys Ile Ile Thr Phe Thr Lys Asn Asn Gln Phe Gln Ala Leu Leu Gln Tyr Gly Asp Pro Val Asn Ala Gln Gln Ala Lys

CTA GCC CTA GAT GGT CAA AAT ATT TAT AAT GCT TGC TGT ACC CTA AGG ATT GAT TTT TCC AAA CTT GTG AAT TTG AAT GTA AAA TAC AAC AAT   847
Leu Ala Leu Asp Gly Gln Asn Ile Tyr Asn Ala Cys Cys Thr Leu Arg Ile Asp Phe Ser Lys Leu Val Asn Leu Asn Val Lys Tyr Asn Asn

GAT AAA AGT AGG GAT TAT ACT CGA CCT GAT CTG CCA TCT GGA GAC GGC CAG CCT GCG TTA GAC CCA GCC ATT GCT GCA GCA TTT GCC AAG GAG   940
Asp Lys Ser Arg Asp Tyr Thr Arg Pro Asp Leu Pro Ser Gly Asp Gly Gln Pro Ala Leu Asp Pro Ala Ile Ala Ala Ala Phe Ala Lys Glu

ACA TCC CTA CTA GCT GTT CCA GGG GCT CTC AGT CCT TTG GCT ATT CCA AAT GCT GCT GCA GCA GCT GCT GCC GCT GCC GCT GGC CGA GTG GGC   1033
Thr Ser Leu Leu Ala Val Pro Gly Ala Leu Ser Pro Leu Ala Ile Pro Asn Ala Ala Ala Ala Ala Ala Ala Ala Ala Ala Gly Arg Val Gly

ATG CCT GGA GTC TCA GCT GGT GGC AAT ACA GTC CTG TTG GTT AGC AAT TTA AAT GAA GAG ATG GTT ACG CCC CAA AGT CTG TTT ACC CTC TTC   1126
Met Pro Gly Val Ser Ala Gly Gly Asn Thr Val Leu Leu Val Ser Asn Leu Asn Glu Glu Met Val Thr Pro Gln Ser Leu Phe Thr Leu Phe

GGT GTT TAT GGA GAT GTG CAG CGC GTG AAG ATT CTG TAC AAT AAG AAA GAC AGT GCT CTG ATA CAG ATG GCT GAT GGG AAC CAG TCC CAG CTC   1219
Gly Val Tyr Gly Asp Val Gln Arg Val Lys Ile Leu Tyr Asn Lys Lys Asp Ser Ala Leu Ile Gln Met Ala Asp Gly Asn Gln Ser Gln Leu

GCC ATG AAT CAT CTT AAT GGG CAG AAG ATG TAT GGA AAA ATT ATT CGT GTT ACT CTC TCT AAA CAT CAG ACT GTG CAA CTA CCT CGA GAG GGA   1312
Ala Met Asn His Leu Asn Gly Gln Lys Met Tyr Gly Lys Ile Ile Arg Val Thr Leu Ser Lys His Gln Thr Val Gln Leu Pro Arg Glu Gly

CTT GAT GAT CAA GGG CTA ACA AAA GAT TTT GGG AAT TCA CCA CTG CAC CGT TTT AAA AAA CCG GGA TCC AAA AAC TTT CAG AAC ATT TTC CCT   1405
Leu Asp Asp Gln Gly Leu Thr Lys Asp Phe Gly Asn Ser Pro Leu His Arg Phe Lys Lys Pro Gly Ser Lys Asn Phe Gln Asn Ile Phe Pro

CCT TCT GCT ACC CTT CAC CTG TCT AAC ATC CCC CCT TCT GTA GCA GAA GAG GAT CTG CGA ACT CTG TTT GCC AAC ACC GGG GGC ACT GTG AAA   1498
Pro Ser Ala Thr Leu His Leu Ser Asn Ile Pro Pro Ser Val Ala Glu Glu Asp Leu Arg Thr Leu Phe Ala Asn Thr Gly Gly Thr Val Lys

GCA TTT AAG TTT TTT CAA AGA GAT CAC AAA ATG GCT CTT CTT CAG ATG GCA ACA GTG GAG GAA GCT ATT CAG GCT TTG ATT GAT CTT CAT AAT   1591
Ala Phe Lys Phe Phe Gln Arg Asp His Lys Met Ala Leu Leu Gln Met Ala Thr Val Glu Glu Ala Ile Gln Ala Leu Ile Asp Leu His Asn

TAT AAC CTT GGA GAA AAC CAT CAT CTG AGA GTG TCT TTC TCC AAG TCA ACA ATC TAA GCACGGGAGATGAAGATGGCGGGCAGATCCCATTGTTGGTGTCATCA   1695
Tyr Asn Leu Gly Glu Asn His His Leu Arg Val Ser Phe Ser Lys Ser Thr Ile ***

CCTATTGACTGTTCAGAAAAGTGGGGGACCAGAGTTTGATTTTTTTCATGCTGTTATCATTCCTTGGTTATAAAATGAAATGGCATATGTAAAGGCAGAGTTGCTAACTGCTGTGTTTCATCT   1818
GTTCAATAGGGAAGCCATTTTTGTCTGTTTAAATTTTTAGTTTAATTTTGCTTTTTTTTTTTTTTTTCCAACTTAGTTGACATACGTGCCTTACAAAGGAAAACTAGTGTTGCTATTCTGCATT   1941
TACTAGGAAAAAGGAATTGGTTGTTTAGGGCACATCGTTATGTGGGAATTAAAATATGTTTGGGCAGGGGTGTGTCAAAAGGTTAAGTTTTTATTTCTCCTGCTTGGAACTTTCTTTGAATTA   2064
TTGGCTTATCACATTTCTTTCTATTTAATCTAATAAGATACTTGATACTGAGAGTATAAAACAGCATTTTTAGTTTTTACTATCTTAGGCTTTATTGCTTTTGAAAACATTGGCCTTTTGTAT   2187
CTCACAAATCTGGTCTAGATTCAGTTATGAATGTAGGCATTAGTTAAAATTAACAAGATGCAGAGTATTAATTTCTTAAGACAAGTGATTTCTGTAAGTTTGAGCCCTATGTGGAAAGCATTG   2310
TGGAACCTTAACCTTTTTGTACACACTCTTGTGGGACGTGTCATATAAATGTCAGCACTAAGTAATGTCTTGTTTGTGGCTGAATATTTTTCGTAGATGTTTTTGAAGTGACATGACTACGTG   2433
CATTTAAATATATATTGCCATCCTTAGTTTGTAATTAAGATTTGGAAAATGGTTGTGGATTTCTGAGCATGTGCAGACTGGTCTAGCTAGTTCAGGAACTGGTGCATGTATTTTTCAAAGACA   2556
AAGAAAGTGTACTGCGAAAACTTGCAGGAAGATTAATTTTGTGGCAGTTTTCTAAAACTGACAACCAGGTGGGACCAAAGTTTATGTGCCTTTAGTCTTAATTTACCTTGCATTGTAATATTC   2679
AGTTTTTAATAAATCTTCAAAATATTTTGTATTTAGGAATAGATCTGACTTTAATAAAAACATGGCTCAGAATCTACAGGTCAAATTTATTTGAACAGTTCTTGTCAATCTGAATTGTTGATTC   2802
TGTTAAATGACCAATACTTTTTGAAATTGATGTACTTAGTTTCAAGATTCATAGATTCTGTTATCTATGTAGACAGAATGGTCATGTATATTTTCTATTAGTTGAGTTTTTACATCTTTAGAA   2925
ATGTAAAATTGTATAGTTTGAAAGCGGCACAATTAAAAATTAATTTTCTAACAAAAAAAAAAAAAAAAAAA                                                       2999
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Figure 2. A sub-family of RRM-type RBPs interacts with Nova.

Alignment of the RRM motifs from mouse brPTB (Figure 1), mouse PTB (Bothwell et al., 1991), human hnRNPL (Pinol-Roma et al., 1989), and rat matrin3 (Belgrader et al., 1991) shows that these proteins comprise a distinct subset of RRM-containing proteins based on the presence of RNP motifs that do not conform to those present in the RRM consensus (Burd and Dreyfuss, 1994). Similar amino acids are color coded and dashes are inserted in the sequence for alignment purposes.

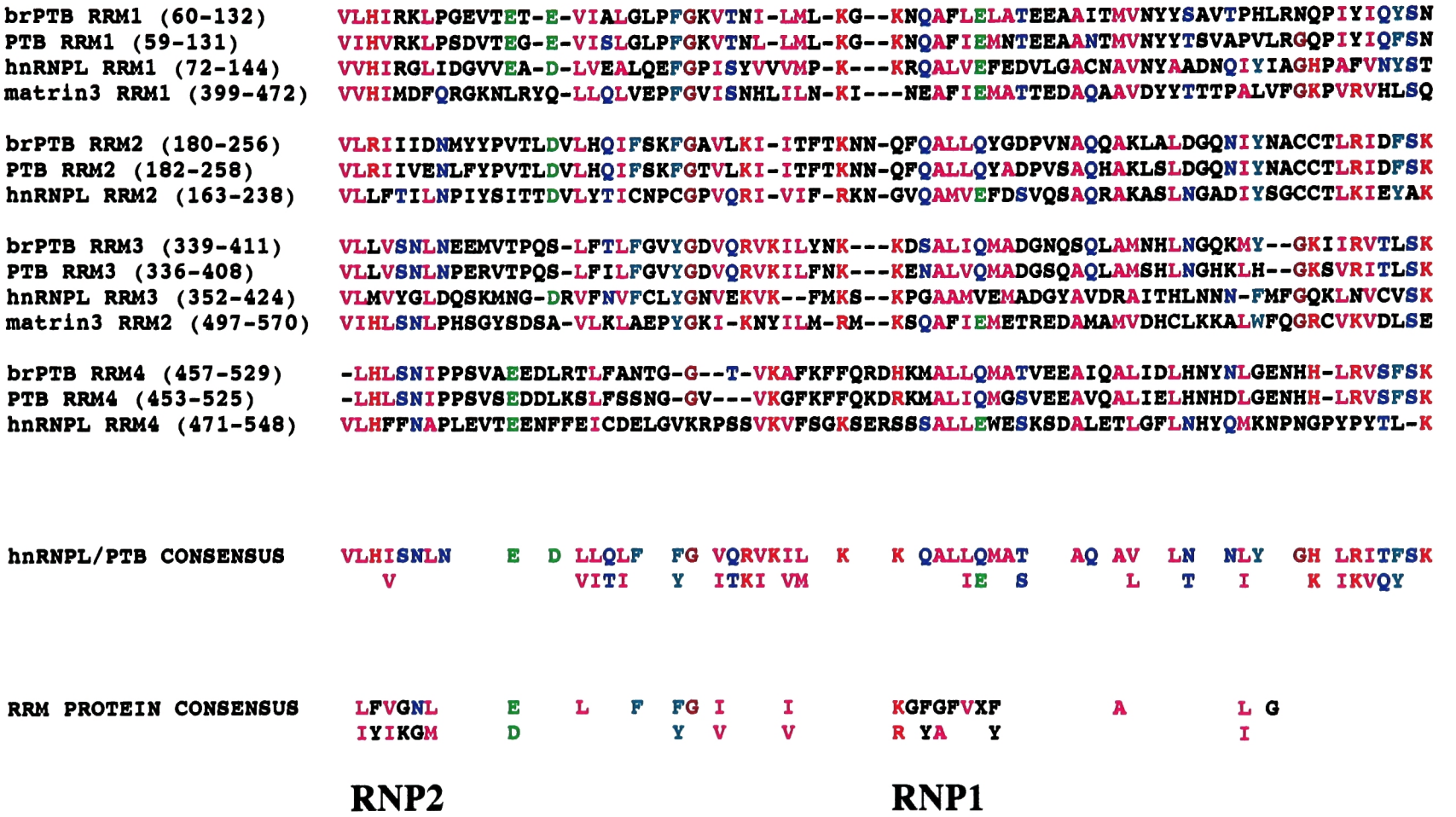

brPTB RRM1 (60-132) VLHIRKLPGEVTET-E-VIALGLPFGKVTNI-LML-KG--KNQAFLELATEEAAITMVNYYSAVTPHLRNQPIYIQYSN
PTB RRM1 (59-131) VIHVRKLPSDVTEG-E-VISLGLPFGKVTNL-LML-KG--KNQAFIEMNTEEAANTMVNYYTSVAPVLRGQPIYIQFSN
hnRNPL RRM1 (72-144) VVHIRGLIDGVVEA-D-LVEALQEFGPISYVVVMP-K---KRQALVEFEDVLGACNAVNYAADNQIYIAGHPAFVNYST
matrin3 RRM1 (399-472) VVHIMDFQRGKNLRYQ-LLQLVEPFGVISNHLILN-KI---NEAFIEMATTEDAQAAVDYYTTTPALVFGKPVRVHLSQ
brPTB RRM2 (180-256) VLRIIIDNMYYPVTLDVLHQIFSKFGAVLKI-ITFTKNN-QFQALLQYGDPVNAQQAKLALDGQNIYNACCTLRIDFSK
PTB RRM2 (182-258) VLRIIVENLFYPVTLDVLHQIFSKFGTVLKI-ITFTKNN-QFQALLQYADPVSAQHAKLSLDGQNIYNACCTLRIDFSK
hnRNPL RRM2 (163-238) VLLFTILNPIYSITTDVLYTICNPCGPVQRI-VIF-RKN-GVQAMVEFDSVQSAQRAKASLNGADIYSGCCTLKIEYAK
brPTB RRM3 (339-411) VLLVSNLNEEMVTPQS-LFTLFGVYGDVQRVKILYNK---KDSALIQMADGNQSQLAMNHLNGQKMY--GKIIRVTLSK
PTB RRM3 (336-408) VLLVSNLNPERVTPQS-LFILFGVYGDVQRVKILFNK---KENALVQMADGSQAQLAMSHLNGHKLH--GKSVRITLSK
hnRNPL RRM3 (352-424) VLMVYGLDQSKMNG-DRVFNVFCLYGNVEKVK--FMKS--KPGAAMVEMADGYAVDRAITHLNNN-FMFGQKLNVCVSK
matrin3 RRM2 (497-570) VIHLSNLPHSGYSDSA-VLKLAEPYGKI-KNYILM-RM--KSQAFIEMETREDAMAMVDHCLKKALWFQGRCVKVDLSE
brPTB RRM4 (457-529) -LHLSNIPPSVAEEDLRTLFANTG-G--T-VKAFKFFQRDHKMALLQMATVEEAIQALIDLHNYNLGENHH-LRVSFSK
PTB RRM4 (453-525) -LHLSNIPPSVSEDDLKSLFSSNG-GV---VKGFKFFQKDRKMALIQMGSVEEAVQALIELHNHDLGENHH-LRVSFSK
hnRNPL RRM4 (471-548) VLHFFNAPLEVTEENFFEICDELGVKRPSSVKVFSGKSERSSSALLEWESKSDALETLGFLNHYQMKNPNGPYPYTL-K
hnRNPL/PTB CONSENSUS VLHISNLN E D LLQLF FG VQRVKIL K K QALLQMAT AQ AV LN NLY GH LRITFSK
V VITI Y ITKI VM IE S L T I K IKVQY
RRM PROTEIN CONSENSUS LFVGNL E L F FG I I KGFGFVXF A L G
IYIKGM D Y V V R YA Y I
RNP2
RNP1

Table 2. Interactions of Nova1 and Nova2 with RRM-type proteins.

LD40 yeast host strains were transformed with the indicated bait (LexA fusion) and prey (GAD fusion) constructs and their interaction was scored by staining for LacZ expression and growth on His^- media. Negative controls included unrelated proteins fused to GAD (TD1) and LexA (TRF1). +++, robust staining and growth; –, no staining or growth; GAD: Gal4 Activation Domain; ND: not determined.

	Bait (LexA)				
	Lac Z			His$^-$	
	TRF1	Nova1	Nova2	Nova1	Nova2
Prey (GAD)					
TD1	—	—	—	—	—
brPTB	—	+++	+++	+++	+++
hnRNP I/PTB	—	ND	+++	ND	+++
hnRNP L	—	ND	+++	ND	+++
matrin 3	—	+++	+++	+++	+++

Figure 3. brPTB mRNA levels are enriched in the brain.

Northern blot of total RNA from the indicated mouse tissues and developmental stages (20 μg/lane), probed with a 1.3 kb DNA probe from the coding sequence of brPTB (top panel). The blot was stripped and reprobed with a 1.2 kb probe from the coding sequence of PTB (bottom panel). The two messages were of approximately the same size (3.6 kb).

spleen
liver
kidney
heart
small intestine
cortex
cerebellum/brainstem
P7 total brain
P0 total brain
E18 total brain
brPTB
PTB

Figure 4. Production of a brPTB-specific polyclonal antibody.

(A) Western blot of total rat cerebellum and brainstem tissue proteins (20 μg/lane), probed with rabbit polyclonal anti-brPTB serum (left panel) or pre-immune serum (right panel, P.I.) from the same animal (see Materials and Methods). A band of approximately 57 kDa is recognized only after the immunization with full length brPTB fusion protein. (B) Western blot of purified recombinant PTB and brPTB fusion proteins as indicated (50 ng/lane), probed with anti-brPTB serum (left panel, 1:5000 dilution). Polyclonal anti-brPTB serum specifically recognizes the brPTB fusion protein but not PTB. A duplicate blot was probed with PTB polyclonal antibody (right panel, 1:500 dilution) and served as a loading control. Molecular weight markers are indicated in the middle (kDa).

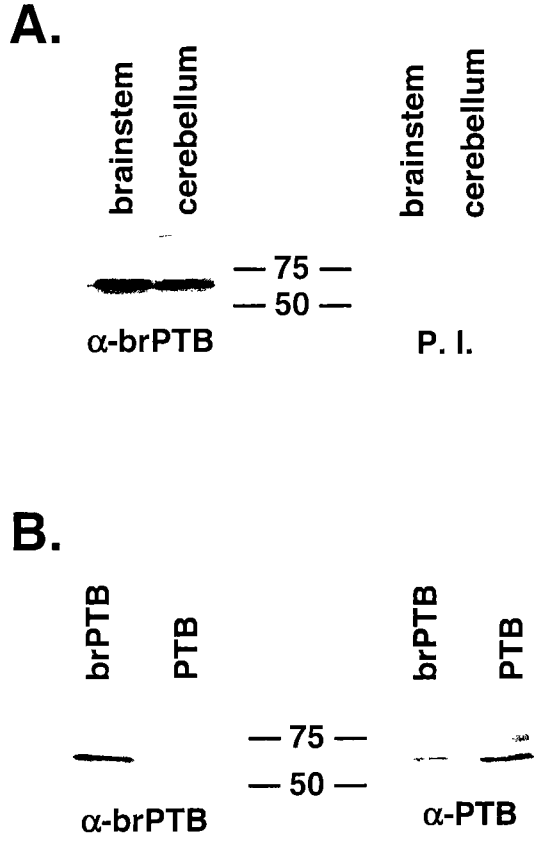

A.
brainstem
cerebellum
brainstem
cerebellum
— 75 —
— 50 —
α-brPTB
P. I.
B.
brPTB
PTB
brPTB
PTB
— 75 —
— 50 —
α-brPTB
α-PTB

Figure 5. brPTB protein levels are enriched in the brain.

Western blot of the indicated mouse tissues (50 μg total protein/lane), probed with various primary antibodies. Molecular weight markers are indicated on the left (kDa). Polyclonal anti-brPTB antibody recognizes a 57 kDa band most prominent in brain tissues and the testis (top panel). POMA patient serum shows that Nova proteins are restricted in the brain (middle panel). A polyclonal anti-HuA antibody recognizes the ubiquitously expressed Hu isoform (Fan and Steitz, 1998a) and served as a loading control (bottom panel).

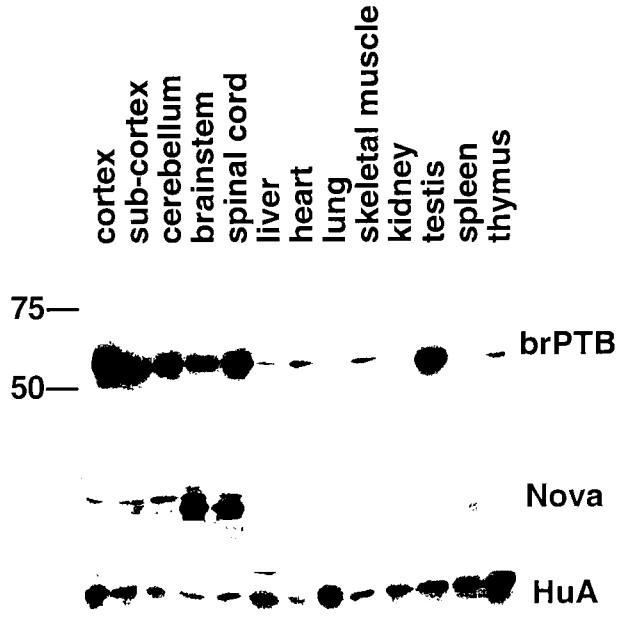

cortex
sub-cortex
cerebellum
brainstem
spinal cord
liver
heart
lung
skeletal muscle
kidney
testis
spleen
thymus
75—
50—
brPTB
Nova
HuA

Figure 6. Immunoreactivity of brPTB in rat and mouse tissues.

(A) Sagittal section from E16 rat, stained with brPTB polyclonal antibody shows reactivity in most cells of the brain, including cerebellum (cb) and brain stem (bs).

(B) brPTB immunoreactivity in rat spinal cord and dorsal root ganglia (arrows).

(C, D) brPTB expression in the developing peripheral nervous system. Immunostaining of E16 rat sagittal sections indicate that brPTB expression is strong in embryonic dorsal root ganglia (C, arrow), trigeminal ganglia (D, arrow), developing adrenal medulla (C, arrowhead) and hypothalamus (D, ht) however very low in liver (C, lv) and adrenal primordium (C, ap).

(E) brPTB staining in P5 mouse cochlea. Note the intensive staining in cochlear spiral ganglion cells (arrow).

(F) brPTB expression in the ganglion cells of the small intestine (E16 rat embryo).

(G) Confocal image of immunofluorescent double exposure for MAP2 (green) and brPTB (red) in a sagittal section of E16 rat cortex.

(H) Confocal image of immunofluorescence double exposure for GFAP (green) and brPTB (red) in a sagittal section of adult rat cerebellum.

Scale bars: 80 μm in A-E; 160 μm in F; 3.4 μm in G; 2.3 μm in H.

Control sections stained with pre-immune serum or with brPTB antibody pre-adsorbed with brPTB fusion protein were negative (data not shown).

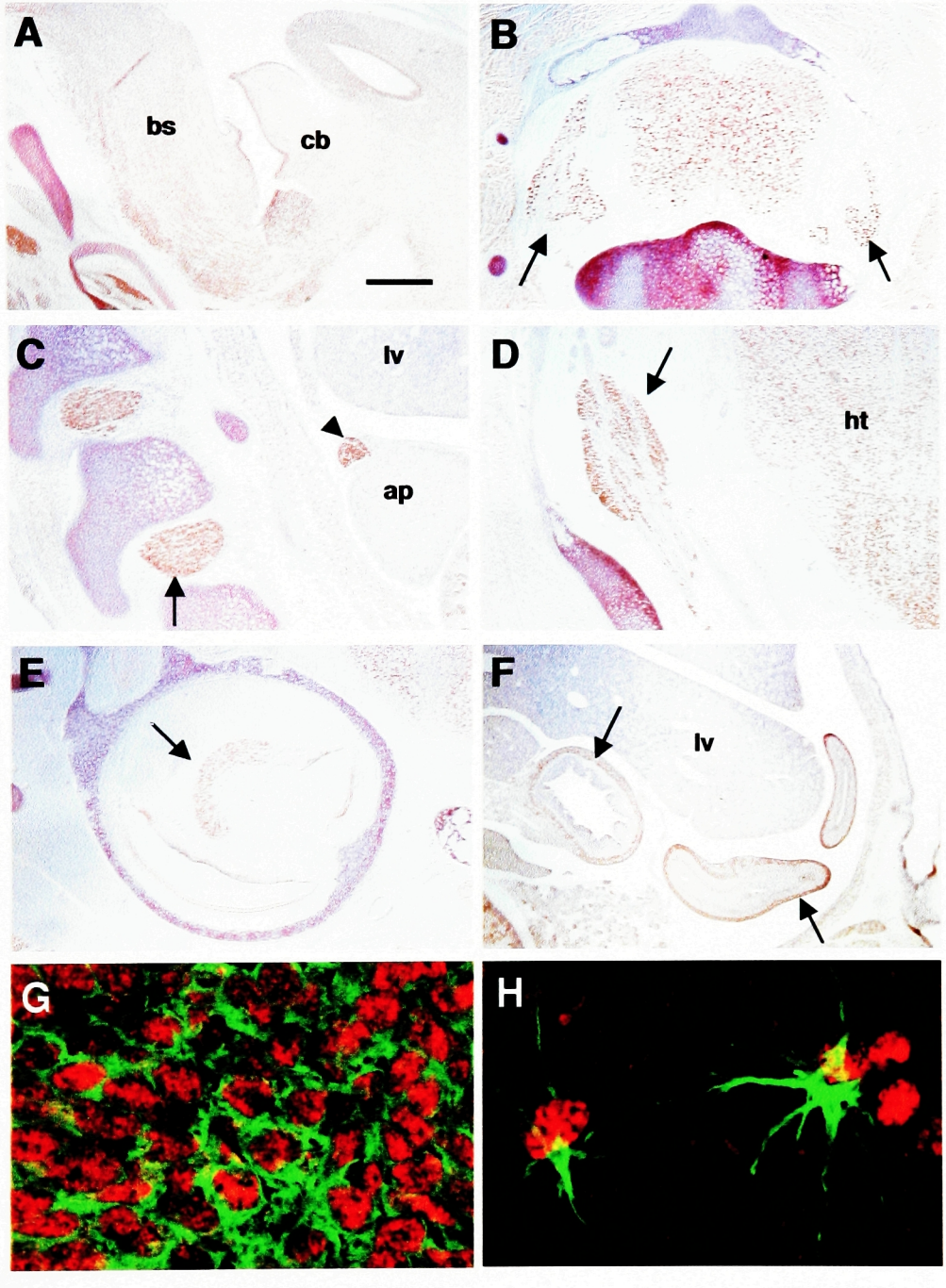
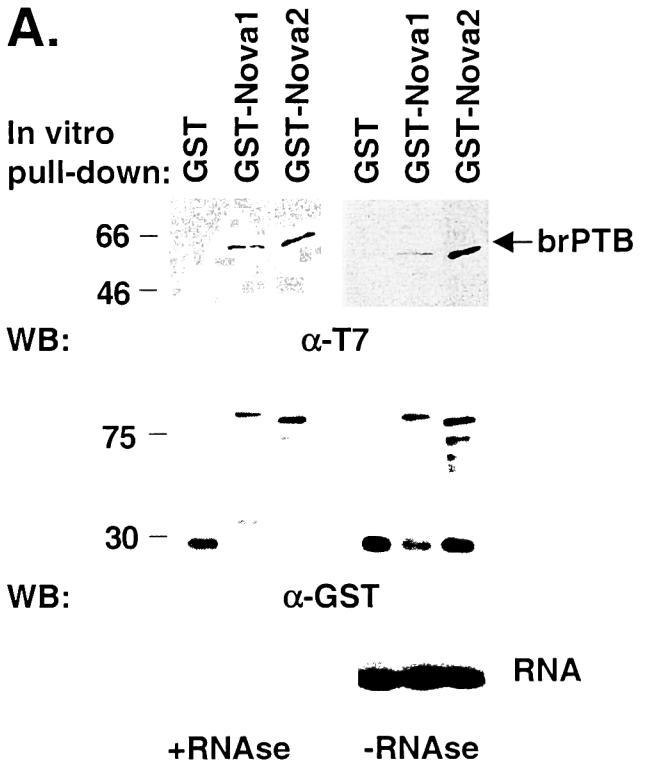

A
bs
cb
B
C
lv
ap
D
ht
E
F
lv
G
H

Figure 7. Nova and brPTB proteins interact *in vitro* and *in vivo*.

(A) *In vitro* GST pul-down assay. Five μg of purified recombinant GST, GST-Nova1 or GST-Nova2 fusion proteins as indicated, were incubated with 2 μg of purified recombinant T7-tagged brPTB fusion protein. The T7-brPTB protein that was precipitated by Glutathione Sepharose beads was assayed by Western Blot using an anti-T7 monoclonal antibody (top panel, α-T7). The presence of equal amounts of GST fusion proteins in each precipitation reaction was confirmed by probing the same blots with an anti-GST polyclonal antibody (middle panel, α-GST). T7-brPTB runs at 59 kDa, GST at 27 kDa, GST-Nova1 at 82 kDa and GST-Nova2 at 80 kDa. To examine whether RNA was needed for the Nova-brPTB interaction, pull down assays were repeated in the presence (left column, +RNAse) or absence (right column, -RNAse) of 1 mg/ml RNAse A. To confirm the activity of the RNAse, reactions were spiked with ^{32}P-labelled RNA and aliquots were run on denaturing urea/polyacrylamide gels and exposed by autoradiography (bottom panel).

(B) *In vivo* GST pull-down assay. Equal amounts of GST, GST-Nova1 and GST-Nova2 eukaryotic expression vectors were transfected into N2A cells as indicated. Cell lysates were incubated with glutathione sepharose beads and the amount of endogenous brPTB precipitated was assayed by Western Blot using the brPTB-specific polyclonal antibody (top panel, α-brPTB). Lysate from untransfected N2A cells (cell lysate) identified the endogenous brPTB band. The same blot was also probed with a polyclonal GST antibody (bottom panel, α-GST) to confirm expression of the transfected GST proteins.


A.
In vitro pull-down:
GST
GST-Nova1
GST-Nova2
GST
GST-Nova1
GST-Nova2
66
46
brPTB
WB:
α-T7
75
30
WB:
α-GST
RNA
+RNAse
-RNAse

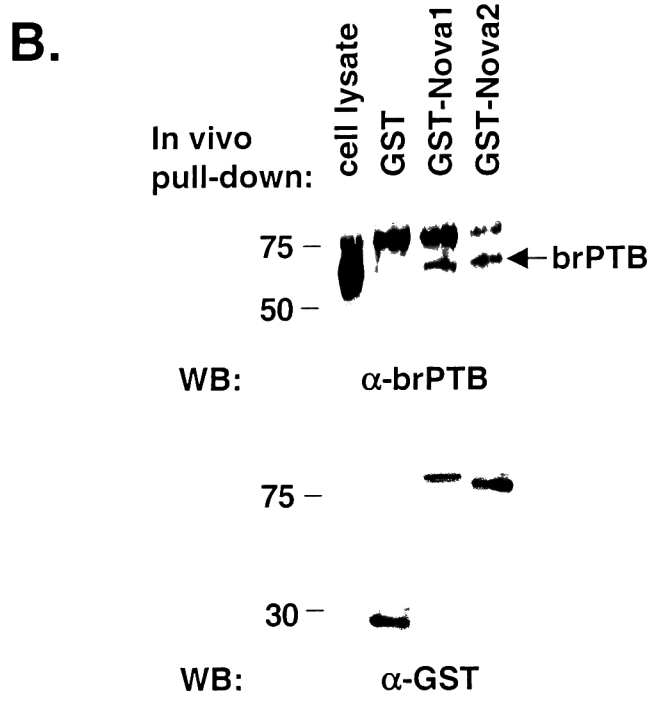
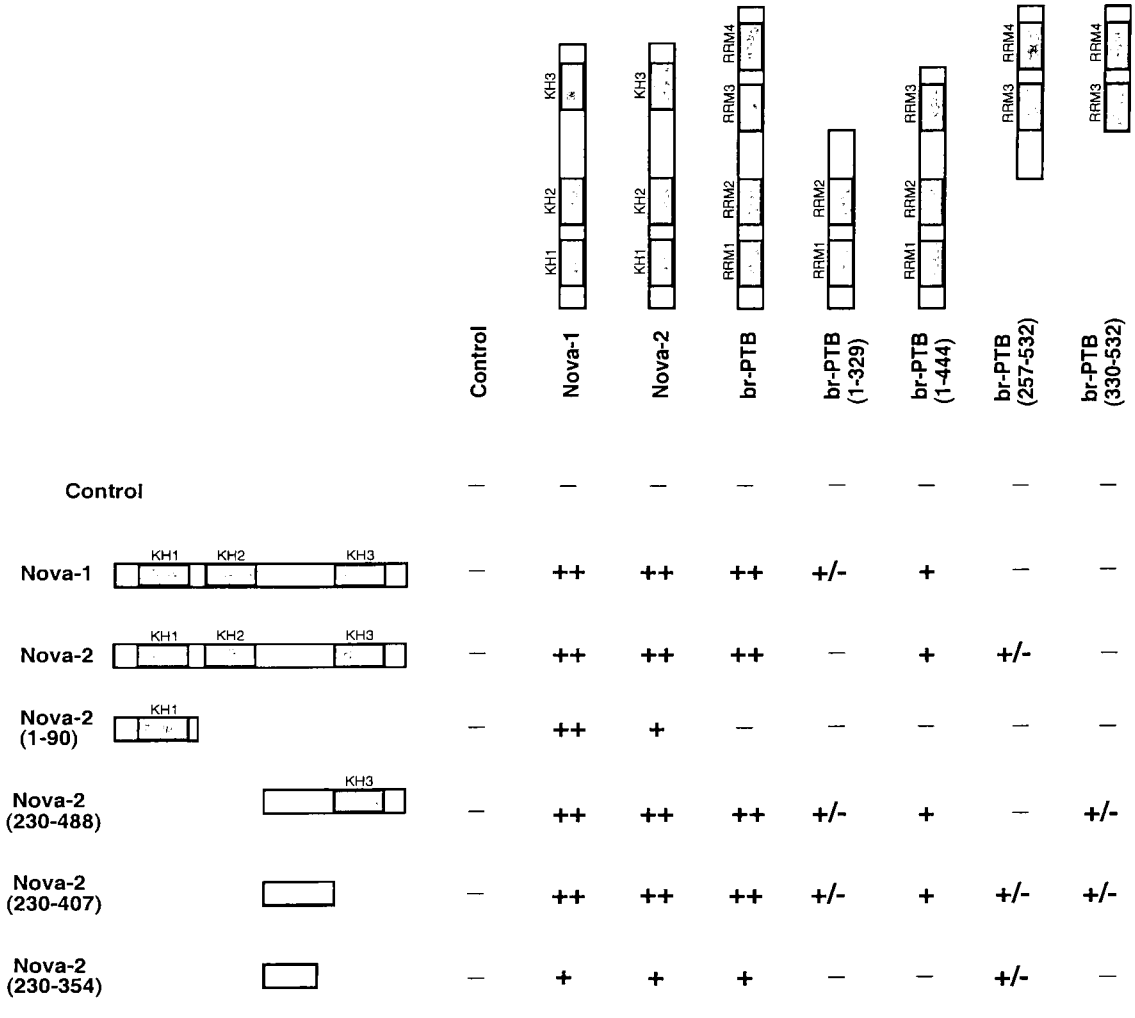

B.
In vivo pull-down:
cell lysate
GST
GST-Nova1
GST-Nova2
75
50
brPTB
WB:
α-brPTB
75
30
WB:
α-GST

Figure 8. Yeast two-hybrid interactions between Nova and brPTB.

A number of bait constructs (LexA fusions) encoding for the indicated Nova1 or Nova2 amino acids (rows) were co-transformed with different Nova1, Nova2 or brPTB activation constructs (GAD fusions) as indicated (columns). Interaction was measured by β-galactosidase activity in a colony lift/X-gal assay (++, very strong staining; +, strong staining; +/-, weak staining or not all colonies positive; , no staining). Control bait (pVA3) and prey (pTD1) transformations exhibited no β-galactosidase activity, as shown.


Control
Nova-1
Nova-2
br-PTB
br-PTB (1-329)
br-PTB (1-444)
br-PTB (257-532)
br-PTB (330-532)
KH1
KH2
KH3
RRM1
RRM2
RRM3
RRM4
Control — — — — — — — —
Nova-1 — ++ ++ ++ +/- + — —
Nova-2 — ++ ++ ++ — + +/- —
Nova-2 (1-90) — ++ + — — — — —
Nova-2 (230-488) — ++ ++ ++ +/- + — +/-
Nova-2 (230-407) — ++ ++ ++ +/- + +/- +/-
Nova-2 (230-354) — + + + — — +/- —

Chapter 4 – brPTB Antagonizes the Action of Nova in Alternative Splicing

Introduction

Alternative splicing is an important mechanism by which cells regulate gene expression and generate diversity (reviewed in Smith and Valcarcel, 2000). Neurons specifically, make great use of alternative splicing in order to regulate functional differences in proteins (reviewed in Dredge et al., 2001; Grabowski and Black, 2001). For example, a wide variety of neurotransmitter receptor activities are regulated by alternative splicing, including regulation of the NR1 NMDA receptor subcellular localization (Ehlers et al., 1995) and its interaction with the neurofilament subunit NF-L (Ehlers et al., 1998), the physiology of the glutamate (Sommer et al., 1990) and NMDA (Hollmann et al., 1993) receptors, and the ability of agrin to induce clustering of acetylcholine receptors (Ferns et al., 1992; Tsim et al., 1992). Moreover, several neurologic diseases such as spinal muscular atrophy, amyotrophic lateral sclerosis and paraneoplastic opsoclonus-myoclonus ataxia (POMA) have been associated with defects in proteins involved in generating the splicing machinery or in the accurate splicing of target RNAs (Fischer et al., 1997; Liu et al., 1997; Jensen et al., 2000a; for review see Dredge et al., 2001).

Since the discovery of tissue-specific splicing of the calcitonin/CGRP transcript in neurons, there has been an extensive search for cis-acting RNA elements and trans-acting factors (RNA-binding proteins) that mediate neuron-specific splicing. The first example of cis-acting regulatory elements in neuronal pre-mRNAs was identified in the

calcitonin/CGRP pre-mRNA (Amara et al., 1982; Emeson et al., 1989) and a number of specific sequences have been identified that are responsible for calcitonin/CGRP tissue-specific processing (Emeson, et al., 1989; Hedjran et al., 1997; Lou et al., 1999). Subsequent work identified regulatory sequences near other neuron-specific exons such as the N1 exon of *src* (Black, 1992) and a 24 nucleotide exon of the $GABA_A$ receptor $\gamma 2$ subunit (Zhang et al., 1996).

The identification of trans-acting factors that regulate neuronal splicing has been a greater challenge. Two general mechanisms might account for the way such factors could mediate regulation of neuronal splicing. Brain-specific variants in splicing patterns could be achieved by differing levels or modifications of ubiquitously expressed splicing factors. For example, antagonism between the hnRNP A1 and ASF/SF2 proteins determines the selection of 5' splice site (Krainer et al., 1990; Ge et al., 1991; Mayeda and Krainer, 1992; Caceres et al., 1994; Horowitz and Krainer, 1994), and varying ratios of these proteins have been shown to affect neuron-specific splicing of clathrin exon EN in cell transfection studies (Caceres, et al., 1994). Additionally, post-translational modifications such as phosphorylation influence the activity of general splicing factors including ASF/SF2 (Colwill et al., 1996; Duncan et al., 1997; Misteli et al., 1997; Xiao and Manley, 1998; Petersen-Mahrt et al., 1999). Such variations might be difficult to detect if they are transient or localized to specific cell types and not reflected in the overall tissue distribution of the proteins. This may be particularly true in the brain where there is great complexity in cell type and number.

A second and perhaps more direct way to regulate pre-mRNA splicing in neurons would be to utilize tissue-restricted regulatory proteins. But evidence for such trans-

acting factors has been limited. Some examples of tissue-restricted splicing events concern the polypyrimidine tract binding protein (PTB; reviewed in Wagner and Garcia-Blanco, 2001). PTB is thought to represses the neuron-specific splicing of calcitonin-CGRP pre-mRNA in non-neuronal cells (Lou et al., 1996; Lou, et al., 1999). Furthermore, PTB has been implicated in tissue-specific alternative splicing events in the α-tropomyocin and α-actinin transcripts, where it is thought to repress exons in a cell-specific manner (Gooding et al., 1998; Southby et al., 1999). Finally, data have emerged that support a model whereby PTB acts as a universal repressor of alternatively spliced exons. For example, in the fibroblast growth factor receptor 2 (FGFR2) transcript, splicing of exon IIIb is actively repressed by PTB, and this inhibition has to be overcome for successful splicing in certain cell types (Carstens et al., 2000).

Some reports have identified a brain-enriched protein recognized by PTB antibodies (Ashiya and Grabowski, 1997; Zhang et al., 1999). Neuron-specific splicing of c-src involves several generally expressed RNA binding proteins (Chan and Black, 1997; Min et al., 1997; Chou et al., 1999), including KSRP which is enriched in brain (Min, et al., 1997). Also, there is data that the neuron-specific RNA binding protein Elav may regulate splice site selection in Drosophila neurons (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001).

Recently, there has been evidence that the neuron-specific RNA-binding protein Nova1 regulates alternative splicing in neurons (Jensen et al., 2000a). *In vitro* RNA selection experiments identified stem-loop RNAs harboring UCAU elements in the loop sequence to which Nova1 and Nova2 proteins bound with sequence-specificity and high affinity (Buckanovich and Darnell, 1997; Yang et al., 1998). The co-crystal structure of

Nova bound to an *in vitro* selected UCAY element has been solved (Lewis et al., 2000), which confirms the specific nature of the Nova-RNA interaction. The Nova1 RNA selection consensus sequence matched an intronic UCAU repeat element in the glycine receptor α2 subunit (GlyRα2) pre-mRNA and identified it as a candidate Nova target. Co-precipitation and cross-linking experiments demonstrated that Nova in brain extracts specifically interacted with the GlyRα2 UCAU repeat element, and co-transfection assays demonstrated that Nova1 enhanced utilization of an adjacent 3' splice site of an alternatively spliced exon, E3A (Buckanovich and Darnell, 1997; Jensen et al., 2000a). Conversely, Nova1-null mice have a specific defect in the utilization of GlyRα2 exon E3A demonstrating that Nova1 is necessary for accurate GlyRα2 exon selection *in vivo* (Jensen et al., 2000a). Furthermore, a second target of Nova1 splicing control, the $GABA_A$ receptor γ2 subunit ($GABA_AR\gamma2$) mRNA, exhibits specific splicing defects of an alternative exon in the absence of Nova1 *in vivo*. In light of these results, experiments were performed to address the effect of the brPTB-Nova interaction in the control of alternative exon selection in the GlyRα2 and $GABA_AR\gamma2$ transcripts, and are presented below.

Results

brPTB downregulates Nova-dependent splicing activation of GlyRα2

It has been previously demonstrated that the Nova protein regulates the alternative splicing of glycine receptor α2 subunit (GlyRα2) pre-mRNA by specifically increasing the inclusion of exon 3A at the expense of exon 3B (Jensen et al., 2000a). Furthermore, this activation of splicing by Nova1 was shown to be dependent on binding of the GlyRα2 RNA at Nova1 sites. To determine whether brPTB is able to affect the ability of Nova to activate GlyRα2 exon 3A inclusion, we co-transfected 293T cells with a GlyRα2 minigene construct (Figure 9A) together with various concentrations of brPTB and Nova.

The amount of protein expressed from the varying doses of transfected DNA was verified by Western blot analysis (Figure 10A). The ratio of exon 3A over 3B in the mRNA transcribed from the minigene was analyzed by an RT-PCR assay taking advantage of a unique SspI restriction site in exon 3A (Jensen et al., 2000a; Figure 10B). The PCR product specifically depended on the presence of reverse transcriptase and the transfection with the minigene construct (Figure 10B; data not shown).

Nova1 transfection alone stimulated exon 3A inclusion by approximately two-fold, as previously reported (Jensen et al., 2000a; Figure 11). When co-transfected, brPTB suppressed the effect of Nova1 in a dose-dependent manner. When the maximum amount of brPTB is co-transfected (6μg), the ratio of exon 3A/3B returns to levels similar to those seen with no Nova1 activation at all. In control experiments, increasing amounts of transfected brPTB DNA in the absence of Nova1 did not alter the splicing pattern of GlyRα2 pre-mRNA (Figure 11). These results suggest that the interaction between brPTB and Nova1 is specific and functional *in vivo*.

Inhibition of GlyRα2 splicing by brPTB requires RNA binding

Earlier work has shown that Nova1 specifically binds to a GlyRα2 UCAU-repeat RNA element located just upstream of exon 3A, the first of the two mutually exclusive exons (Figure 9B). Interestingly, a consensus sequence binding site for PTB, consisting of a UCUU element within a polypyrimidine tract, exists proximal to the Nova-binding element. Given the high degree of sequence identity between brPTB and PTB, it was proposed that brPTB binds to this PTB consensus site on the GlyRα2 intronic element.

Filter binding assays were performed with brPTB and a radiolabeled 90 nucleotide RNA that included the Nova1 and PTB binding sites (Figures 9B and 12). brPTB bound to this RNA, but with approximately a 12-fold reduced affinity (Kd = 559 nM) relative to Nova1 (Kd = 46 nM). Nonetheless, this binding is specific since no detectable binding was observed to an unrelated piece of ribosomal RNA of similar length. Furthermore, when the putative brPTB binding site on this 90 nt RNA fragment was mutated (cytidine to thymidine; Figure 9B) its binding was reduced to the rRNA background levels (C->T mutant in Figure 12).

When the co-transfection/splicing assays were repeated using a mutant GlyRα2 minigene harboring the C->T mutation that abrogated brPTB binding to isolated RNA (as described above), brPTB was no longer able to block the ability of Nova to stimulate exon 3A inclusion (Figure 13). The ratios of exon 3A/3B stayed at the high, Nova1-induced levels and increasing amounts of co-transfected brPTB did not bring them down to baseline. Again, the presence of synthesized brPTB and Nova1 proteins in levels related to the amount of transfected DNA in these experiments was confirmed by Western blot analysis (data not shown). Taken together, these results suggest that brPTB

inhibits the Nova1-dependent activation of exon 3A inclusion and that this action is mediated, at least in part, by brPTB binding to the GlyRα2 pre-mRNA upstream of the Nova1 binding site, since when binding of the brPTB protein is abolished, so is the inhibitory effect on splicing.

brPTB and Nova1 form a complex on GlyRα2 intronic RNA

In order to study the interaction between brPTB and Nova1 in relation to this RNA target, we performed electrophoretic mobility shift assays with the same radiolabeled GlyRα2 intronic RNA fragment and purified recombinant Nova1 and brPTB fusion proteins. When each protein was independently added to the GlyRα2 RNA fragment to a final concentration of 100 nM the mobility of the RNA was shifted by Nova1 but not by brPTB (arrow in top panel of Figure 14, compare lanes 3 and 5).

This was consistent with the results of the filter binding assays that showed Nova1 affinity for this RNA piece to be at least ten-fold higher than brPTB and was relatively equivalent in terms of binding constants. Nova1 protein had a Kd of 46 nM in filter binding assays and was able to band shift the RNA complex at concentrations of 100 nM (and to a lesser degree at 50 nM). Conversely, brPTB protein with a Kd of 559 nM in the filter binding assays did not result in a band shift at 50 or 100 nM, as expected.

However, when concentrations of brPTB that did not bind RNA individually (50-100 nM) were added to 100 nM of Nova1, a supershifted complex was seen (arrowhead in top panel of Figure 14, compare lanes 8 and 9 with 4 and 5) suggesting that if Nova1 is already present on the RNA, brPTB can bind resulting in a brPTB-Nova-RNA complex.

As a control, the same concentration of an unrelated protein (TRF2) did not affect the mobility of the Nova-RNA complex (data not shown).

To examine whether the ability of brPTB to mediate this change in mobility was dependent on Nova-RNA binding, we repeated these assays using a GlyRα2 90 nt RNA fragment in which the Nova1-binding UCAU repeat elements were mutated to UAAU (Figure 14 bottom panel). Using this mutant RNA, Nova1 binding was abolished and no supershifted complex was observed, consistent with previous data showing that Nova1 does not bind to this RNA (Buckanovich and Darnell. 1997; Jensen et al., 2000a). Taken together, these results suggest that Nova1 and brPTB form a complex on GlyRα2 target RNA upstream of the alternatively spliced exon 3A and that this complex is crucially dependent on Nova1 binding the UCAU intronic RNA element.

brPTB inhibits splicing activation of $GABA_AR\gamma2$

Similarly with the results obtained for the glycine receptor α2 subunit mRNA, Nova has been shown to regulate the alternative splicing of $GABA_A$ receptor γ2 subunit (Jensen et al., 2000a). In this case, exon 9 is alternatively spliced and its inclusion depends on the presence of Nova1 as shown by splicing assays comparing RNA obtained from wild type and Nova1 knock-out mice. Furthermore, in splicing assays, the inclusion of exon 9 from a $GABA_AR\gamma2$ minigene (Figure 15A) is directly proportional to the amount of Nova1 synthesized from a co-transfected construct (Dredge et al., work in progress; this work).

In 293T cells co-transfected with the $GABA_AR\gamma2$ minigene construct, Nova1 increased the inclusion of exon 9 by approximately four-fold (Figure 16). These assays were analogous to the ones performed with the GlyRα2 minigene and were based on RT-

PCR analysis of RNA obtained from various transfected cell lines. The amount of protein synthesized from the transfected constructs was verified by Western blot (data not shown), and the relative ratios of long form (including exon 9) over short form (excluding exon 9) of the $GABA_AR\gamma2$ mRNA were determined by Phosphorimager analysis. As can be seen in Figure 16, the increase in exon 9 inclusion obtained by co-transfection of Nova1 is eliminated by simultaneous co-transfection of increasing amounts of br-PTB. When there is no Nova1 present, brPTB still has an inhibitory effect on the inclusion of exon 9, however much less than in the presence of Nova1. This suggests that in this case, as opposed to GlyRα2, brPTB plays a general repressive role in the inclusion of exon 9 that is not strictly dependent on the action of Nova1.

In contrast to GlyRα2, Nova1 binding sites in the $GABA_AR\gamma2$ pre-mRNA have not been defined in detail (K. Dredge and R. Darnell, unpublished observations). However, given the results with GlyRα2, we examined the intronic sequence upstream of alternatively spliced exon 9 in the $GABA_AR\gamma2$ transcript. An 85 nucleotide fragment immediately upstream of exon 9 contains three sites that seemed promising in terms of binding brPTB (Figure 15B). These included a total of three UCUU sequences in the context of polypyrimidine tracts. Two of them were closer to each other and were mutated together (RNA mutant GA2, Figure 15B). The other was mutated alone (RNA mutant GA1, Figure 15B). However, whether one (GA1) or two (GA2) of these putative brPTB sites were mutated, the net effect observed in binding assays was negligible (Figure 17). These experiments failed to identify binding sites for brPTB immediately upstream of the alternatively spliced exon in the $GABA_AR\gamma2$ pre-mRNA.

Consistent with this data, when 293T cells were transfected with $GABA_AR\gamma2$ minigene constructs containing these mutations, the inhibitory effect of brPTB remained substantial (shown in Figure 18 for RNA mutant GA2; similar results obtained with mutant GA1 are not shown). Therefore, brPTB inhibits Nova1 in the inclusion of $GABA_AR\gamma2$ exon 9, but this effect is not mediated by intronic UCUU RNA sequences present immediately upstream of the alternative spliced exon.

brPTB has no effect on Nova-dependent alternative splicing in N2A cells

To examine the interaction of Nova and brPTB in the control of alternative splicing in an environment that more closely resembles neurons *in vivo*, we performed the co-transfection/splicing assays in cultured N2A cells, a neuroblastoma cell line. Since N2A cells contain endogenous Nova and brPTB (see below), the ratio of exon inclusion in untransfected cells was expected to be different than 293T cells which do not contain Nova1. Indeed, the background level of the ratio of exon 3A over 3B with the GlyRα2 minigene was higher in N2A cells than in 293T cells (Figure 19; compare to Figure 11). Expression of additional, transfected Nova1 increased the inclusion of exon 3A approximately two-fold. The 3A/3B exon ratios obtained in this experiment are slightly lower than the ones previously observed (Jensen et al., 2000a) and could be due to sub-optimal splicing from this minigene. However, the two-fold increase in the ratio upon addition of Nova1 is consistent with previous results. Co-transfection of increasing amounts of brPTB, has no effect on the ratio of exon 3A over 3B that stays in the Nova1-induced levels (Figure 19).

Similarly, in assays with the $GABA_AR\gamma 2$ minigene, there is a ten-fold increase in the inclusion of exon 9 compared to the ratio obtained with 293T cells (Figure 20; compare to Figure 16) and it was closer to the ratios obtained from wild type tissue RNA samples (Jensen et al., 2000a). The effect of Nova1 co-transfection is again striking, increasing the inclusion of exon 9 by four-fold. However, the transfection of brPTB expression vectors does not significantly inhibit the effect of Nova1 (Figure 20).

PTB inhibits Nova-dependent splicing activation of GlyRα2

To test whether the original PTB homologue has a similar effect on the increase of exon inclusion mediated by Nova1, co-transfection/splicing assays were performed in 293T cells with the wild type GlyRα2 minigene. As shown in Figure 21, PTB has the same, if not a stronger effect than brPTB in inhibiting Nova1-dependent exon 3A inclusion. The transfection constructs for the two PTB homologues were based on the same vector with the same promoters and polyadenyation sites but containing different epitope tags. Thus, while Western blot analysis showed that both proteins were synthesized in increasing levels with more DNA transfected, as before, it was impossible to compare the relative levels of the two protein homologues as they were recognized by different primary antibodies.

Discussion

The action of brPTB to inhibit Nova-dependent activation of exon inclusion is consistent with the general role described for PTB in suppressing exon inclusion in pre-mRNA splicing. This is believed to be mediated by PTB binding to polypyrimidine rich elements in intronic sequences and blocking the assembly of a splicing competent complex in a manner analogous to the action of Sxl in blocking $U2AF^{65}$ binding of the polypyrimidine tract (Valcarcel et al., 1993). For example, PTB acts to repress smooth muscle-specific inclusion of alternatively spliced exons in the α-tropomyosin and α-actinin pre-mRNAs, by binding to regulatory elements upstream of the 3' splice site (Lin and Patton, 1995; Perez et al., 1997; Southby et al, 1999).

In neuronal cells, PTB inhibits inclusion of the c-src exon, in part by an action on 3' splice-site selection that can be competed by $U2AF^{65}$ (Chan and Black, 1997). Similarly, previous studies with p59/nPTB as well as current studies on brPTB, all point to an inhibitory action on splice site selection. Even in the single instance where PTB enhances exon inclusion in the alternative splicing of the calcitonin-CGRP pre-mRNA, it is believed to act by disrupting U2AF recognition of an enhancer pseudo-exon, thereby indirectly promoting correct exon utilization (Lou et al., 1999).

Given several instances in which PTB acts on alternatively spliced pre-mRNAs to inhibit the inclusion of neuronal exons, it has been suggested that a general action of PTB might be to inhibit neuron-specific exon utilization (Valcarcel and Gebauer, 1997; Grabowski, 1998). In previous reports, the presence of the brain enriched form of PTB was correlated with the presence of neuron-specific splicing *in vitro*, while the addition of the general form of PTB inhibited neuron-specific exon utilization (Ashiya and

Grabowski, 1997; Zhang et al., 1999). These results had suggested the possibility that the inhibitory action of PTB is replaced in the brain by a permissive or stimulatory action of the brain-PTB isoform (Grabowski, 1998; Wagner and Garcia-Blanco, 2001). However, the proposal that brPTB promotes neuron-specific splicing is not supported by the experiments presented here, where brPTB antagonizes Nova1 action in promoting neuron-specific exon inclusion.

There are several possible mechanisms by which brPTB could regulate Nova activity in splicing. One model is that brPTB competes with the ability of Nova to bind the GlyRα2 UCAU element, in a manner reminiscent of the action of PTB to compete out U2AF binding. However, we find that brPTB binds to the isolated GlyR 90nt RNA with a 12-fold weaker affinity than Nova1. Moreover, brPTB appears to supershift a Nova-RNA complex, suggesting that it does not interfere with the ability of Nova to bind the RNA. Therefore, it is unlikely that brPTB and Nova compete for the same site on the RNA and that this competition inhibits the ability of Nova to enhance exon inclusion.

Notably, immediately upstream of the GlyRα2 UCAU repeat element exists a potential PTB consensus binding element, as defined by Patton and colleagues (Perez et al., 1997): a UCUU motif in the context of a pyrimidine rich element. Although this sequence does not clearly define a PTB binding site (for example see Singh et al., 1995), and brPTB and PTB binding specificities may differ, our results suggest that Nova and brPTB may bind adjacent intronic sequence elements. However, based on this data, we cannot rule out the possibility that brPTB binds exclusively to the RNA target. We find that brPTB and Nova proteins interact and that brPTB binds the GlyRα2 RNA weakly but with sequence specificity. Furthermore, binding of the RNA is necessary for the

inhibitory action of brPTB on Nova1 function. The simplest model consistent with this data is that brPTB binds the GlyRα2 pre-mRNA at a site adjacent to Nova and mediates an inhibitory effect on Nova-dependent exon inclusion through protein-protein interactions, perhaps by preventing assembly of a multiprotein complex necessary to activate splicing.

A core sequence termed the Downstream Control Sequence (DCS) is necessary for enhancement of c-src splicing *in vitro* (Modafferi and Black, 1997; Modafferi and Black, 1999). Repression of c-src splicing in non-neuronal cells requires both the upstream element and sequences within and adjacent to the DCS. The DCS RNA element has been used to clone trans-acting factors that may bind to brPTB in a manner analogous to the proposed Nova-brPTB interaction to regulate neuronal splicing. For example, a complex of at least six proteins cross links to DCS RNA, including a positive acting factor related to Nova by the presence of multiple KH type RNA binding domains, termed KSRP (Min et al., 1997). In addition, the same brPTB protein identified here was recently shown to bind DCS RNA as part of a larger complex including KSRP and hnRNP H, and to exhibit weak repression of c-src splicing (Markovtsov et al., 2000). These observations and our observations on Nova and brPTB suggest that protein-RNA complexes acting to regulate splice site selection may include brPTB, and that brPTB acts as an antagonist of neuron-specific splicing mediated by positive acting factors.

In the case of the $GABA_AR\gamma2$ mRNA, the situation appears to be slightly more complicated. Nova1 increases the inclusion of alternative exon 9 by a margin that is larger than the effect on GlyRα2 (Jensen et al, 2000a; this work). However, the contribution of brPTB in inhibiting this effect seems less specific. First, there is a slight

inhibition of exon 9 inclusion even in cells that express no Nova (i.e. 293Ts; Figure 16). While this result is consistent with the role of PTB as a splicing inhibitor, it also contradicts previous work that has suggested that the neural isoform of PTB (i.e. brPTB) plays a permissive role in the splicing of neuronal transcripts (Ashiya and Grabowski, 1997; Zhang et al., 1999). Significantly, the inhibition of brPTB on $GABA_AR\gamma2$ splicing in the absence of Nova in neuronal N2A cells is less pronounced, indicating that the behavior of brPTB in neuronal cells can be different, perhaps depending on the presence of other factors.

Mutations in what ostensibly appeared to be brPTB sites upstream of alternative exon 9 in $GABA_AR\gamma2$ mRNA, had no effect on binding or on the inhibition of splicing. Preliminary data suggests that the Nova1 binding site controlling the splicing increase in this exon is located within a downstream intron and work is currently being done to establish whether brPTB also affects $GABA_AR\gamma2$ splicing through other sites nearby (Dredge et al., work in progress). So, while the location for the Nova and brPTB sites might be different than $GlyR\alpha2$, the mechanism of exon selection regulation could be similar (i.e. competition for the recruitment of splicing factors) only occurring at the downstream intron as has been proposed for the c-src transcript (see below).

Splicing inhibition by brPTB in cell lines that more closely resemble neurons (N2A cells) is significantly reduced, suggesting that other cell type-specific factors required for this effect might be interacting with and negatively regulating Nova and/or brPTB proteins. brPTB is not a strictly neuron-specific protein, as its presence has been detected in tissues other than the brain, such as the heart and testis (Polydorides et al., 2000; Markovtsov et al., 2000; Lillevali et al., 2001). Thus, it is hard to imagine that

brPTB independently acts to derepress neuron-specific splicing patterns, as it has been suggested (Ashiya and Grabowski, 1997; Zhang et al., 1999). Instead, it is more likely that brPTB participates in the control of splicing in a complex with other factors some of which can be cell-specific, such as Nova, and might explain the variation in the results obtained here in the splicing assay with different cell types.

In light of these results, the model proposed for the tissue-specific regulation of splicing in Drosophila should probably be replaced in the case of mammalian cells with a more dynamic model where proteins with antagonistic effects in splicing are in a constant battle. The final outcome of this battle is one that depends on the relative levels of the factors involved as well the specific circumstances that could be influenced by a multitude of other factors that are temporally and spatially regulated.

In support of this model, recent data has furthered the idea that different isoforms of PTB can have distinct activities in splicing inhibition (Markovtsov et al., 2000; Wollerton et al., 2001). Alternatively spliced isoforms of the PTB transcript itself result from the insertion of short amino acid stretches between RRM2 and RRM3 (Gil et al. 1991; Patton et al., 1991). The various PTB isoforms have different effects in the splicing repression of the alternative exon in the α-tropomyosin mRNA *in vitro* and *in vivo* (Wollerton et al., 2001). However this effect was not observed in the repression of the α-actinin transcript where all PTB isoforms were equally effective in splicing repression. Together with the result that the nPTB homologue is less repressive than PTB in c-src splicing (Markovtsov et al., 2000) this suggests that tissue-specific splicing regulation can be achieved by variations in the levels of homologues or otherwise processed isoforms of splicing factors.

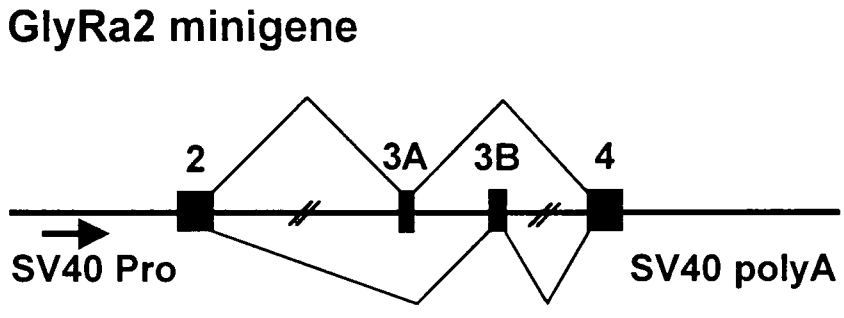
In conclusion, the work presented here extends our understanding of the mechanism by which neurons differentially regulate alternative splicing. It is suggested that the function of the neuron-specific protein Nova to promote exon inclusion can be modulated by interaction with additional proteins. The brPTB protein is not strictly neuron-specific as is the Nova protein: brPTB is also expressed in glial cells, and to a lesser but significant extent, in cells outside of the nervous system, particularly the heart and testis. Nonetheless, the existence of a brain-enriched protein interacting with a strictly neuron-specific protein suggests a layer of neuronal regulation different from a model in which tissue-specific alternative splicing is regulated by varying levels or modifications of general splicing factors (such ASF/SF2).

Figure 9. Schematic of the glycine receptor α2 subunit minigene.

(A) The glycine receptor α2 subunit (GlyRα2) minigene construct used in the *in vivo* splicing assays. It contains alternative exons 3A and 3B flanked by exons 2 and 4 and most of the intronic sequence in between, under the control of an SV40 promoter (SV40 Pro) and with the addition of an SV40 polyadenylation sequence (SV40 polyA).

(B) DNA sequence of a 90 nt fragment of intronic GlyRα2 RNA upstream of exon 3A that contains Nova binding sites (UCAU; solid underline) and a candidate binding site for brPTB (UCUU in pyrimidine context; dashed overline). The C->T mutation in the brPTB binding site is indicated. RNA contains U instead of T. Numbering of base pairs in intron 2 starts at the junction with exon 3A, upstream is negative.

A.


GlyRa2 minigene
2
3A
3B
4
SV40 Pro
SV40 polyA

B.

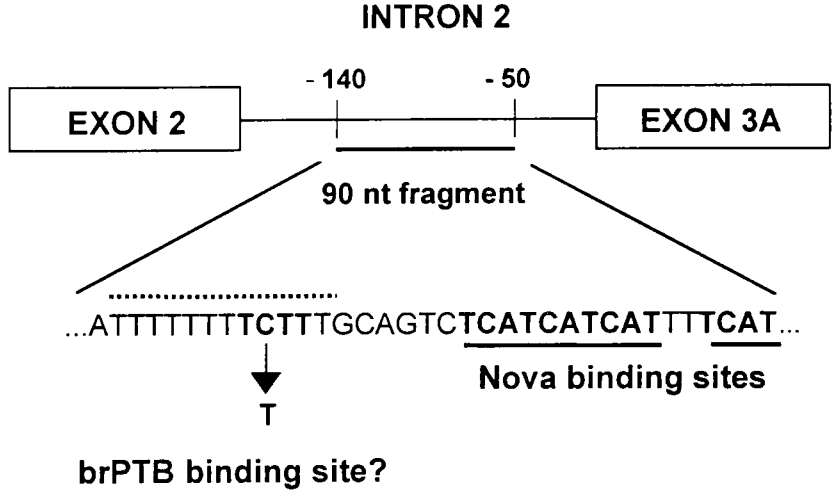
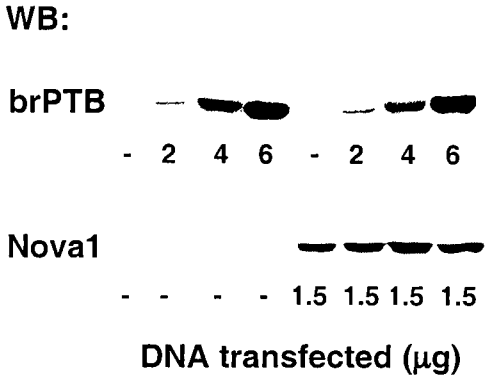

INTRON 2
- 140
- 50
EXON 2
EXON 3A
90 nt fragment
...ATTTTTTTTCTTTGCAGTCTCATCATCATTTTCAT...
Nova binding sites
T
brPTB binding site?

Figure 10. Altered splicing patterns in GlyRα2 minigene assays.

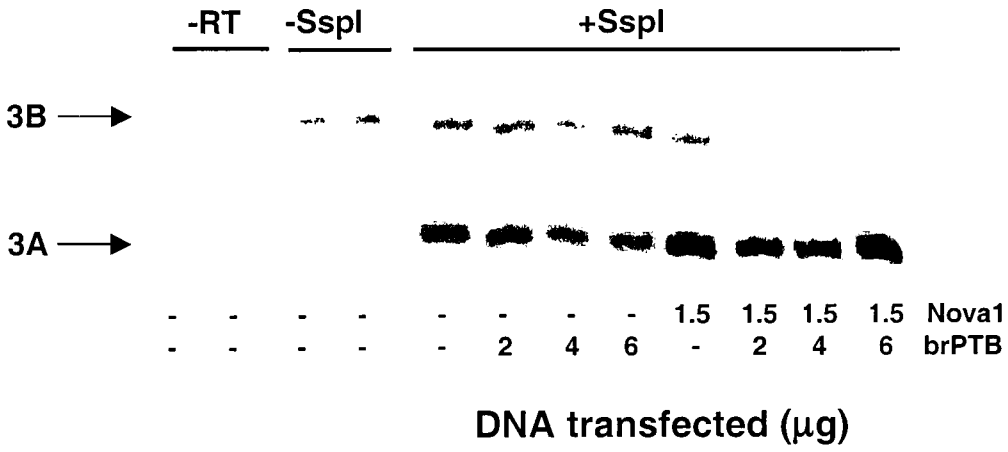
(A) Western Blot analysis of cell extracts. One twentieth the amount of protein in each transfection was separated on SDS-PAGE, followed by immunoblotting with anti-flag monoclonal antibody (brPTB) or POMA patient serum (Nova1) to confirm that increasing amounts of transfected DNA correlated with proportionally increasing amount of protein synthesized by the cells.

(B) Sample autoradiogram of RT-PCR assay in brPTB/Nova1 co-transfections in 293T cells. Digestion with SspI only cuts the 2->3A->4 splice product, leaving the 2->3B->4 band intact within the same lane. The relative intensity of the bands (ratio 3A/3B) was quantified using a Phosphorimager (Molecular Dynamics) and calculated with ImageQuant software (as described in Jensen et al., 2000a; see Materials and Methods). This data represents a single experiment with the C->T mutant GlyRa2 minigene as presented in Figure 13.

A.



B.



Phosphorimage Data
Quantitation (3A/3B) 2.57 2.87 2.80 2.40 5.00 5.01 5.22 5.30

Figure 11. brPTB antagonizes Nova1 in GlyRα2 splicing.

Bar graph of the ratio of exon 3A to exon 3B in 293T cells transfected with the indicated amounts of Nova1 and brPTB expression vectors plus the GlyRα2 minigene containing exons 2 to 4 (Jensen et al 2000a; see Figure 10). Ratios of included exon 3A over 3B were determined by RT-PCR, followed by SspI digestion (which only cuts within exon 3A; see Figure 10) and quantified using a Phosphorimager (see Materials and Methods). Each point represents the average of four transfections, and error bars indicate the standard deviation. For a representative data set, see the Appendix.

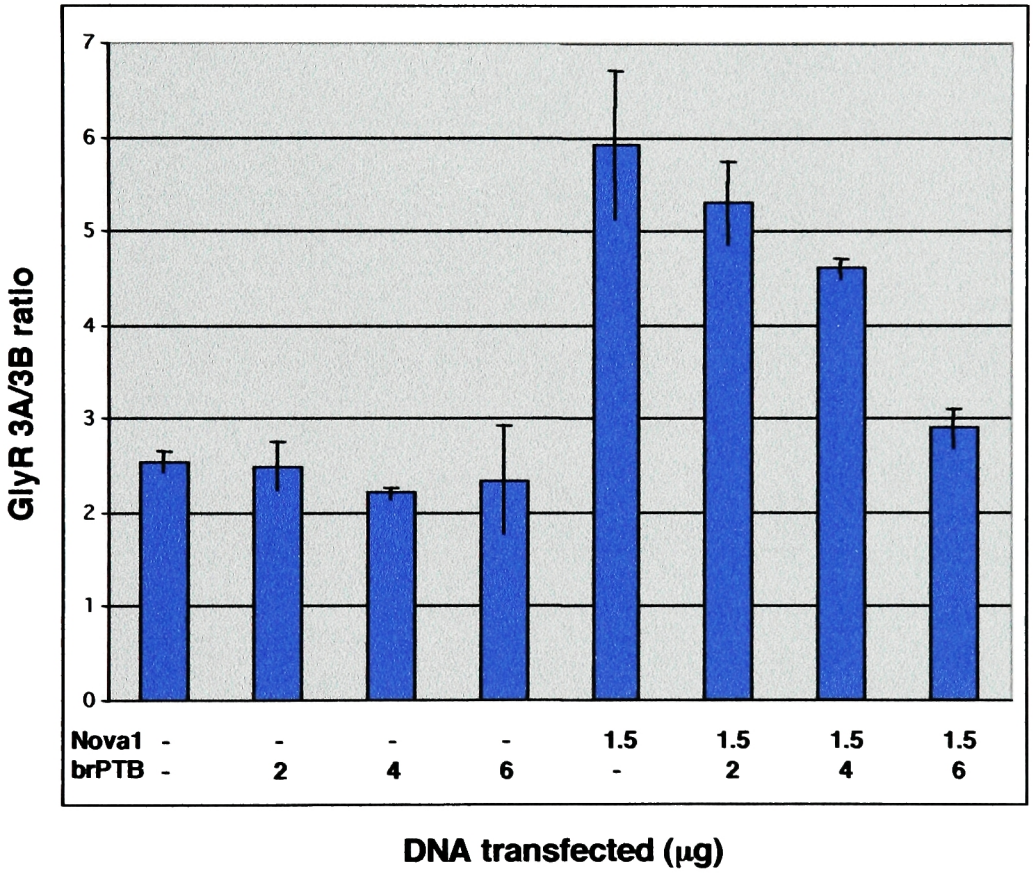

GlyR 3A/3B ratio
7
6
5
4
3
2
1
0
Nova1 - - - - 1.5 1.5 1.5 1.5
brPTB - 2 4 6 - 2 4 6
DNA transfected (μg)

Figure 12. C->T mutation in GlyRα2 RNA diminishes brPTB binding.

Filter binding assays of purified recombinant fusion proteins Nova1 and brPTB, as indicated, with wild type (WT) and C->T mutant (C->T) GlyRα2 RNA and an unrelated fragment of ribosomal RNA (rRNA) of equal length. Mutating the brPTB binding site (as shown in Figure 9) reduced its binding to background levels (compare with rRNA curve) while it had no effect on Nova1 binding (data not shown).

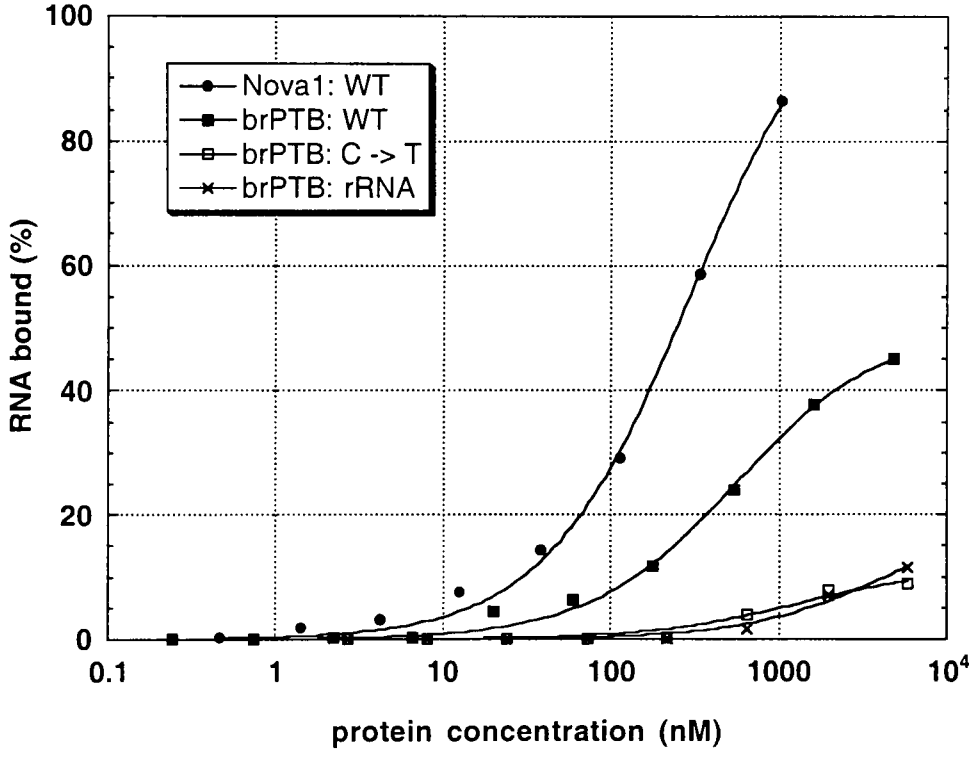

Nova1: WT
brPTB: WT
brPTB: C -> T
brPTB: rRNA
RNA bound (%)
100
80
60
40
20
0
0.1
1
10
100
1000
10^4
protein concentration (nM)

Figure 13. GlyRα2 binding is necessary for brPTB splicing inhibition.

Bar graph of the ratio of exon 3A to exon 3B in 293T cells transfected with the indicated amounts of Nova1 and brPTB expression vectors plus the GlyRα2 minigene carrying the C->T mutation (C->T) that prevented brPTB binding *in vitro* (Figure 12). Each point represents the average of four different transfections and error bars indicate the standard deviation (purple bars). The results obtained with the original minigene construct (WT) are included here for comparison (blue bars; Figure 11). For a representative data set, see the Appendix.

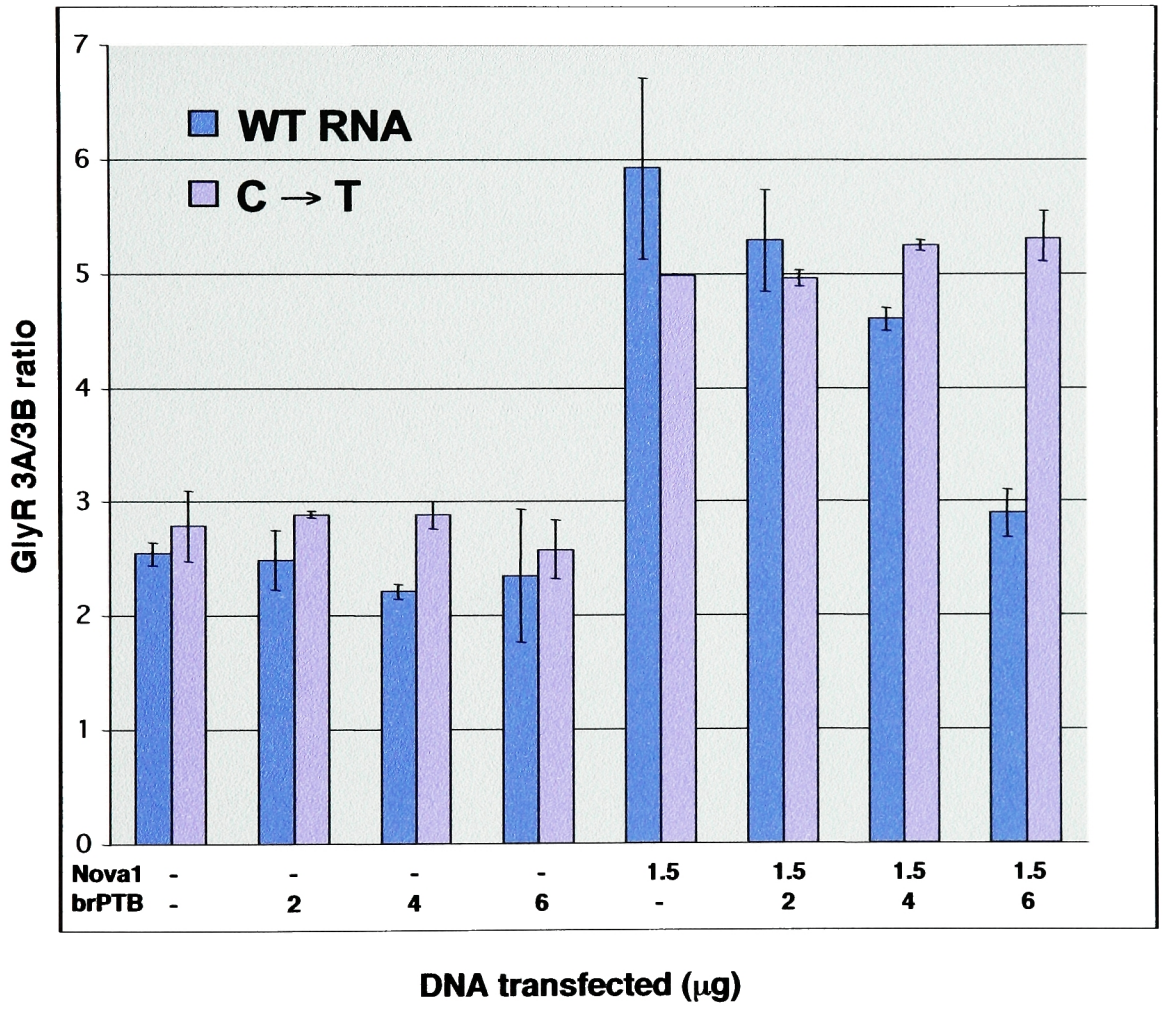
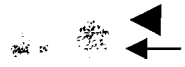

WT RNA
C → T
GlyR 3A/3B ratio
0
1
2
3
4
5
6
7
Nova1 - - - - 1.5 1.5 1.5 1.5
brPTB - 2 4 6 - 2 4 6
DNA transfected (μg)

Figure 14. brPTB and Nova1 form a complex on GlyRα2 intronic RNA.

Electrophoretic mobility shift assays of brPTB and Nova1 proteins on GlyRα2 RNA. Purified, recombinant fusion proteins in the concentrations indicated (nM) were incubated with a radioactively labeled wild type or mutant GlyRα2 RNA that does not bind Nova1 in filter binding assays (data not shown). Nova1 is able to band-shift the wild type GlyRα2 RNA (top panel, arrow) while brPTB does not. When both proteins are present, the RNA is super-shifted (top panel, arrowhead). Nova1 does not mediate a gel-shift with the mutant GlyRα2 RNA (bottom panel), and neither does brPTB. The presence of an unrelated protein (TRF2) at the same concentration (100 nM) did not affect the binding of either Nova1 or brPTB (data not shown).

Wild Type RNA



Nova1	—	50	100	—	—	50	50	100	100
brPTB	—	—	—	50	100	50	100	50	100

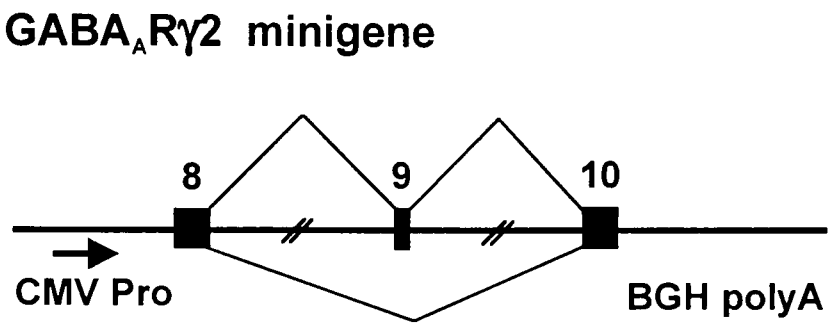
Mutant RNA

Figure 15. Schematic of the $GABA_A$ receptor γ2 subunit minigene.

(A) The $GABA_A$ receptor γ2 subunit ($GABA_A\gamma2$) minigene construct used in co-transfection/splicing assays. It contains alternatively spliced exon 9 flanked by exons 8 and 10 and most of the intronic sequence in between, under the control of a CMV promoter (CMV Pro) and with the addition of a polyadenylation sequence from Bovine Growth Hormone (BGH polyA).

(B) In the 85 nucleotide intronic sequence upstream of alternative exon 9, there are 3 candidate binding sites for brPTB (UCUU in a pyrimidine context; dashed underline). The UC->GA mutations in the putative binding sites (two in Mutant RNA GA2 and one in Mutant RNA GA1) of brPTB on $GABA_A\gamma2$ are shown. Numbering of base pairs in intron 8 starts at junction with exon 9, upstream is negative.

A.


GABA$_A$Rγ2 minigene
8
9
10
CMV Pro
BGH polyA

B.

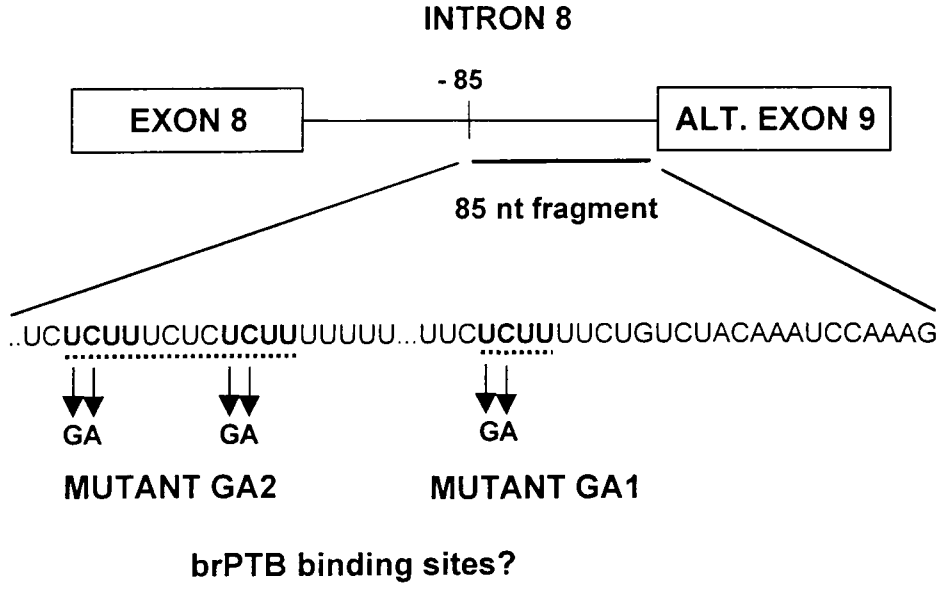

INTRON 8
- 85
EXON 8
ALT. EXON 9
85 nt fragment
..UCUCUUUCUCUCUUUUUUU...UUCUCUUUUCUGUCUACAAAUCCAAAG
GA
GA
GA
MUTANT GA2
MUTANT GA1
brPTB binding sites?

Figure 16. brPTB antagonizes Nova1 in $GABA_AR\gamma2$ splicing.

Bar graph of the ratio of exon 3A to exon 3B in 293T cells transfected with the indicated amounts of Nova1 and brPTB expression vectors plus the $GABA_A\gamma2$ minigene containing exons 8 to 10 (Figure 15). Ratios (L/S) of RNAs including alternative exon 9 (long form, L) over those excluding exon 9 (short form, S) were determined by RT-PCR, quantified with a Phosphorimager (Molecular Dynamics) and calculated using ImageQuant software (see Materials and Methods). Each point represents the average of eight transfections and error bars indicate the standard deviation. The amount of protein expressed in each transfection was verified by Western Blot (data not shown). For a representative data set, see the Appendix.

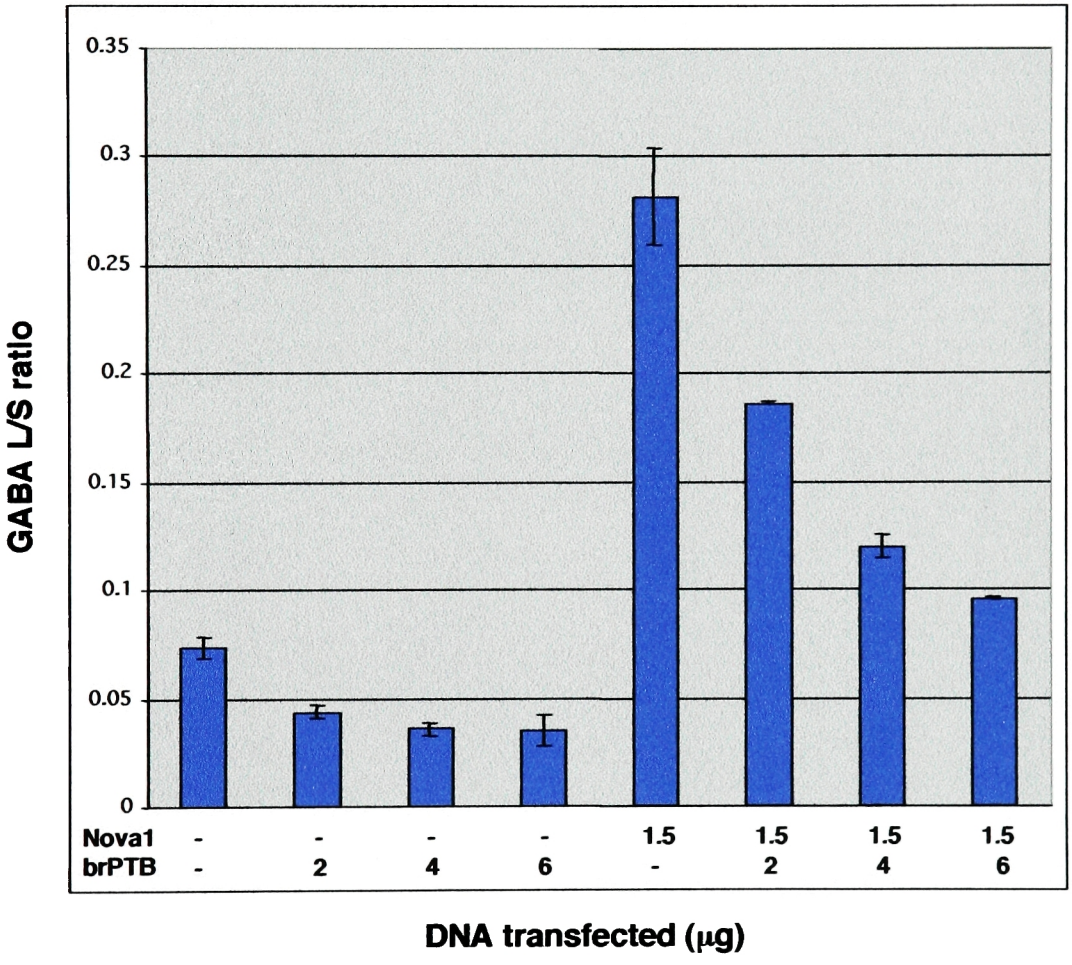

GABA L/S ratio
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Nova1 - - - - 1.5 1.5 1.5 1.5
brPTB - 2 4 6 - 2 4 6
DNA transfected (μg)

Figure 17. Mutations in $GABA_A\gamma2$ RNA do not affect brPTB binding.

Filter binding assays of purified recombinant brPTB fusion protein with wild type (WT GABA) and two mutant (GABA GA2 and GA1; Figure 15) $GABA_A\gamma2$ RNAs and an unrelated fragment of ribosomal RNA (rRNA). As shown, mutating the putative brPTB binding sites did not substantially reduce its binding.

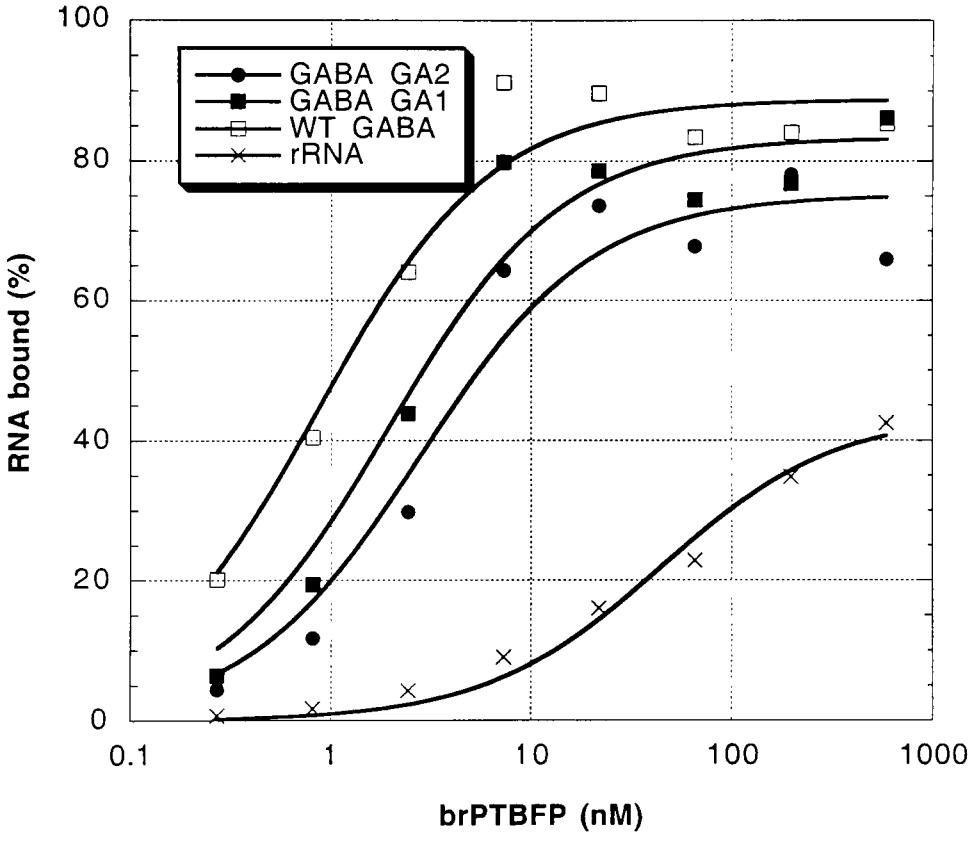

GABA GA2
GABA GA1
WT GABA
rRNA
RNA bound (%)
100
80
60
40
20
0
0.1
1
10
100
1000
brPTBFP (nM)

Figure 18. Mutations in $GABA_AR\gamma2$ RNA do not affect brPTB splicing.

Bar graph of the ratio of exon 3A to exon 3B in 293T cells transfected with the indicated amounts of Nova1 and brPTB expression vectors plus the Mutant GA2 $GABA_A\gamma2$ minigene (as shown in Figure 15). Ratios (L/S) were calculated as before (Figure 16). Each point represents the average of four transfections and error bars indicate the standard deviation. The amount of protein expressed in each transfection was verified by Western blot (data not shown). For a representative data set, see the Appendix.

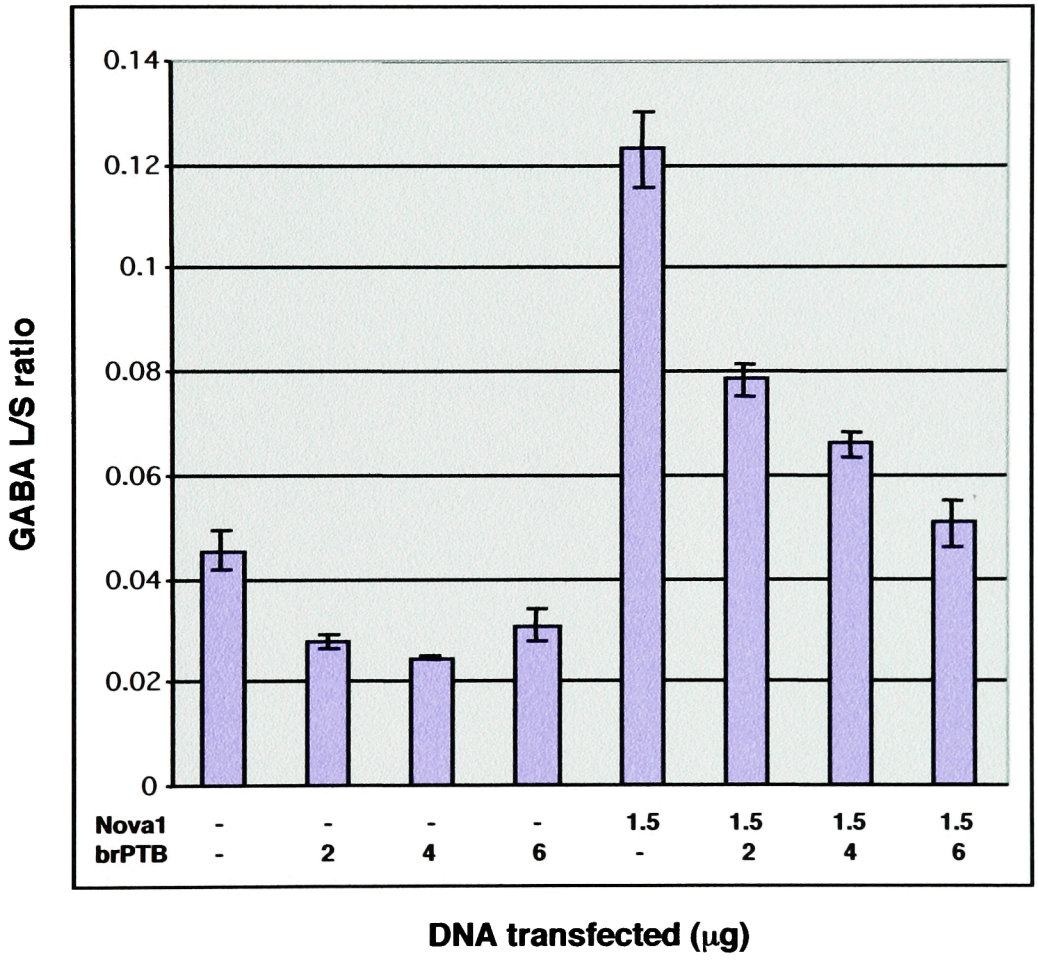

GABA L/S ratio
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Nova1 - - - - 1.5 1.5 1.5 1.5
brPTB - 2 4 6 - 2 4 6
DNA transfected (μg)

Figure 19. brPTB has no effect on the splicing of GlyRα2 in N2As.

Bar graph of the ratio of exon 3A to exon 3B in N2A cells transfected with the indicated amounts of Nova1 and brPTB expression vectors plus the wild type GlyRα2 minigene. Ratios of included exon 3A over exon 3B were calculated as before (Figure 10). Each point represents the average of four transfections and error bars indicate the standard deviation. The amount of protein expressed in each transfection was verified by Western blot (data not shown). For a representative data set, see the Appendix.

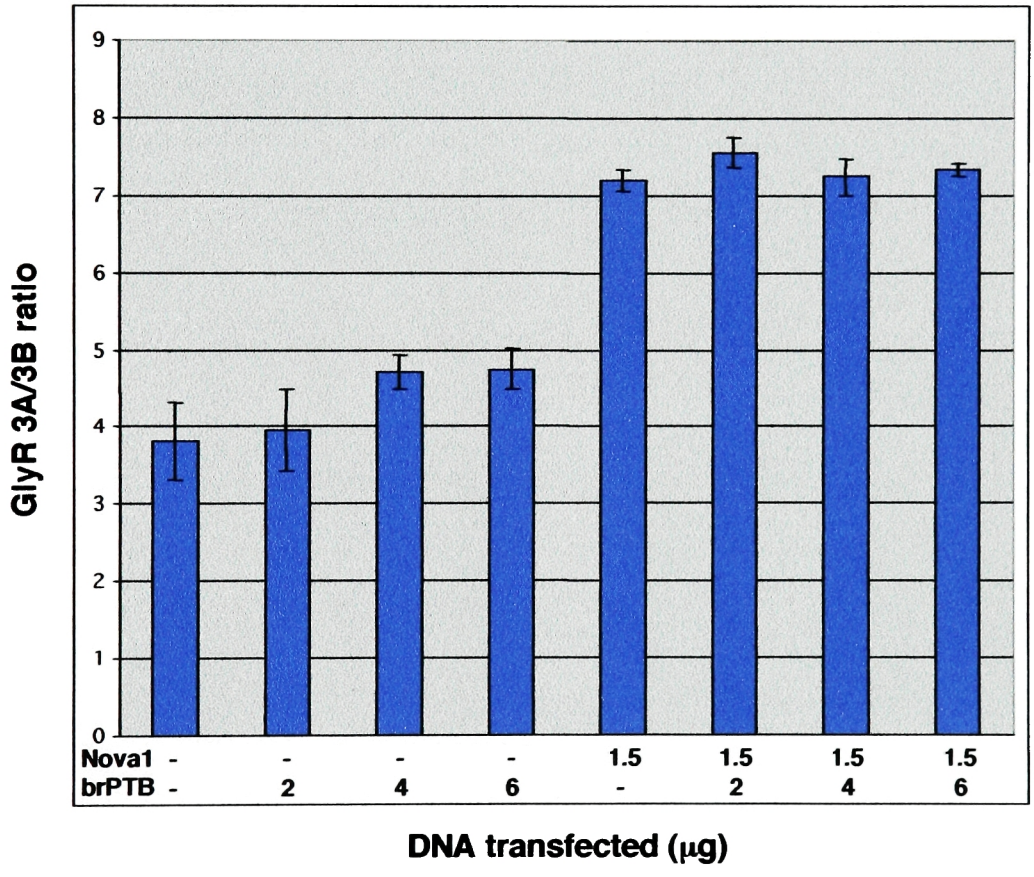

GlyR 3A/3B ratio
9
8
7
6
5
4
3
2
1
0
Nova1 - - - - 1.5 1.5 1.5 1.5
brPTB - 2 4 6 - 2 4 6
DNA transfected (μg)

Figure 20. brPTB has no effect on the splicing of $GABA_AR\gamma2$ in N2As.

Bar graph of the ratio of exon 3A to exon 3B in N2A cells transfected with the indicated amounts of Nova1 and brPTB expression vectors plus the wild type $GABA_AR\gamma2$ minigene. Ratios of included exon 9 were calculated as before (Figure 16). Each point represents the average of four transfections and error bars indicate the standard deviation. The amount of protein expressed in each transfection was verified by Western blot (data not shown). For a representative data set, see the Appendix.

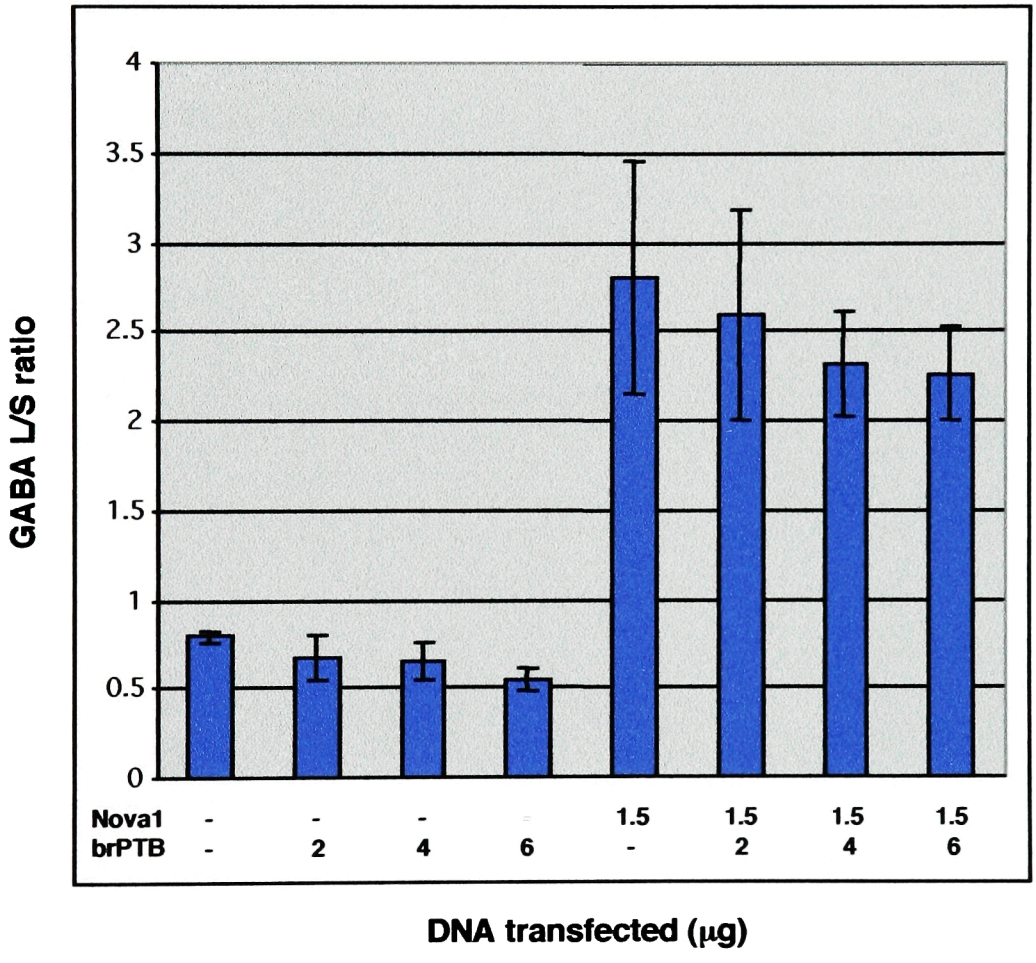
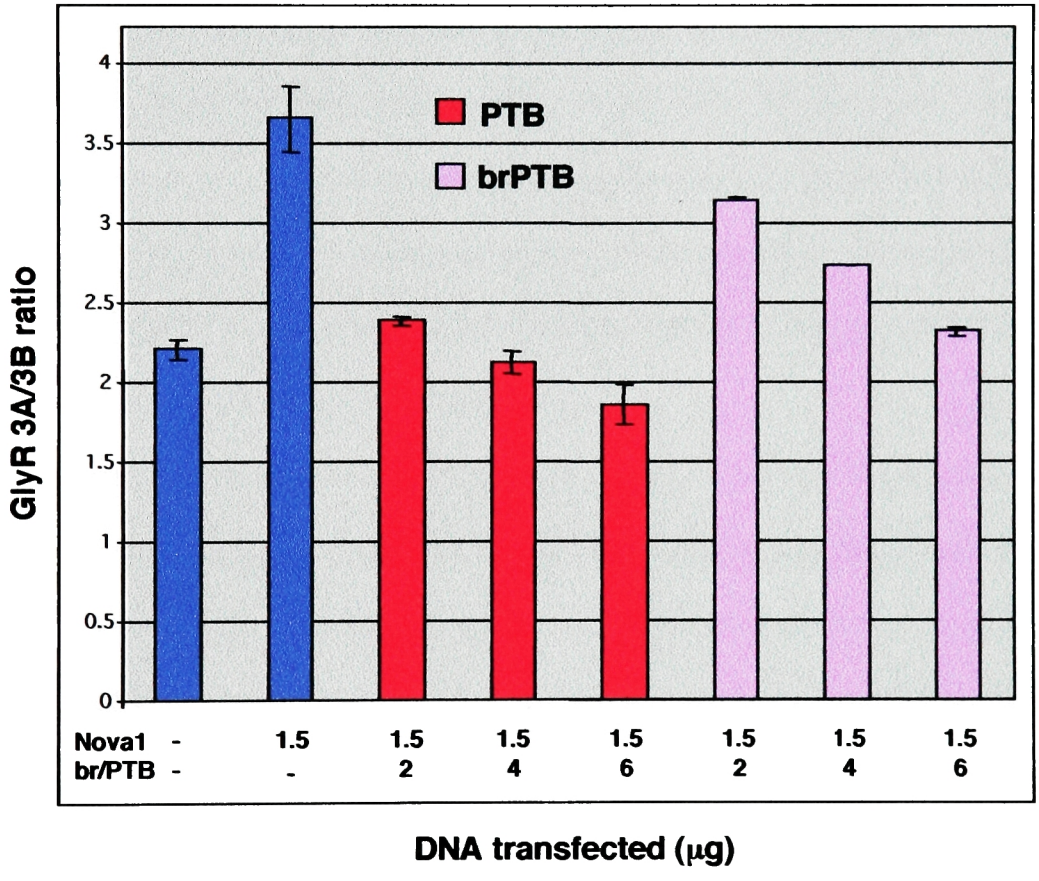

GABA L/S ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
Nova1 - - - 1.5 1.5 1.5 1.5
brPTB - 2 4 6 - 2 4 6
DNA transfected (μg)

Figure 21. PTB and brPTB have similar effects on Nova inhibition.

Bar graph of the ratio of exon 3A to exon 3B in 293T cells transfected with the indicated amounts of Nova1, PTB and brPTB expression vectors and the wild type GlyRα2 minigene. Ratios of included exon 3A over 3B were determined as before (Figure 10). Each point represents the average of four transfections and error bars indicate the standard deviation. The amount of protein expressed in each transfection was verified by Western blot (data not shown). For a representative data set, see the Appendix.


GlyR 3A/3B ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
PTB
brPTB
Nova1 - 1.5 1.5 1.5 1.5 1.5 1.5 1.5
br/PTB - - 2 4 6 2 4 6
DNA transfected (μg)

Chapter 5 – brPTB and Nova proteins co-localize in the nuclei of neuronal cells

Introduction

The coupling of biochemical steps in RNA metabolism is a common and useful method to achieve multi-step control and regulation of various processes in tandem. RNA-binding proteins are uniquely positioned to play an important role in this coupling by virtue of their specific association with target RNAs and their localization in particular sub-compartments of the cell where such events are taking place. Thus, examining the localization of RBPs is necessary in order to understand their function and to study the possible causes of dysfunction in human disease.

Initially, it was hypothesized that the spatial organization of the nucleus occurred in static, morphologically distinct areas that functioned to compartmentalize nuclear processes in a manner analogous to cytoplasmic organelles (reviewed in Misteli and Spector, 1998; Sleeman and Lamond, 1999; Lewis and Tollervey, 2000). However, while most cytoplasmic organelles are membrane-bound, sub-nuclear structures involved in RNA metabolism are lacking a membrane suggesting that the nucleus is a more fluid structure allowing for greater movement and communication between its compartments. In fact, recent studies have shown that, when visualized in living cells, these compartments are highly dynamic and subject to reorganization upon changes in transcriptional activity (Huang and Spector, 1996; Misteli et al., 1997; Sleeman et al., 1998). Thus, it is more likely that the spatial organization of the nucleus *in vivo* (and not

when fixed for observation) reflects an intricate pattern of interactions with functional significance and consequences.

It may seem obvious that the cellular location of each protein is dependent on its functional activity and vice versa. However, information is still lacking on many fronts in deciphering the dynamics and regulation of this connection between localization and function. In the nucleus, there is still debate whether some structures represent inactive storage sites or the settings of active RNA processing. For example, alteration in staining techniques results in the redistribution of splicing factors from previously considered storage sites to sites of active RNA transcription suggesting that functional requirements guide localization rather than the need for compartmentalization (Neugebauer and Roth, 1997; Mintz and Spector, 2000). The situation is further complicated by the existence of the nuclear matrix whose composition and function are not completely understood and it is not known whether its role in the localization and movement of nuclear proteins is one of hindrance or assistance. The advantages of concentrating protein factors to specific areas of functional significance are many and include conservation of energy, increased efficiency and ease of regulation. Finally, the question remains whether the existence of distinct sub-nuclear domains and the distribution of nuclear factors is the cause or the consequence of specific biological processes and changes in the cellular steady state.

The eukaryotic nucleus contains an array of morphologically distinct substructures (reviewed in Lamond and Earnshaw, 1998; Misteli and Spector, 1998). While the function of many of these nuclear bodies remains unclear, their organization and constitution is thought to be in a dynamic state and to even depend on a range of pathological cellular conditions including viral infection, oncogene expression and

disease phenotype. Rather than providing purely structural roles, many of these domains contain factors that are required in the cellular processes that take place in the nucleus, namely chromosomal organization, DNA replication, transcription, RNA processing and transport. Therefore, as morphological observations on the appearance and biochemical evidence on the composition of these bodies grow more prevalent, it becomes evident that the localization and function of cellular macromolecules are inexorably linked.

Perhaps the most prominent of nuclear structures, chromosomal DNA assumes varied function-dependent states during the cell cycle. For example, the interplay between heterochromatin and euchromatin has profound effects on gene expression and is possibly regulated by proteins localized in either structure (e.g. the polycomb-group proteins). The remainder of the nucleus, loosely defined as interchromatin space, contains various nuclear bodies that are better studied during interphase, the relatively steady state of the cell cycle (Table 3). The largest structure among those is the nucleolus, where ribosomal RNA (rRNA) is synthesized, processed and assembled into ribosomes. The nucleolus is a dynamic structure that is transcription- and cell cycle-dependent and also interacts with other sub-nuclear structures that it is in close contact with, such as coiled bodies and the perinucleolar compartment (PNC; see below).

One of the very first nuclear organelles to be described, coiled bodies (also called Cajal bodies) are thought to be involved in the assembly of the nuclear transcription machinery and are best characterized by the marker protein p80-coilin (Gall et al., 1999; reviewed in Gall, 2000; Gall, 2001). The capacity of p80-coilin for self-association, phosphorylation and shuttling are believed to be key factors in the formation and function of coiled bodies, including the proposition that they play a role in a nucleo-cytoplasmic

transport system (Alliegro and Alliegro, 1998; Bellini and Gall, 1999; Hebert and Matera, 2000). Among the proteins that localize to coiled bodies besides p80-coilin are Nopp140 (a nucleolar shuttling protein) and snRNPs (key components of the splicing apparatus).

Related to but not coinciding with coiled bodies are structures called Gemini of coiled bodies (or gems) that contain the SMN protein and its interacting partner SIP1 (Liu and Dreyfuss, 1996; Liu et al., 1997). Gems are thought to participate in snRNP biogenesis, maturation and trafficking, perhaps through their association with coiled bodies (Fischer et al., 1997). Importantly, the disease spinal muscular atrophy (SMA) which is caused by mutations in the SMN gene, is manifested on a cellular level by the disruption of a critical protein interaction of SMN and its lack of localization to gems (Gangwani et al., 2001; Matera and Hebert, 2001). Specifically, the localization of SMN to nuclear bodies depends on its interaction with the zinc-finger protein ZRP1 and this interaction is disrupted by mutations of SMN in SMA patients. This data is in concert with the hypothesis that the localization of SMN in gems is one of functional significance. Furthermore, and possibly because of improper SMN localization, snRNP biogenesis is disrupted and pre-mRNA splicing is affected when SMN is mutated (Pellizzoni et al., 1998).

While the biochemical steps of RNA processing from DNA to protein have been extensively characterized, the localization of these processes lacks critical evidence and is plagued by the problem of identifying and separating active sites from inactive storage areas (reviewed in Lewis and Tollervey, 2000). Active transcription and perhaps splicing as well are thought to take place in perichromatin fibrils (Zhang et al., 1994). Consistent with the idea of coupling between pre-mRNA processing and transcription, the staining

of spliceosomal components (general splicing factors) matches the distribution of nascent transcripts. In addition, PML bodies are nuclear domains where the protein involved in pro-myelocytic leukaemia is localized and may play a role in transcription regulation and, since they are targets of viral transformation, in oncogenesis (Zhong et al., 2000).

One of the first splicing factors found to localize in discrete regions within the nucleus was SC35, whose staining pattern was found to be distinct from that of snRNPs (Fu and Maniatis, 1990). It was hypothesized that SC35 plays an active role in splicing and is thus localized in perichromatin fibrils, whereas snRNPs and SR proteins are mostly localized in so-called nuclear speckles. Speckles are concentrations of proteins that produce a punctate pattern when analyzed by immunofluorescence and are also called interchromatin granule clusters when visualized by electron microscopy (reviewed in Huang and Spector, 1992; Sleeman and Lamond, 1999). Speckles do not co-localize with sites of active transcription (nascent transcripts) or splicing (snRNAs, SC35 protein) and were thus thought to be areas of assembly and storage of inactive components of the splicing machinery (Pombo and Cook, 1996; Mintz and Spector, 2000). However, the localization of spicing factors in nuclear speckles is thought to be a highly dynamic process and depends on gene activation, RNA transcription and pre-mRNA splicing (Spector, 1996; Misteli et al., 1997; Phair and Misteli, 2000).

Another nuclear structure that has been recently characterized is the perinucleolar compartment (PNC). As the name suggests, it is localized in the periphery of the nucleolus, and contains small RNAs transcribed by RNA polymerase III (e.g. Y RNAs), and PTB/hnRNP I (Ghetti et al., 1992; Matera et al., 1995; Huang et al., 1997). The PNC does not coincide with any of the known nuclear sub-domains and a functional role for it

has not been suggested. A protein named PTB-Associated Splicing Factor (PSF) has been found to interact with PTB and to be an essential splicing factor participating early in spliceosome formation (Patton et el., 1993; Gozani et al., 1994). PSF localization in the nucleus occurs in a punctate pattern that coincides with PTB staining only when the salt-soluble PTB fraction is removed, suggesting that the remaining, more stable fraction interacts with PSF, possibly as part of a larger spliceosomal complex (Meissner et al., 2000). Interestingly, PSF localizes to the PNC in a manner that is dependent on its second RRM and redistributes from speckles to the PNC upon inhibition of transcription (Dye and Patton, 2001). This is in contrast to other splicing factors, which upon transcription inhibition generally accumulate in speckles that presumably function as storage depots. This provides evidence for the functional link between splicing and transcription and suggests that the role of PSF in the PNC is not one of active splicing. The localization of matrin3, which is also a member of the PTB sub-family of RRM-type RNA-binding proteins, has not been extensively studied other than the observation that it co-fractionates with the nuclear matrix (Nakayasu and Berezney, 1991).

Earlier experiments have identified the Nova proteins as mostly nuclear. In order to examine the sub-nuclear localization of Nova in more detail and get insight into the specific protein interactions that might be guiding this pattern, immunofluorescence studies were undertaken in cell lines and tissue sections. Additionally, the localization pattern of brPTB was studied since it has been shown to interact with Nova *in vitro* and *in vivo*, and in light of the discrete nuclear structures occupied by PTB in the PNC. The functional aspect of the brPTB-Nova interaction in the regulation of alternative splicing prompted the examination of the nuclear staining pattern of the two proteins.

Results

Nova proteins localize in distinct sub-nuclear structures

In order to examine the sub-cellular distribution pattern of Nova proteins, N2A cells were stained with POMA patient serum. This serum has been previously shown to recognize both Nova1 and Nova2 (Buckanovich et al., 1993; Yang et al., 1998). N2A cells are a mouse neuroblastoma cell line that expresses endogenous Nova (Jensen et el., 2000a; data not shown). Approximately 4 to 5 distinct structures can be seen in the cell nuclei that are specific to the anti-Nova serum (Figure 22A). These foci do not appear with secondary antibody alone, or in other cells that do not express Nova (data not shown). When N2A cells were stained with an affinity-purified Nova2-specific antibody (Yang, 1997), a similar staining pattern emerged (Figure 22B). Since the anti-Nova patient serum recognizes both Nova proteins, the possibility that Nova2 is responsible for the signal in both of these experiments cannot be excluded. However, the slight difference in the staining pattern obtained with the two antibodies and the larger size of the structures visualized with the anti-Nova serum suggest that both Nova1 and Nova2 proteins localize in distinct nuclear foci. When mouse brain sections were stained with anti-Nova serum, neuronal cells in the cerebellum (most likely Purkinje cells) exhibited a similar, albeit not as sharp, staining pattern (Figure 22C). The localization of endogenous Nova proteins in neurons consists of concentrations in approximately four to five discrete foci per nucleus.

Nova proteins do not co-localize with any known sub-nuclear domains

Nova proteins play a role in pre-mRNA splicing and quite possibly in other aspects of RNA metabolism (Jensen et al., 2000). Since these functions of Nova could be mediated

by interactions with other proteins and could occur in these nuclear foci, it was important to test whether the localization of Nova coincided with any of the known nuclear structural domains many of which are defined by RNA-binding proteins (see Table 3). To that end, a series of monoclonal and polyclonal antibodies that identified most of these sub-nuclear structures were used to examine possible co-localization with Nova.

As seen in Figure 23, N2A cells were doubly stained with anti-Nova serum (green, second column) and the indicated antibodies (red, first column; see Materials and Methods). Panels demonstrating the staining pattern of each antibody are shown and correlate with the staining previously observed for these proteins (see this Introduction for references). In the third column, the merged panels clearly show that Nova does not co-localize with any of these proteins as the green and red foci remain distinct. From these results it can be concluded that the concentrations of Nova in neuronal nuclei do not coincide with coiled bodies (p80-coilin and Nopp140 protein), gems (SIP-1 protein), speckles (Sm and SR proteins), or perichromatin fibrils (SC35, RNA polymerase II).

Nova proteins co-localize with brPTB in N2A nuclei

Since a functional interaction of Nova with brPTB has been suggested by the work presented this far, it was of importance to examine whether the two proteins co-localize, especially since both proteins stain mostly neuronal nuclei (Buckanovich et al., 1996; Polydorides et al., 2000; this work). N2A cells contain both proteins endogenously and were doubly stained with anti-Nova patient serum and the brPTB-specific polyclonal antibody (see above). Figure 24A shows that the two proteins display a similar pattern of punctate staining in the nucleus, consisting of approximately five distinct foci (brPTB in

red, Nova in green). Importantly, when the panels are merged, the foci co-localize, as manifested by the yellow color.

To verify that this co-localization is real and not the result of bleed-through between the two filters used to detect the Cy2- and Cy3-conjugated secondary antibodies, single staining with Nova or brPTB was performed in N2A cells. As shown in Figure 24B, there is no signal when the red filter is used to excite cells that have been stained with anti-Nova serum and a Cy2-conjugated anti-human secondary antibody (which is normally excited by the green filter). Correspondingly, there is no signal when the green filter is used to excite cells stained with anti-brPTB polyclonal serum and a Cy3-conjugated anti-rabbit secondary antibody. Furthermore, there is no cross-reactivity between the two antibodies in Western blot assays (data not shown). Thus, the staining observed and the co-localization between Nova and brPTB is not the result of aberrant excitation but rather the coincidence of two antibodies staining for two different proteins.

To clarify the issue of whether the staining pattern observed so far with the anti-Nova patient serum is the result of Nova1 localization, Nova2 or both, N2A cells were transfected with eukaryotic GFP-fusion expression constructs of full length Nova1 and Nova2 proteins and in addition stained the cells with anti-brPTB (Figure 25A). Both GFP-Nova1 and GFP-Nova2 exhibit the same localization pattern observed with endogenous Nova, namely four to five foci per nucleus. Furthermore, these foci co-localize with endogenous brPTB when the panels are merged (third row, Figure 25A). Thus, both endogenous and transfected Nova proteins, and both Nova1 and Nova2 co-localize with endogenous brPTB in N2A nuclei.

In order to confirm that brPTB is indeed the protein that co-localizes with Nova and to control for background immunoreactivity with the anti-brPTB antibody, N2A cells were transfected with a flag-tagged brPTB expression construct. These cells were then stained with a monoclonal anti-flag antibody as well as anti-Nova patient serum and the appropriate secondary antibodies in order to visualize the proteins. As can be seen in the left column of Figure 25B, transfected brPTB co-localizes with endogenous Nova, witnessed by the yellow color when the panels are merged. Again, the pattern of brPTB expression seems to be broader than that of Nova but there still exist foci of increased protein concentration and those co-localize with Nova. Appropriately, in the case of some multi-nucleated cells in the left column of Figure 25B, more foci are present.

Nova proteins co-localize with PTB in N2A nuclei

N2A cells were transfected with a myc-tagged PTB expression construct and stained with a monoclonal anti-myc antibody and anti-Nova serum. In cells that expressed PTB, it co-localized with endogenous Nova in a pattern similar to that of transfected brPTB (middle column in Figure 25B). Thus, while the endogenous PTB family member that is recognized by the anti-brPTB antibody in N2A cells is most likely brPTB (in Figures 24A and 25A), transfected PTB in N2A cells behaves in the same way by co-localizing with Nova proteins in a specific nuclear punctate pattern.

However, when N2A cells were stained with a polyclonal anti-matrin3 antibody, the result was unexpected. Even though matrin3 is one of the proteins isolated in the yeast-two-hybrid screen with both Nova1 and Nova2 (Table1), and its interaction with the Nova proteins is as robust as the other members of the PTB sub-family (Table 2), it

did not co-localize with endogenous Nova in N2A cells (Figure 25B, right column). This observation could be due to the pattern obtained with this matrin3 antibody that did not result in a clear, punctate staining in the nucleus and was instead more diffuse.

Nova proteins do not co-localize with brPTB in non-neuronal cell lines

Since the functional interaction between Nova1 and brPTB varied among different cell lines in the splicing assay presented in Chapter 4, it was important to examine their localization pattern in cell lines other than neuronal N2As. For that purpose, 293T cells (derived from human kidney epithelium), that do not express brPTB or Nova proteins endogenously (data not shown), were transfected with a GFP-brPTB expression construct and either a Nova1 or a Nova2 expression construct (Figure 26A). In either case, when the panels were merged, it became obvious that Nova proteins do not co-localize with brPTB in this cell line. The same result was obtained when GFP-Nova1 or GFP-Nova2 were co-transfected with a flag-tagged brPTB construct and then stained with an anti-flag monoclonal antibody (Figure 26B).

Nova proteins do not co-localize with PTB in non-neuronal cell lines

When GFP-Nova1 and GFP-Nova2 expression constructs were transfected into 293T cells that were stained with anti-brPTB antibody, there was no co-localization (Figure 27A). Since PTB is most likely recognized by this antibody (now used at a higher concentration it cross-reacts with PTB), and because there is no brPTB protein present in 293T cells data not shown), the slightly lower level of staining of PTB can be explained.

To verify this result, and to make sure that PTB did not co-localize with Nova proteins in 293T cells, a myc-tagged PTB construct was co-transfected with either GFP-Nova1 or GFP-Nova2 (Figure 27B). Again, no co-localization is observed. Finally, the same result was obtained in HeLa cells transfected with either GFP-Nova1 or GFP-Nova2 and stained with anti-brPTB antibody (again presumably staining the endogenous PTB protein): no co-localization was evident (Figure 28). Given that the localization of brPTB and PTB proteins in cell nuclei is diffusely nucleoplasmic, the co-localization with Nova proteins could be the result of this widespread nuclear staining. However, as seen in Figure 24A, brPTB is concentrated in distinct foci and these coincide with the Nova concentrations.

Nova proteins co-localize with brPTB in tissue sections

To examine the localization pattern of Nova and brPTB in tissues, immunohistochemistry was performed in rat and mouse spinal cords (Figure 29A). Sagital sections of formalin-perfused tissues were stained with Nova and brPTB sera and the appropriate secondary antibodies and were observed under confocal microscopy. The results demonstrate that the co-localization observed in cell cultures corresponds to a biologically significant process observable in the context of the whole brain. Both mouse and rat tissues stained for Nova and brPTB and the localization pattern was similar to that obtained in cell culture, but with higher background staining. Even though this background staining made it more difficult to pinpoint the nuclear inclusions of Nova and brPTB, the merged planes demonstrated the existence of co-localization in neuronal nuclei.

To examine the co-localization pattern of Nova2 with brPTB, Nova1 knock out mice were used (described in Jensen et al., 2000a). When stained with anti-Nova human serum (now only recognizing the Nova2 protein) and brPTB polyclonal antibody, cerebellar sections of null mice and their wild type litter mates both showed similar staining patterns. While the staining was slightly reduced in the case of the knock out mice (in wild type animals both Nova proteins are present and the staining should be stronger), co-localization of Nova2 with brPTB was nevertheless still observed in the mouse cerebellum (Figure 29B).

Identifying localization domains in Nova1

The multiple peptide domains present in Nova proteins have been studied in terms of their contribution to overall function as examined for protein interactions, RNA binding, structural considerations and splicing activity (Lewis et al., 2000; Jensen et al., 2000b; this work; Dredge and Darnell, personal communication). To test whether different Nova elements are responsible for its nuclear localization and to identify what those may be, fusion constructs of Nova1 deletions with the green fluorescent protein (GFP) were transfected into N2A cells and their localization was examined under live conditions (for complete amino acid listings of these constructs see Chapter 2, Materials and Methods). Full-length Nova1 and Nova2 proteins, when fused to GFP, exhibit an expression pattern similar to that observed for endogenous proteins or otherwise tagged and transfected constructs in various cell lines (N2A, 293T and HeLa cells; see above). In live N2A cells too, this pattern consists of four to five concentrated domains per nucleus, clearly visible even when GFP-fusion proteins are overexpressed (Figure 30).

Nova proteins contain near their amino terminus a sequence that matches the consensus bipartite NLS element (amino acids 25-41), similar to that identified in the Nucleoplasmin and hnRNP K proteins (Pinol-Roma and Dreyfuss, 1992; Siomi et al., 1993; Michael et al., 1997). This element consists of approximately 16 amino acids with basic residues (arginines and lysines) on both ends. Moreover, it is highly conserved between Nova1 and Nova2, suggesting that it is a functional domain. To test the capacity of this domain in mediating the nuclear localization of Nova, a GFP fusion construct of the putative Nova1 NLS sequence was transfected into N2A cells. The localization of this construct was decidedly nuclear, but not punctate (GFP-NLS, Figure 30) as opposed to GFP alone which was diffusely observed throughout the cell (GFP vector).

When two of the basic residues in this NLS (lysines in positions 27 and 40) were mutated, first individually and then in concert, to negatively charged glutamates, effectively destroying the basic nature of the signal, the GFP-NLS fusion constructs failed to localize to N2A nuclei (GFP-NLS K27E, K40E, and K27,40E). This result implies that amino acids 25-41 of the Nova1 protein are sufficient for nuclear localization, functioning as an NLS, and that, furthermore, substituting lysine in position 27 or 40 of this sequence with glutamate successfully eliminates this signal.

However, when GFP construct of full-length Nova1 protein containing these mutations were transfected into N2A cells, they were able to partly localize to the nucleus and, furthermore, exhibited the punctate staining pattern seen with the wild type protein (GFP-Nova1 K27E, K40E, and K27,40E in Figure 30). This suggests that the NLS of Nova1, while sufficient, is not necessary for nuclear localization and, more importantly, it does not mediate the distinct, punctate nuclear staining pattern of Nova. Consistent with

this data, a GFP construct completely lacking the entire NLS sequence of Nova1 (GFP-Nova1 ΔNLS, Figure 30) was still able to partly localize in nuclear foci. Thus, it can be concluded from these results that other domains in the Nova protein are responsible for its punctate staining pattern.

In order to identify these domains, more Nova1 deletion constructs were made and tested for their contribution to its localization pattern. A GFP fusion construct of the first and second KH domains (GFP-Nova1 KH1/2, Figure 30) was completely nuclear as expected, since it contains the NLS sequence. Nevertheless, it did not exhibit a punctate pattern within the nucleus, suggesting that the domain responsible for this may be located in the carboxy terminal half of the protein.

Indeed, when a construct containing the spacer and KH3 domains of Nova1 was tested (GFP-Nova1 sp/KH3, Figure 30), the staining observed was mostly nuclear and appeared to be forming discrete concentrations suggestive of the foci described thus far. Therefore, a domain guiding the localization of Nova to the nucleus is present in the second half of the protein and it could be responsible for its concentration in the sub-nuclear structures observed. An attempt to pinpoint this domain by examining the localization of a full-length and a truncated form of the spacer (GFP-Nova1 spacer and GFP-Nova1 t.spacer, respectively, Figure 30) was not successful as these constructs did not differ appreciably from the diffuse cellular staining obtained with the empty vector.

Discussion

The study of a protein's cellular localization can provide valuable clues as to other proteins it may interact and co-localize with ultimately leading to an understanding of its function. The paraneoplastic disease antigen Nova is an RNA-binding protein that has been shown to be essential for the development and survival of neurons, possibly through its role in regulating alternative splicing (Jensen et al., 2000a). Many RBPs and specifically splicing factors have been shown to localize to distinct sub-nuclear structures (reviewed in Sleeman and Lamond, 1999; Lewis and Tollervey, 2000). In this study, the nuclear localization of Nova was examined, both in cell lines and tissue sections with the purpose of gathering more information about its function in neuronal RNA processing.

The results presented identify discrete foci where both Nova1 and Nova2 concentrate in neuronal nuclei and where none of the other RBPs whose nuclear localization has been described in some detail can be found. These Nova foci amount to between four and five structures per nucleus which would place them in a similar category with coiled bodies and gems, based on size and number (see Table 3). Importantly, brPTB, a protein identified by virtue of its interaction with Nova, co-localizes with it in these foci (Polydorides et al., 2000; this work).

The absence of co-localization of Nova with proteins that occupy speckles (Sm and SR proteins) or perichromatin fibrils (SC35, RNA polymerase II) is perhaps surprising, given its role as a splicing activator. However, not all splicing factors localize in speckles. Furthermore, speckles have been described as storage sites for splicing factors, while the functional role of the nuclear concentrations of Nova has not been elucidated yet and might not be one of inactive storage. Many of the active sites of

transcription that have been loosely described as perichromatin fibrils, are hard to detect, only appear after special treatment, and do not necessarily conform to a given pattern (Huang and Spector, 1992; Misteli and Spector, 1998). Thus, if Nova played a role in splicing regulation, and this was coupled to active transcription, it could still be possible that the Nova nuclear foci are sites of active splicing and do no co-localize with the proteins tested here. In that respect, the Nova sites could be the setting of splicing for specific target RNAs (hence their small number in the nucleus) and not sites where the general splicing machinery (e.g. RNA polymerase II, SC35, etc.) is localized.

The co-localization of Nova with brPTB was observed with endogenous and transfected proteins in N2A cells but not in other cell lines, suggesting that this interaction is neuron-specific. The simple *in vitro* protein-protein interaction between Nova and brPTB did not require the presence of other protein or RNA co-factors (see Chapter 3). When transfected in 293T or HeLa cells, both brPTB and Nova proteins can individually localize in a pattern similar to the endogenous pattern seen in N2A cells, but they do not co-localize (Figures 26, 27, and 28). This result insinuates that another neuronal-specific co-factor is required for this co-localization while no such factor is necessary for their *in vitro* GST pull-down interaction. Importantly, the *in vivo* GST pull-down was performed with N2A cell extracts and was not attempted in 293T or HeLa cells. Alternatively, factors that restrict this interaction could exist in 293T or HeLa cells only and not in N2As, thereby forbidding co-localization in these cells.

It is important to note the apparent contradiction between the results presented here (i.e. the absence of co-localization between brPTB and Nova proteins in 293T cells) and the effect of brPTB on Nova1-dependent exon selection discussed in Chapter 4. The

gross pattern observed in these co-localization studies depends on antibody recognition of large amounts of proteins in fixed, permeabilized cells. In contrast, the splicing assays presented in Chapter 4 concern individual proteins on a molecular level and their functional interaction over a single RNA molecule. While a significant amount of splicing events would be required to explain the differences observed in exon selection, the RT-PCR assay is much more sensitive than immunofluorescent techniques. Similarly, while Nova and brPTB co-localize in N2A cells, there seem to be no brPTB-mediated inhibition of Nova1 splicing activation. The implications of these results for the proposed functional significance of the interaction between brPTB and Nova proteins will be discussed in more detail in Chapter 6.

It is not clear from these results whether the localization of one of the two proteins is required first, in order to serve as an anchor for the localization of the second. brPTB contains a putative NLS as well, and it has not been possible to map the interaction between Nova and brPTB to specific domains. While an RNA factor is not required for the *in vitro* interaction, it is entirely possible that the co-localization, which would probably require larger amounts of proteins in order to be detectable by immunoreactive methods, occurs over an RNA target that both proteins bind to. For example, in the case of the GlyRα2 mRNA, the binding sites that have been identified for Nova and brPTB are not overlapping and could serve as the anchor for bringing these proteins together in the nucleus (see Chapter 4).

As previously discussed, the perinucleolar compartment is a newly recognized structural entity in the nucleus that contains the PTB, PSF, RNA polymerase II and KSRP proteins (Matera et al., 1995; Huang et al., 1997; Black et al., RNA 2001 meeting poster

presentation). No information is available on the differential composition of the PNC between various tissues. In the results described here, PTB co-localized with Nova only in N2A cells, yet when examined in other cells lines, it displayed a pattern that could very well be described as perinucleolar (for example, see Figure 28). Nova also seemed to be perinucleolar in those cases but it did not coincide with PTB. However, Nova did not co-localize with RNA pol II which has been detected in the PNC (Huang et al., 1997), while its co-localization with KSRP and PSF was not tested. It remains to be seen whether the co-localization of Nova with brPTB in neuronal nuclei occurs within the context of the PNC and whether this structure contains different proteins among various cell types.

It is evident that the staining pattern of brPTB is not as punctate as the one for Nova. Besides staining the cytoplasm, brPTB stains the nucleus more broadly than Nova does, yet can still be seen in distinct concentrations of higher intensity that coincide with Nova staining. The broader staining of brPTB could be the result of background reactivity due to the polyclonal antibody and not due to more widespread expression of the protein. However, this is unlikely as transfection of GFP- or flag-tagged brPTB constructs result in a similar staining pattern. Moreover, a general caveat of experiments such as these, involves the problem of proteins being over-expressed from transfected constructs. Nevertheless, since the localization pattern of Nova and brPTB was also examined in the case of endogenous proteins with immunostaining techniques, this is unlikely to account for the data presented.

Some of the domains of Nova1 that could mediate nuclear and sub-nuclear localization involve regions of sequence homology with domains of known function from other proteins. These include a classic NLS signal similar to the one present in the

hnRNP K protein, and an NES sequence, homologous to the one present in the HIV protein Rev. As the experiments presented here show, the Nova1 NLS is sufficient but not necessary for nuclear localization. Once deleted, Nova1 is still able to be imported into the nucleus and, once there, it can localize in a punctate pattern. Save the possibility of diffusion of this construct into the nucleus, this would suggest that another domain exists in Nova1 that is able to guide it into the nucleus where it can localize in the distinct pattern observed (see below).

Similar results have been observed with the localization pattern of the closest, evolutionary, protein to Nova, hnRNP K. Deletion of the classical NLS in hnRNP K still allows it to localize to the nucleus (Michael et al., 1997). However, in that case, nuclear import becomes transcription-dependent, supporting the evidence that shuttling and nuclear localization of these RBPs is contingent on their function. This line of evidence led to the discovery of KNS, a novel shuttling domain that can carry hnRNP K in and out of the nucleus even when the classical NLS is absent. While Nova has not been shown to shuttle yet, insight from the results with hnRNP K can prove helpful in elucidating the mechanism by which, even in the absence of a functional NLS, Nova can localize in the foci observed with full-length protein.

As implied by the above, the sub-nuclear localization of Nova proteins could be the result of actual RNA binding, as is the case for other RBPs. This would entail the function of its three KH domains. However, when constructs containing the first two KH domains of Nova1 were tested, no discernible sub-nuclear localization pattern emerged (Figure 30). When the spacer/KH3 construct was tested some foci materialized, but none as pronounced as in the case of the full-length protein. The spacer domain has been

hypothesized to play a role in homotypic or heterotypic dimer formation that could facilitate RNA binding. Some preliminary evidence has hinted that the third KH domain of Nova plays an important role in RNA binding and perhaps could be the main RNA binding motif (Lewis et al., 2000; Jensen et al., 2000b; Dredge et al., work in progress).

This would suggest that the sub-nuclear structural formations of Nova are sites of active RNA-binding and perhaps of functional activity (i.e. splicing regulation) as well. In concert with this hypothesis, experiments with actinomycin D (a drug that inhibits transcription) reduce the appearance of Nova foci suggesting that those are active sites related to ongoing gene expression and not storage facilities of inactive proteins as has been proposed for splicing factors and speckles which get bigger with such treatment (Jensen, Dredge and Darnell, personal communication). However, Nova does not localize in sites where presumably transcription and splicing are taking place (i.e. perichromatin fibrils).

It would be interesting to repeat the actinomycin D experiment with the Nova construct lacking the NLS sequence, which has been shown here to still be able to localize to the nucleus and, furthermore, concentrate in sub-nuclear foci. If in fact the Nova ΔNLS constructs are still responsive to actinomycin D treatment, as they are in the case of hnRNP K, it would suggest that other domains in Nova are responsible for its ability to enter the nucleus and that the classical NLS identified here is not part of a possible shuttling domain in Nova, but rather exists in addition to it.

It will be instructive to ascertain whether Nova proteins shuttle between the nucleus and the cytoplasm and delineate the requirements for this shuttling (e.g. transcription dependence, domains, receptors, etc.). Along these lines, it will also be

important to examine the function of the Rev-like NES sequence present in Nova1 and Nova2. Importantly, the NES present in Nova2 contains one less leucine residue and preliminary evidence suggests that the localization of Nova2 is predominantly nuclear whereas Nova1 can also be found in the cytoplasm (Yang, 1997). Together with some evidence suggesting that Nova1 and Nova2 form dimers (Yang, 1997; this work), the hypothesis that Nova1 helps guide Nova2 out of the nucleus can be tested in hetero-karyon assays and transgenic mice.

The actual functional implication of these Nova/brPTB bodies remains to be seen. Important experiments that will help elucidate this, involve in situ hybridization with Nova RNA targets (genes whose splicing is regulated by Nova, such as GlyRα2 and $GABA_AR\gamma2$) and concomitant visualization of the Nova and brPTB proteins. These studies will determine whether Nova foci contain RNA and furthermore whether this interaction (RNA binding in the sub-nuclear localizations) reflects the role of Nova in splicing activation.

The interaction between Nova and brPTB and its importance in their nuclear co-localization can be further studied with the generation of dominant negative constructs. For example, Nova mutants that do not bind RNA but can mediate protein-protein interactions or conversely, that still bind RNA targets but do not interact with brPTB *in vitro*, can be used to determine whether the co-localization in the nucleus depends on RNA binding or protein interaction with brPTB. Finally, the generation of a brPTB knock out mouse will also shed light on its requirement for the nuclear localization of Nova as well as determine whether brPTB (and by extension hnRNP I/PTB) is an essential splicing factor and/or necessary for the development of the nervous systems.

Table 3. Summary of structural domains in the interphase nucleus.

Summary table of the most prominent and best-characterized structural features in the nucleus during interphase. Included are the name of the structure, the number of bodies per nucleus (No.), the proposed function, and some of the proteins known to localize within these bodies. For references, see text. NK: not known.

Structural Domains in the Interphase Nucleus

Structure	No.	Function	Proteins
Nucleolus	1-3	rRNA transcription, processing	RNA pol I, III
Coiled (Cajal) bodies (CB)	1-10	snRNP biogenesis, trafficking and regeneration, RNA processing	Coilin p80, Nopp140, ELAV, pigpen (hnRNP P2), snRNPs (Sm), U2AF65/35, cdk2-cyclinE, fibrillarin
Gemini of CBs	3-10	Cytoplasmic snRNP biogenesis	SMN, SIP-1
Perichromatin fibrils	>100	Transcription/splicing sites	RNA pol II, some SR proteins (SC35)
Speckles (Inter-chromatin Granules)	20-50	Splicing factor storage sites	snRNPs (Sm), SR proteins, stable polyA RNA (no pre-mRNA)
PML bodies	10-50	Transcription regulation, apoptosis	PML, Rb, Sumo-1, Sp100
hnRNP clusters	NK	NK	eg. hnRNP L
Perinucleolar Compartment (PNC)	NK	NK	RNA pol II, PTB, PSF, KSRP
Others: Heat shock factor 1 (HSF1), Polycomb-group proteins (PcG), Transcription factor clusters (GATA-1 bodies), Cleavage/polyadenylation bodies, OPT domain (Oct1, PTF, Transcription)			

Figure 22. Nova localizes in distinct sub-nuclear structures.

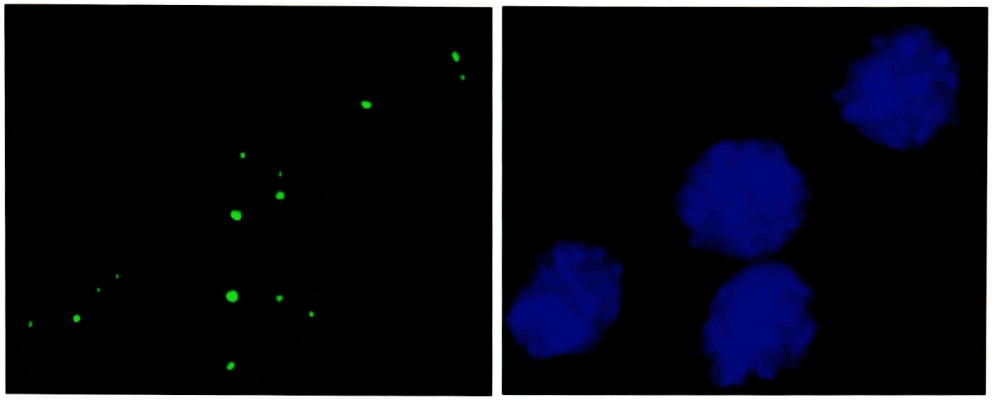
(A) N2A cells were fixed and stained with anti-Nova human serum and visualized with Cy2-conjugated anti-human secondary antibody (green). Notice the four to five distinct, bright-staining foci per cell nucleus.

(B) N2A cells were stained with Nova2-specific affinity-purified polyclonal antibody (Yang, 1997) and Cy3-conjugated anti-rabbit secondary antibody (red). Similar staining to Nova1 was observed.

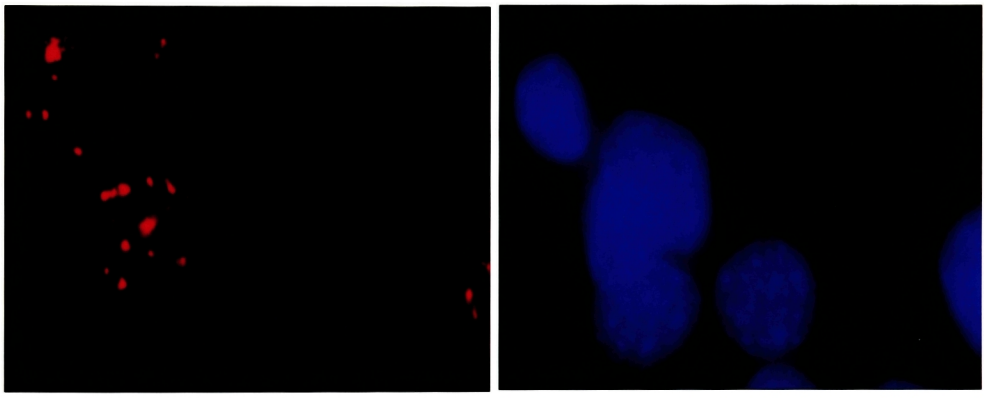
(C) Mouse brain sagital sections were stained with anti-Nova human serum and visualized with Cy2-conjugated anti-human secondary antibody (green, see Materials and Methods).

DAPI stain was used in all slides to visualize cell nuclei.

A.



B.



C.

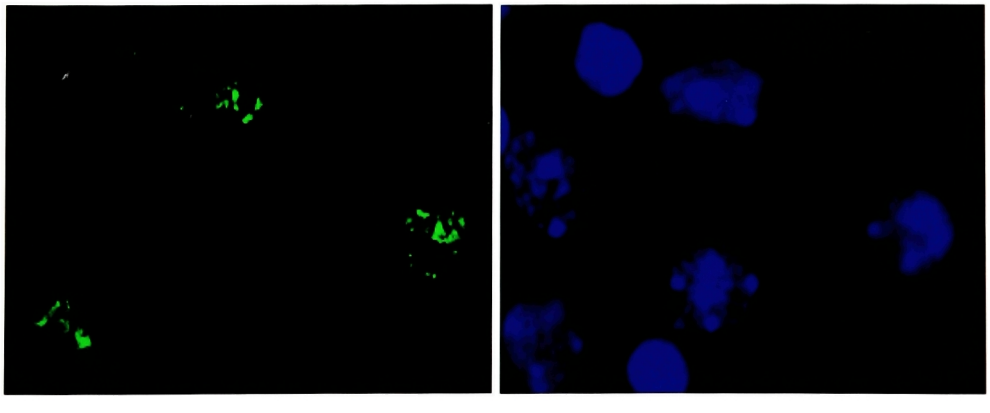
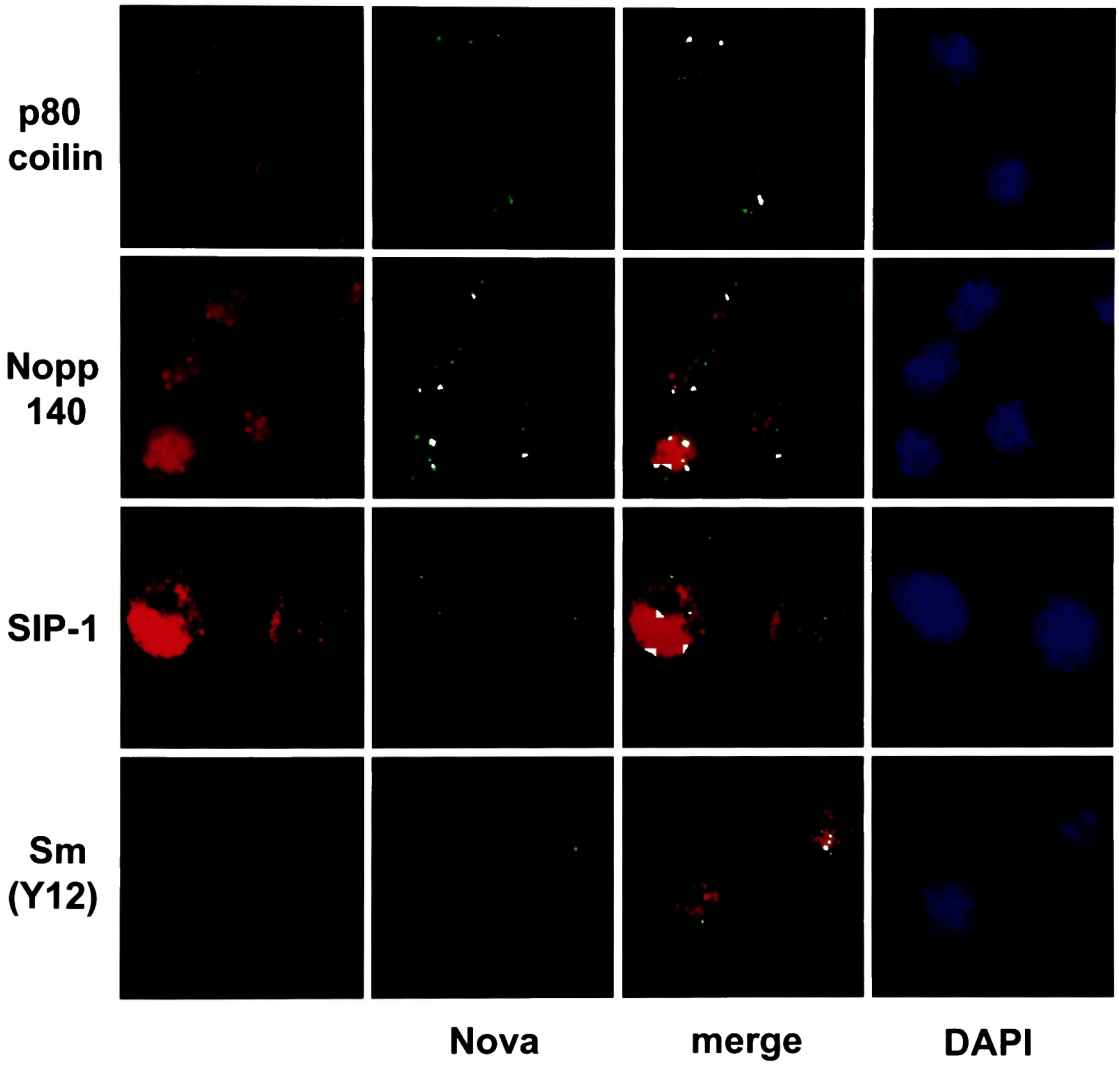


Figure 23. Nova does not colocalize with known sub-nuclear structures.

N2A cells were fixed and stained with the indicated primary antibodies and human anti-Nova serum. The appropriate Cy3-conjugated secondary antibody was used to visualize each protein (red, first column). Cy2-conjugated anti-human secondary antibody was used to visualize Nova (green, second column). The two planes were then merged to examine co-localization (third column). The cells were also stained for DAPI to visualize cell nuclei (blue, fourth column). For the antibodies used and the specific proteins recognized by each, see Materials and Methods.


p80
coilin
Nopp
140
SIP-1
Sm
(Y12)
Nova
merge
DAPI

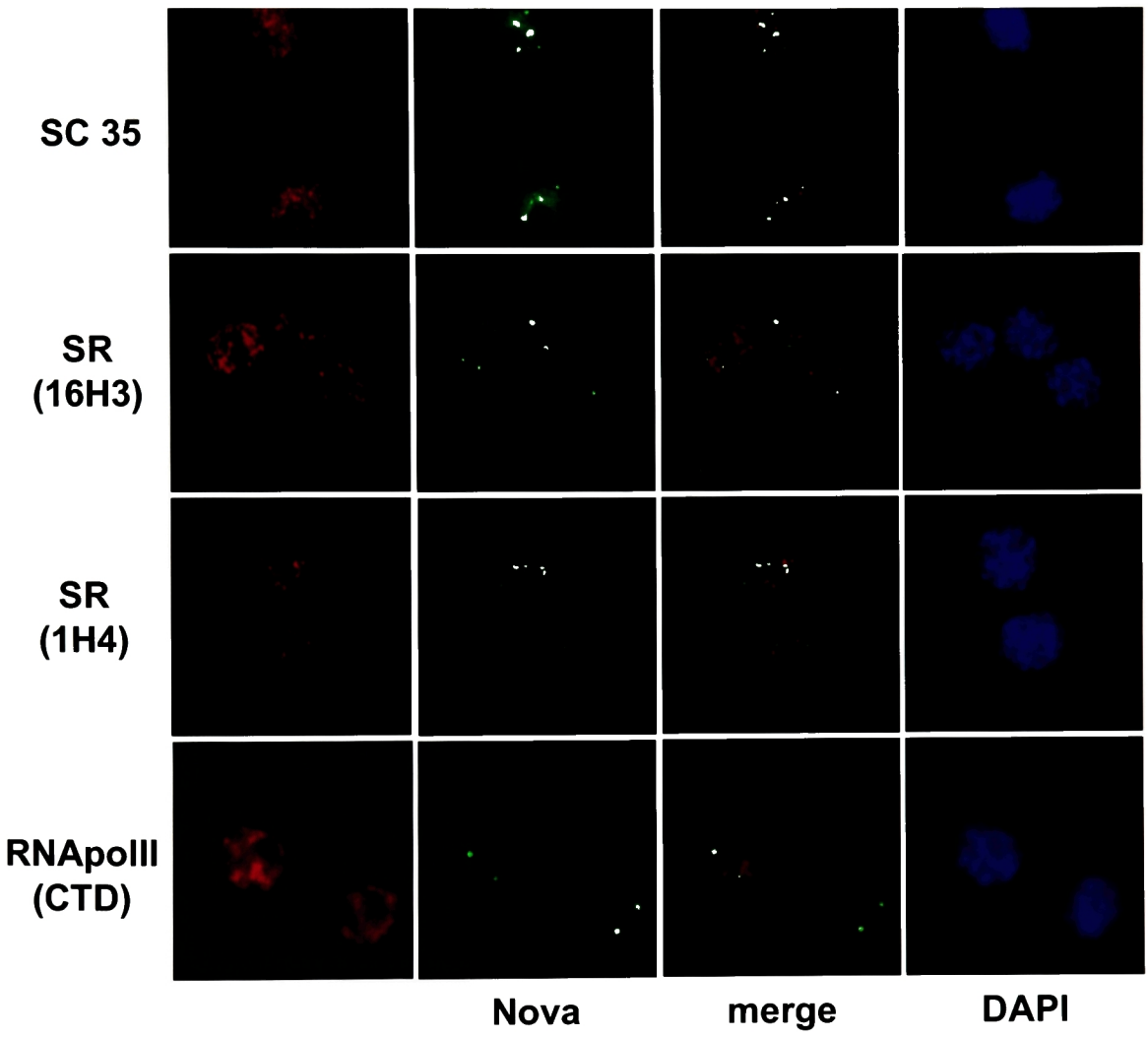

SC 35
SR
(16H3)
SR
(1H4)
RNApolII
(CTD)
Nova
merge
DAPI

Figure 24. Endogenous Nova and brPTB co-localize in N2A cell nuclei.

(A) N2A cells were fixed and stained with rabbit polyclonal anti-brPTB serum (top left panel) and human anti-Nova patient serum (top right panel). Cy2-conjugated anti-human (green) and Cy3-conjugated anti-rabbit (red) secondary antibodies were used to visualize the proteins, respectively. The panels were merged to examine co-localization, evident by the yellow color (bottom left panel). Cells were also stained with DAPI to delineate the nuclei (blue, bottom right panel).

(B) Bleedthrough Controls. N2A cells were stained individually for Nova (top row) or brPTB (bottom row) as before, the indicated secondary antibodies, and DAPI. When observed with the red filter, Cy-3 conjugated anti-human antibody did not cause any bleed-through (top row, middle panel). Similarly, the Cy2-conjugated anti-rabbit antibody used to visualize brPTB protein did not light up with the green filter (bottom row, left panel) demonstrating that the co-localization patterns observed were not due to secondary antibodies emitting signal with the wrong filter.

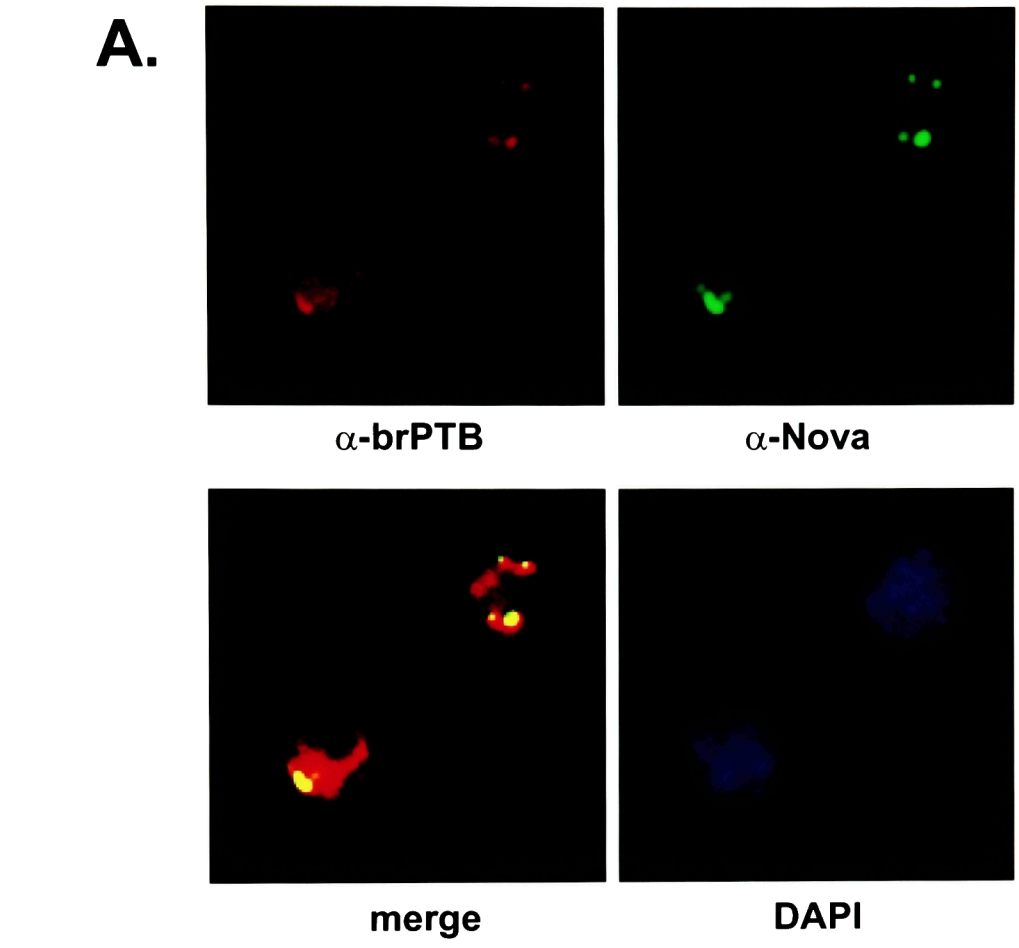
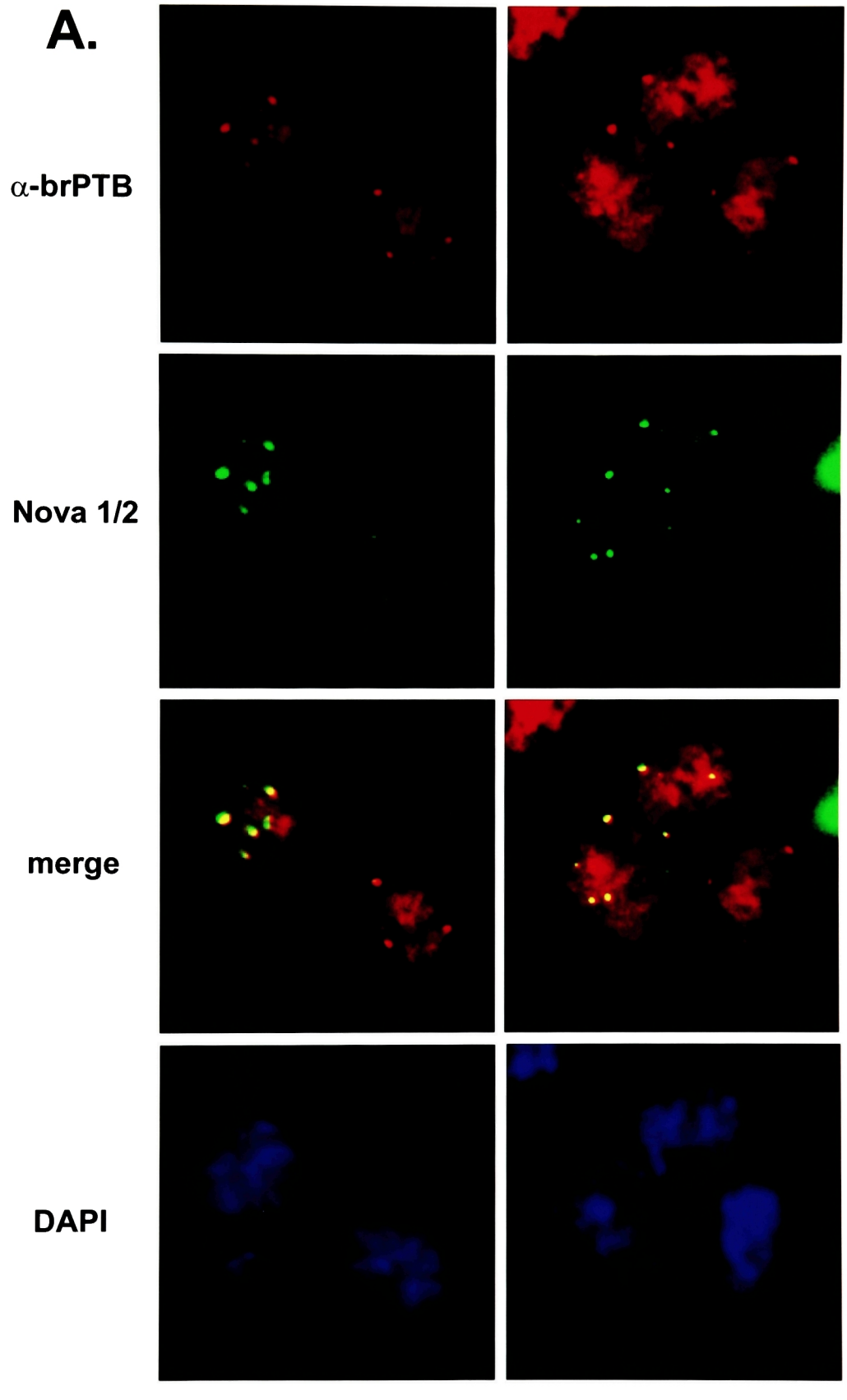

A.
α-brPTB
α-Nova
merge
DAPI
B.
α-Nova
Cy3 α-human
DAPI
α-brPTB
Cy2 α-rabbit
DAPI
green filter
red filter
blue filter

Figure 25. Transfected brPTB and PTB co-localize with Nova in N2As.

(A) GFP-Nova1 (left) and GFP-Nova2 (right) eukaryotic expression constructs were transfected into N2A cells. After fixation, cells were stained with anti-brPTB serum and Cy2-conjugated secondary antibody (red, first row panels). GFP-Nova1 (left column, second row) and GFP-Nova2 (right column, second row) were visualized with the green filter. The merged images (third row) demonstrate co-localization, as seen by the yellow-colored foci. DAPI stain was used to delineate cell nuclei (blue, fourth row).

(B) Eukaryotic expression constructs for flag-tagged brPTB (left column) and myc-tagged PTB (middle column) were transfected into N2A cells that were subsequently fixed and stained with anti-Nova serum and anti-flag or anti-myc monoclonal antibodies, respectively. Alternatively, anti-matrin3 polyclonal chicken antibody was used together with anti-Nova serum (right column). Cy2-conjugated anti-human secondary antibody was used to visualize Nova proteins (green, second row) and Cy3-conjugated anti-mouse or anti-chicken secondary antibodies were used to visualize brPTB, PTB and matrin3 (red, first row). Merged panels (third row) show that both transfected brPTB and PTB are able to co-localize with endogenous Nova proteins in N2A cells. In contrast, endogenous matrin3 protein, perhaps because its staining is more diffuse, does not co-localize with Nova. DAPI was used to stain cell nuclei (blue, fourth row).


A.
α-brPTB
Nova 1/2
merge
DAPI

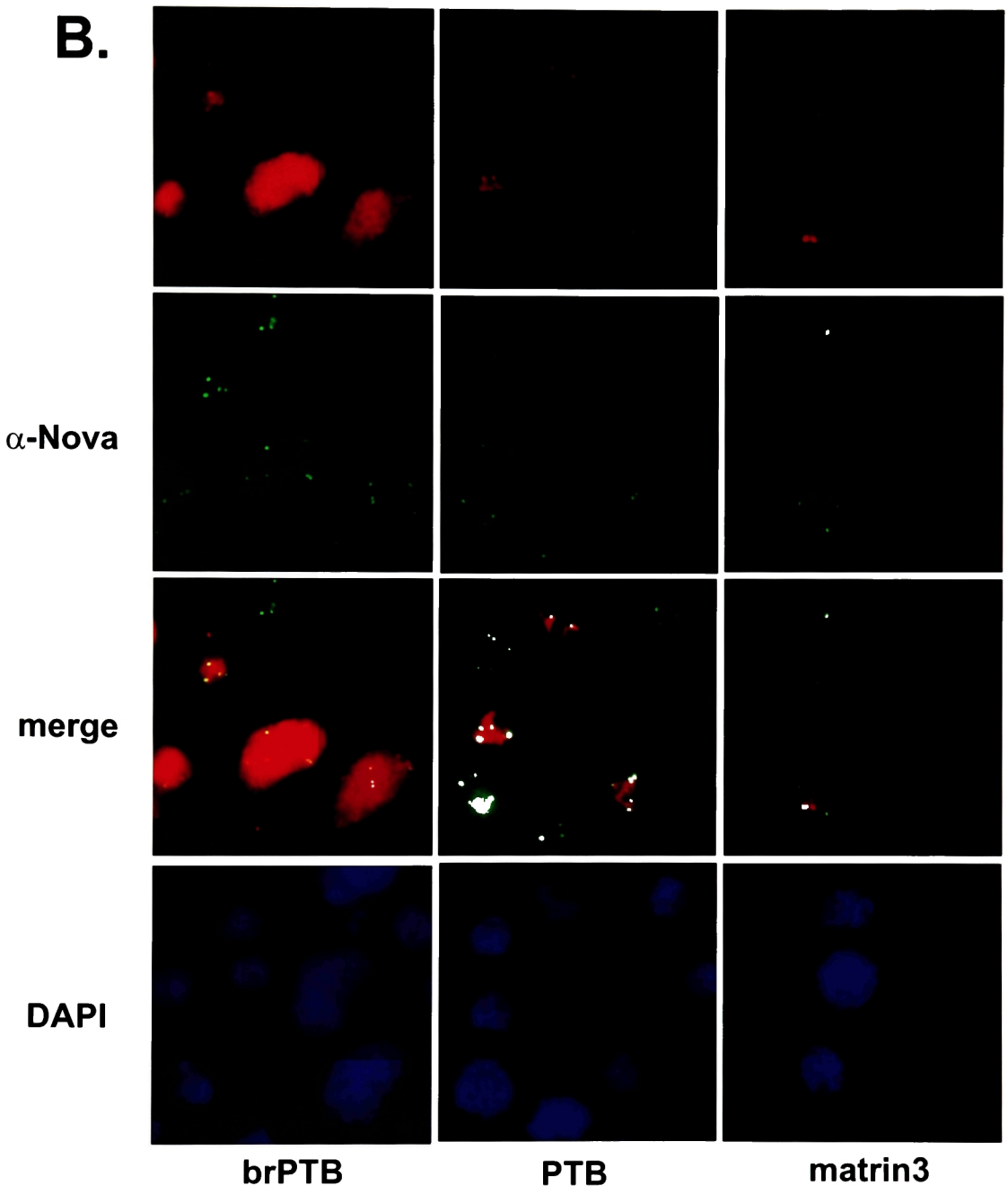
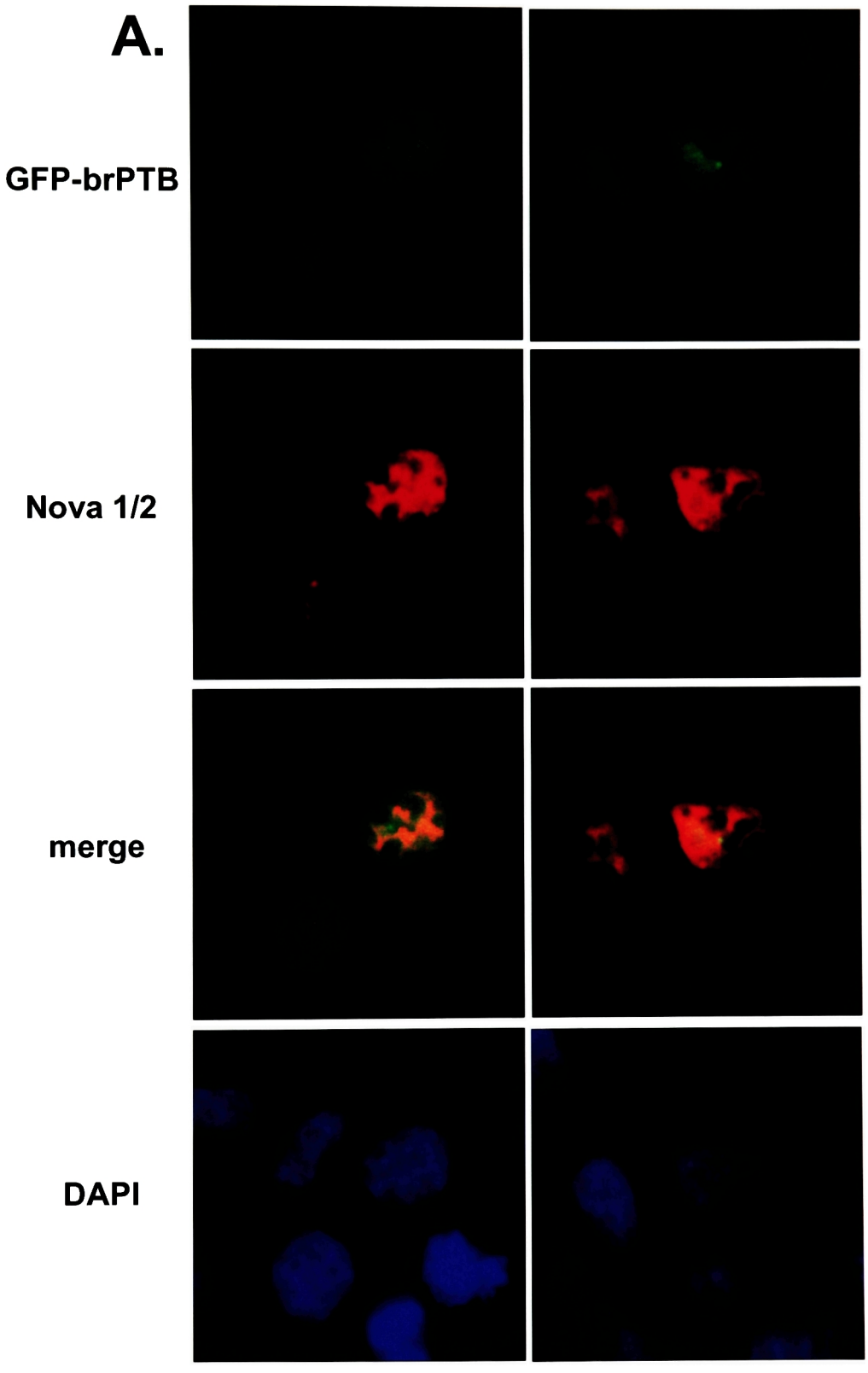

B.
α-Nova
merge
DAPI
brPTB
PTB
matrin3

Figure 26. Transfected brPTB does not co-localize with Nova in 293Ts.

(A) 293T cells were transfected with T7-tagged Nova1 (left column) and GST-tagged Nova2 (right column) as well as brPTB eukaryotic expression constructs. The cells were fixed and stained with the appropriate primary monoclonal antibodies. GFP-brPTB protein was visualized with the green filter (first row) and Nova proteins were visualized with Cy3-conjugated anti-mouse secondary antibodies (red, second row). Merged images (third row) show no co-localization. DAPI was used to delineate cell nuclei (blue, fourth row).

(B) 293T cells were transfected with GFP-Nova1 (left column) or GFP-Nova2 (right column) and flag-tagged brPTB. brPTB was visualized with anti-flag primary antibody and Cy3-conjugated anti-mouse secondary antibody (red, first row) while Nova proteins were visualized with the green filter (second row). No co-localization is observed when the panels are merged (third row). DAPI was used to stain cell nuclei (blue, fourth row).


A.
GFP-brPTB
Nova 1/2
merge
DAPI

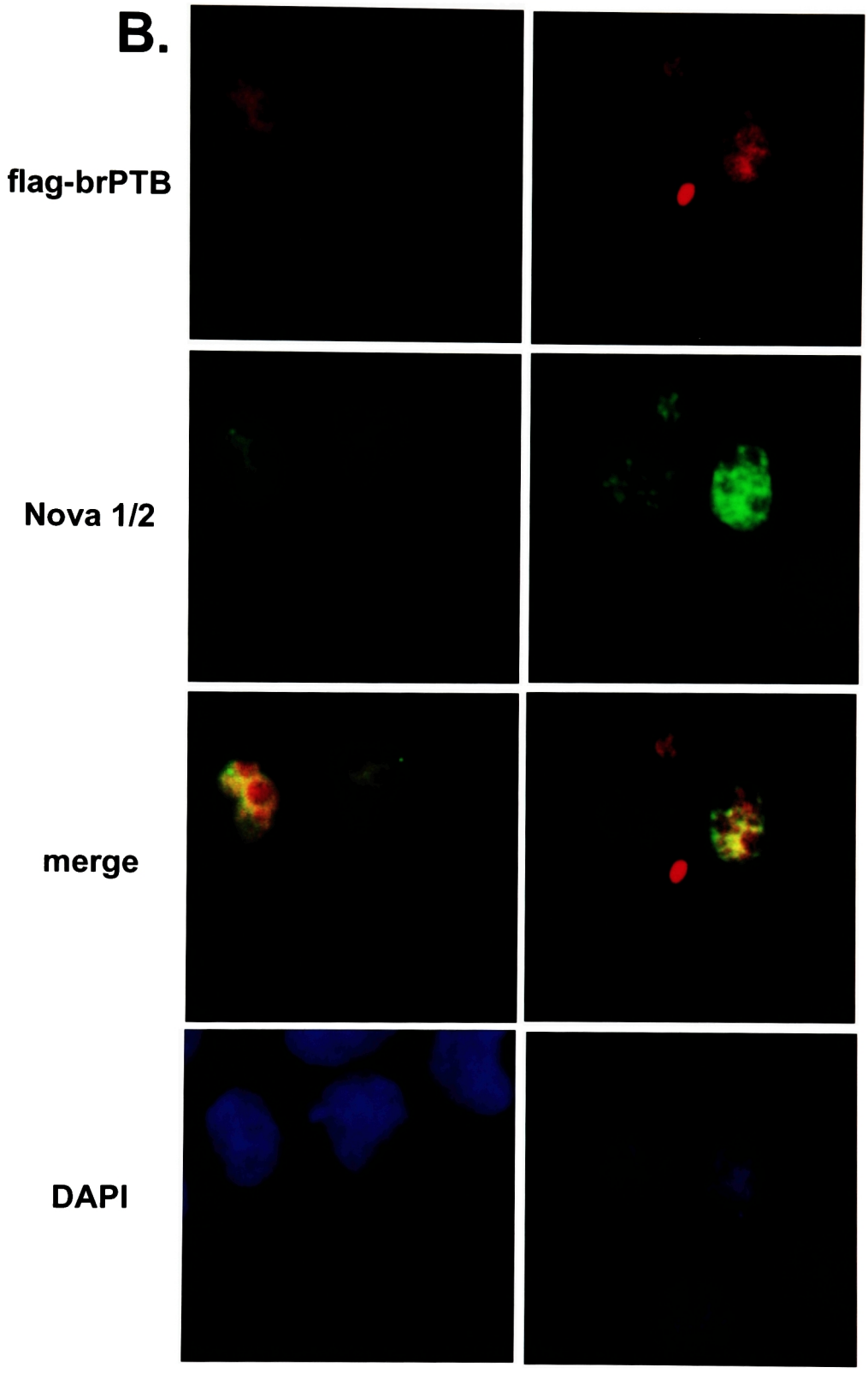
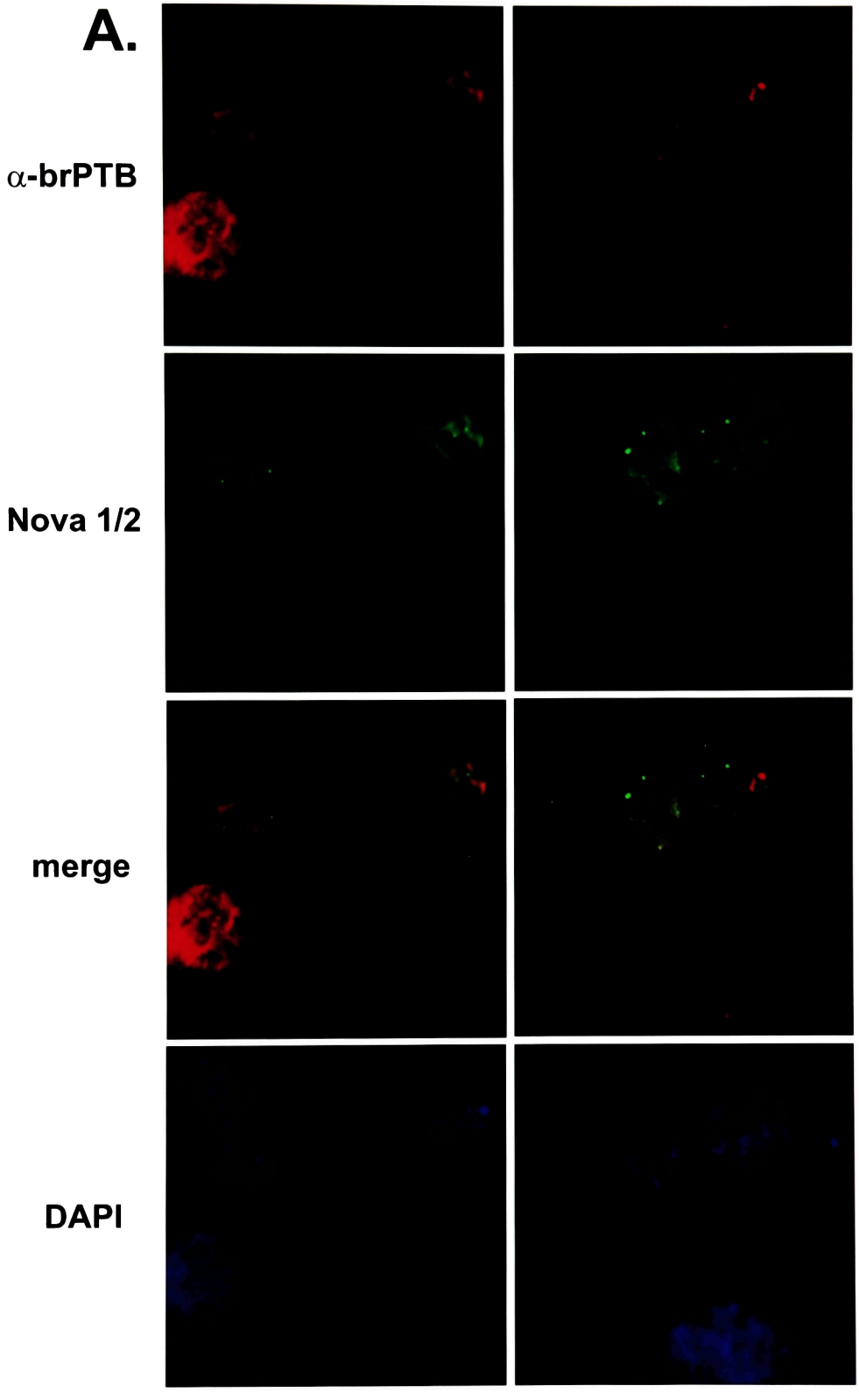

B.
flag-brPTB
Nova 1/2
merge
DAPI

Figure 27. PTB does not co-localize with Nova in 293T cells.

(A) 293T cells were transfected with GFP-Nova1 (left column) or GFP-Nova2 (right column), visualized with the green filter (second row). Anti-brPTB antibody was used to stain PTB proteins present in 293T cells and was visualized with Cy3-conjugated anti-rabbit secondary antibody (red, first row). Merged images (third row) show no co-localization. DAPI was used to delineate cell nuclei (blue, fourth row).

(B) 293T cells were transfected with myc-tagged PTB constructs and again GFP-Nova1 (left column) or GFP-Nova2 (right column). PTB was visualized with anti-myc primary antibody and Cy3-conjugated anti-mouse secondary antibody (red, first row) and Nova proteins were visualized with the green filter (second row). No co-localization is observed when the panels are merged (third row). DAPI was used to stain cell nuclei (blue, fourth row).


A.
α-brPTB
Nova 1/2
merge
DAPI

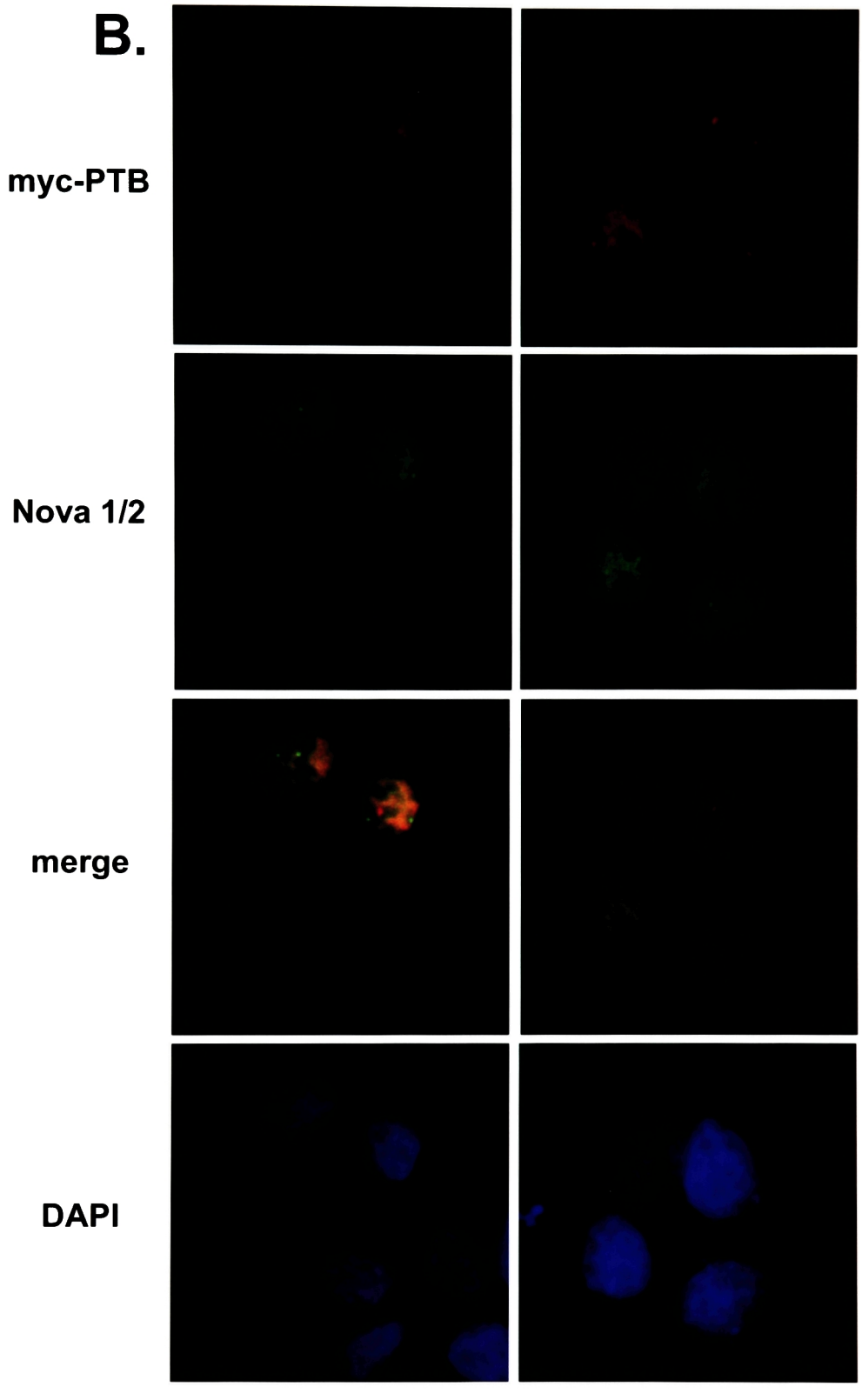

B.
myc-PTB
Nova 1/2
merge
DAPI

Figure 28. PTB proteins do not co-localize with Nova in HeLa cells.

HeLa cells were transfected with GFP-Nova1 (left column) or GFP-Nova2 (right column). After fixation, cells were incubated with anti-brPTB antibody. PTB was visualized with Cy3-conjugated anti-rabbit secondary antibody (red, first row) and Nova proteins were visualized with the green filter (second row). Merged images (third row) show no co-localization. DAPI was used to stain cell nuclei (blue, fourth row).

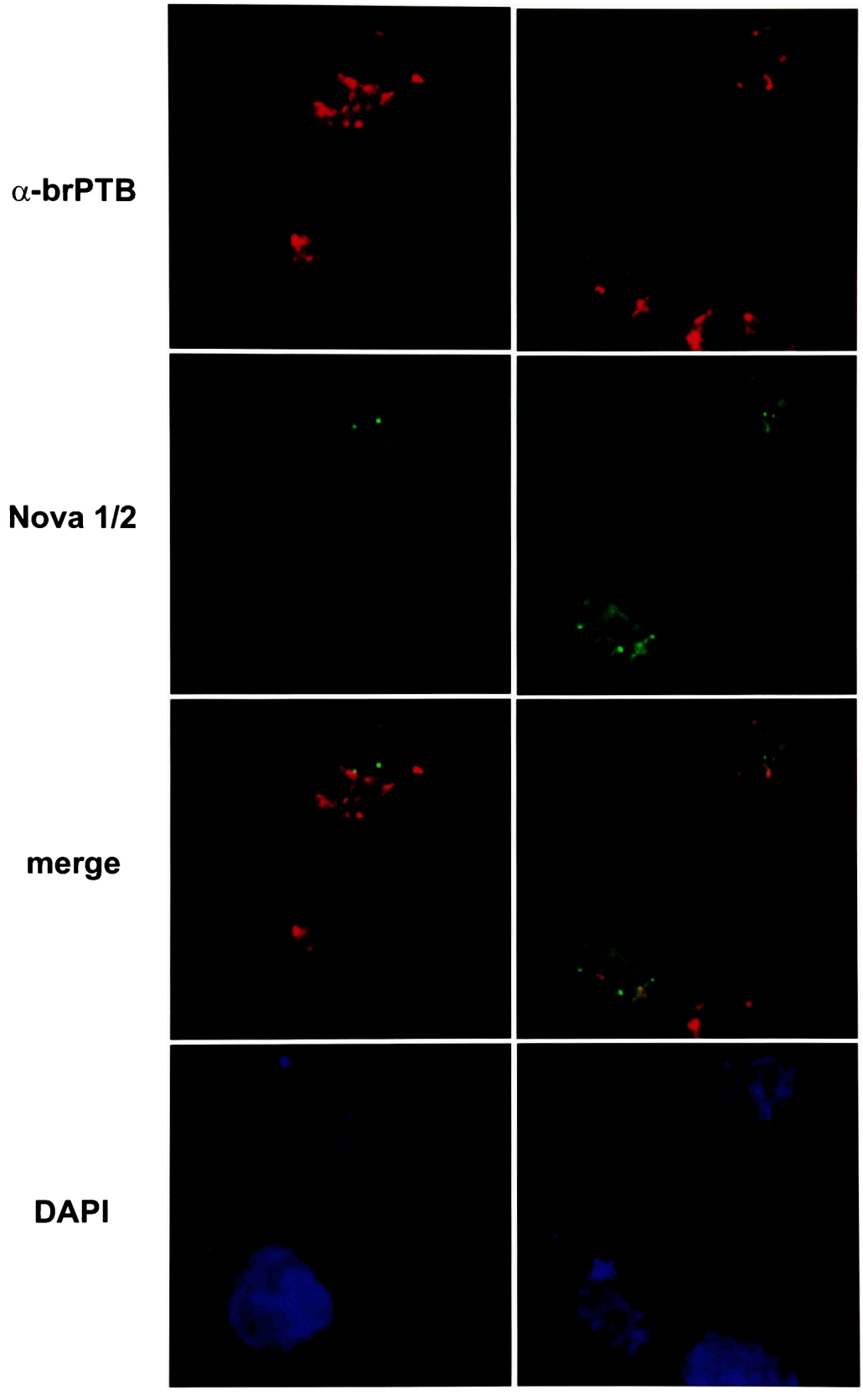
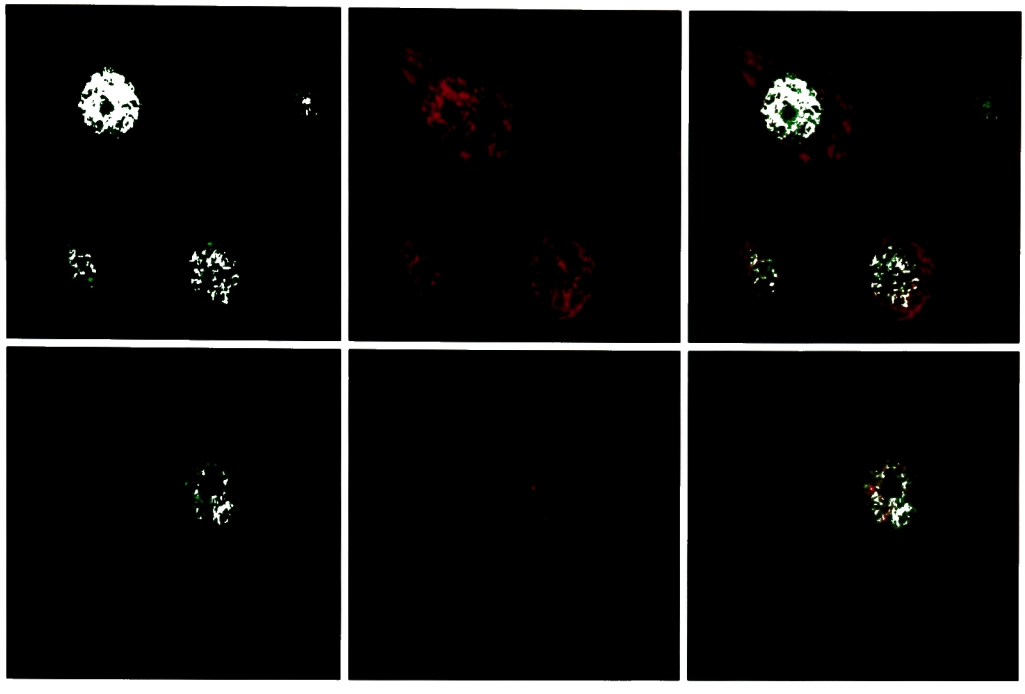

α-brPTB
Nova 1/2
merge
DAPI

Figure 29. Nova and brPTB proteins co-localize in tissue sections.

(A) Confocal laser images of Nova and brPTB expression patterns in P5 mouse spinal cord horizontal sections (top row) and adult rat spinal cord horizontal sections (bottom row). Nova proteins (green, left column) were visualized with patient serum and Cy-5-conjugated anti-human secondary antibody. In the same optical section, brPTB protein (red, middle column) was visualized with anti-brPTB antibody and Cy-2-conjugated anti-rabbit secondary antibody. Merged panels (right column) show co-localization of brPTB and Nova proteins within nuclei of motor neurons. Some cells (presumably glia) stain only with anti-brPTB antibody and not with anti-Nova serum.

(B) Immunohistochemical studies of cerebellar sections of P5 Nova1 knockout mice (KO, bottom row) and their wild type litter mates (WT, top row). Sections were stained with anti-Nova serum (green, left column) and anti-brPTB antibody (red, middle column) as well as Cy2-conjugated anti-human and Cy3-conjugated anti-rabbit secondary antibodies. Merged panels (right column) show co-localization.

A.



B.

WT

KO

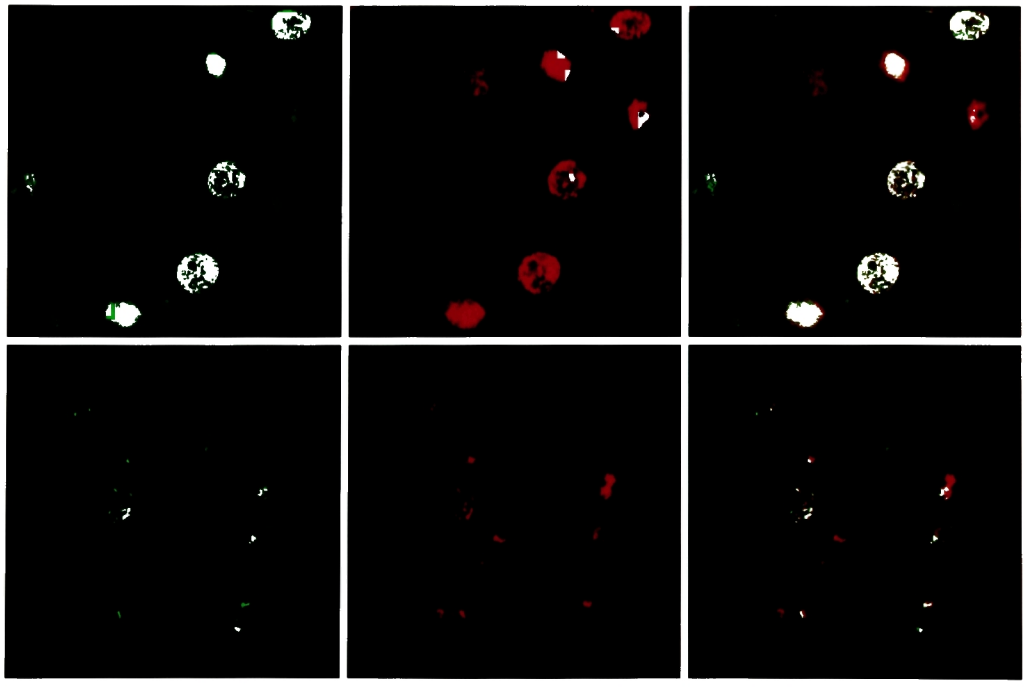
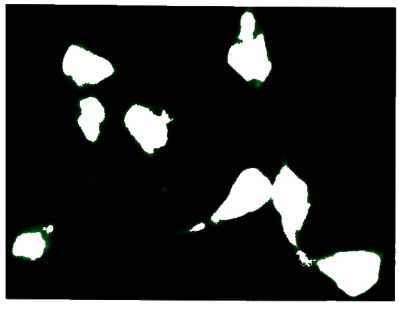
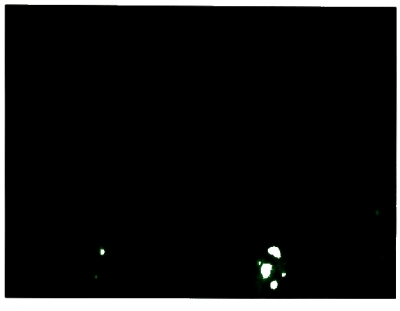


Figure 30. Localization of Nova1 deletion mutants in live N2A cells.

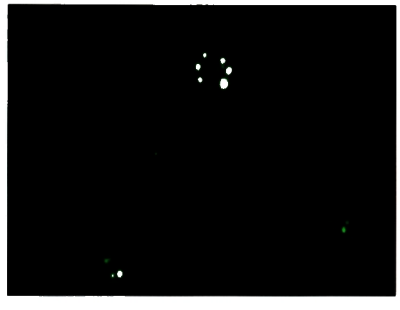
N2A cells were transfected with the indicated GFP fusion eukaryotic expression constructs and were observed live in an inverted microscope under a mercury lamp. All deletion constructs (except for the indicated full-length Nova2) were of the Nova1 protein (see Materials and Methods for an analytical description of the constructs).



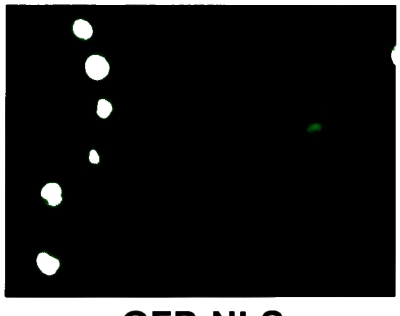
GFP vector



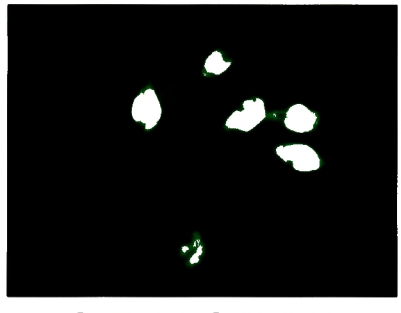
GFP-Nova1



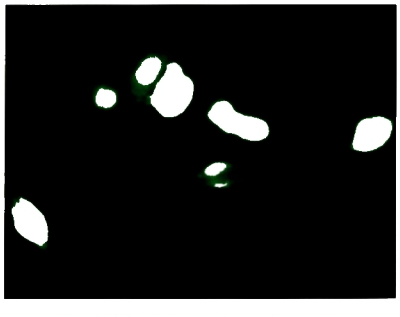
GFP-Nova2



GFP-NLS

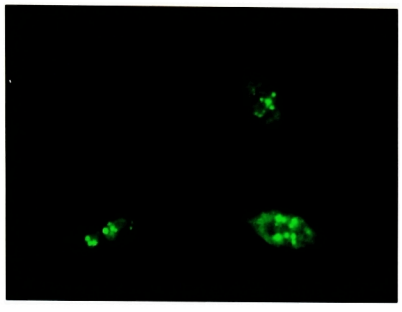


GFP-NLS K27E

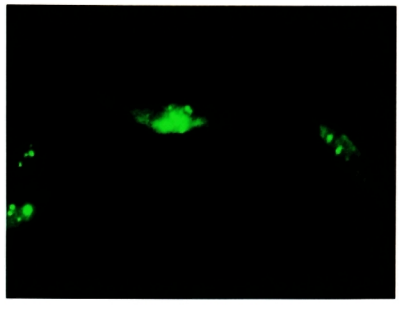


GFP-NLS K40E

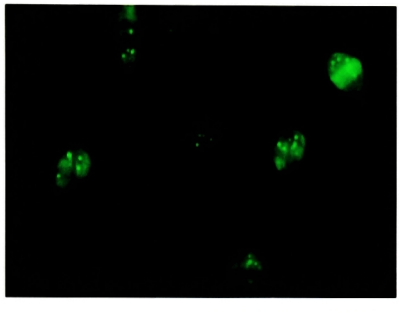
GFP-NLS K27,40E



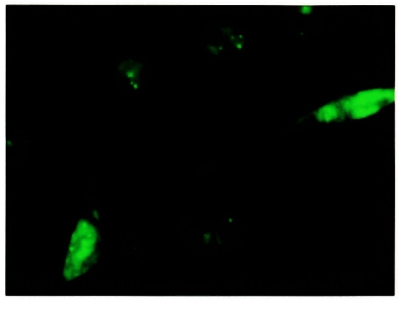
GFP-Nova1 K27E



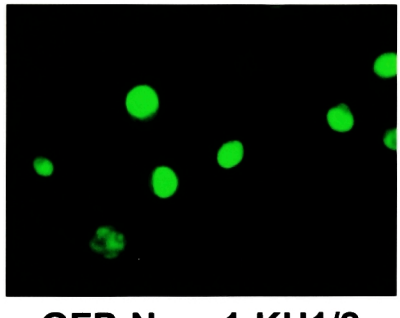
GFP-Nova1 K40E



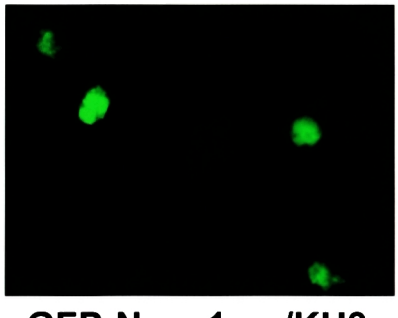
GFP-Nova1 K27,40E



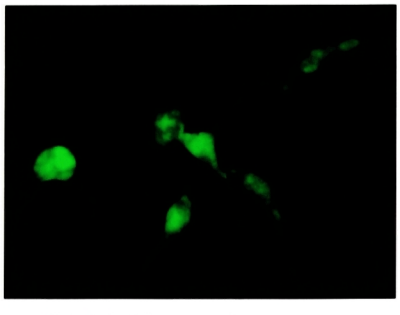
GFP-Nova1 ΔNLS



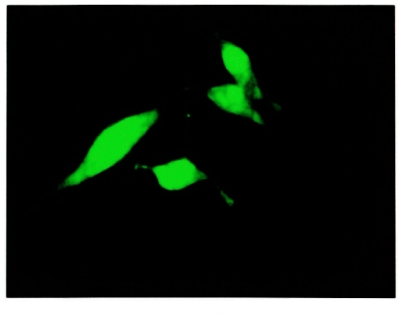
GFP-Nova1 KH1/2



GFP-Nova1 sp/KH3



GFP-Nova1 spacer



GFP-Nova1 t.spacer

Chapter 6 – General Discussion

RNA-binding proteins and neuron-specific alternative splicing

Approaches to examine the regulation of transcripts that are differentially spliced in the nervous system have made significant contributions to our basic understanding of the mechanisms by which neuron- and, by extension, cell-specific splicing, is achieved. Collective data from the study of genes implicated in neuronal diseases suggest that efficient regulation of such a tissue-specific cellular process requires several levels of control (reviewed in Dredge et al., 2001). First, specific cis-acting regulatory sequences are likely to be present near differentially spliced exons, as has been demonstrated for the c-src and $GABA_AR\gamma2$ transcripts. In general, splice sites in alternatively regulated exons are weak compared to the consensus and are poorly recognized by the basal splicing machinery. Second, trans-acting RNA-binding proteins, such as Nova, hnRNP A1 and hnRNP I/PTB need to recognize and interact with these sequences. In the case of weak splice sites in regulated exons, binding by hnRNPs and other factors could help "strengthen" them by recruiting components of the splicing machinery. Finally, there must be key protein-protein interactions whether positive or negative, direct or indirect. When these interactions occur between sequence- and gene-specific RBPs recognizing individual binding sites in exons or introns, they set the stage for "exon definition". On the other hand, when these RBPs interact with and recruit components of the general splicing machinery in the recognition of splice sites, they help form the spliceosomal "commitment complex" and provide for an additional level of splicing regulation.

The search for transcripts that undergo neuron-specific splicing has generated an important set of pre-mRNAs and splice variants (reviewed in Dredge et al., 2001; Grabowski and Black, 2001). Studies of the neuron-specific splicing of the c-src, $GABA_AR\gamma2$ subunit, NMDA receptor NR1 subunit and clathrin light chain B mRNAs have identified cis-acting repressor elements that mediate the exclusion of the neuron-specific exon in other tissues (Chan and Black, 1997; Ashiya and Grabowski, 1997; Zhang et al., 1999). These repressor elements were mostly pyrimidine-rich and, when present as competitors *in vitro*, were able to derepress splicing back to the neural pattern.

At the same time, a variety of biochemical approaches including UV cross-linking and immunoprecipitation experiments identified the polypyrimidine tract-binding protein (PTB) as an RBP that binds many of these repressor elements. (Chan and Black, 1997; Ashiya and Grabowski, 1997; reviewed in Wagner and Garcia-Blanco, 2001). *In vitro* splicing assays after immunodepletion and adding-back of recombinant protein have established that PTB protein is able to repress neuron-specific exon inclusion. In contrast, several splicing enhancers have been found to bind a regulatory sequence downstream of the neuron-specific exon in c-src, including hnRNP H, hnRNP F, and a protein named KSRP, a new splicing regulator that may be enriched in neuronal cell lines (Min et al., 1997; Chou et al., 1999).

One interpretation of such results is to view neuron-specific splicing as a default process, selectively repressed in non-neuronal tissues by trans-acting RNA-binding proteins such as the ubiquitous factor PTB. In neural cells, this repression is proposed to be relieved by the presence of positive regulators that would compete by simple mass action for the same or overlapping binding sites on the mRNA (Zhang et al., 1999;

reviewed in Grabowski, 1998). In support of a role for PTB in splicing repression, an antagonism between PTB and the U2AF splicing factor has been implied (Lin and Patton, 1995; Singh et al., 1995; Lou et al., 1999), that is mechanistically similar to the antagonistic binding of Sxl protein and U2AF on the transformer mRNA in Drosophila (Valcarcel et al., 1993). Due to the proximity of poly-pyrimidine tracts to the branch point intronic sequence, an interference by PTB in spliceosome assembly at the U2 snRNP step has also been suggested, in a manner parallel to the antagonism between ASF/SF2 and hnRNP A1 at the 5' splice site (Caceres et al., 1994). However, the ubiquitous expression of these factors (PTB, U2AF) has precluded any conclusions about their role in tissue-specific splicing regulation.

As mentioned above, the study of neurologic disease has been particularly powerful in terms of identifying key players in the regulation of neuron-specific splicing. The regulation of splicing in GlyRα2 by Nova has emerged as a clear example of the interplay between different RNA-binding proteins in the control of splicing. In this study, a brain enriched form of PTB named brPTB was cloned by virtue of its interaction with Nova and was shown to specifically antagonize the neuron-specific increase in GlyRα2 and $GABA_AR\gamma2$ exon inclusion mediated by Nova.

The presence of a neuronal form of PTB had been hinted at by UV crosslinking studies of both the $GABA_AR\gamma2$ subunit and the c-src m-RNAs, and a possible permissive role in splicing had been proposed to replace the repressive action of ubiquitous PTB (Chan and Black, 1997; Ashiya and Grabowski, 1997). However, brPTB does not de-repress splicing in neurons as had been suggested, and also does not appear to antagonize the positive effect of Nova in splicing by a simple displacement mechanism, since it has a

lower affinity for the target RNA than did Nova (Polydorides et al., 2000; this work). Furthermore, in the experiments presented here, PTB had a similar, if not more pronounced, effect in antagonizing the Nova1-dependent splicing increase.

More recently, it was shown that in neurons, ATP, most likely via another unidentified factor, removes PTB from repressor sequence elements present in the c-src transcript and thus allows for neural splicing to occur (Chou et al., 2000). Moreover, it was suggested that the behavior of brPTB was different than PTB in terms of RNA binding and splicing repression. While this model would still require the presence of a neuron-specific factor to mediate the effect of ATP, it provides insight as to the regulatory mechanisms of such a process. PTB may prevent the assembly of U2 snRNP on the RNA in non-neuronal cells and removal of PTB from the RNA in an ATP-dependent fashion leads to derepression of splicing in neurons. In this model, brPTB may add an additional level of splicing repression in neuronal cells.

Post-translational modifications of RNA-binding factors have also been described as a means of regulating their function. Specifically, phosphorylation of SR proteins has been shown to be required for their splicing enhancer or repressor function and additional proteins that regulate this modification have been identified (Cao, et al., 1997; Xiao et al., 1997; Kanopka et al., 1998; Petersen-Mahrt et al., 1999). However, there has been no evidence that any of these modifications, or the proteins that mediate them, are tissue or cell-type specific. Therefore, until such data emerges, the effect of phosphorylation and other post-translational modifications in the regulation of neuron-specific splicing will remain unclear. The interaction of Nova with p32/YL2 in the yeast-two-hybrid screen presented here and the suggestion that p32 negatively regulates ASF/SF2 by inhibiting its

phosphorylation (Petersen-Mahrt et al., 1999), suggest that the Nova splicing factor may also be regulated in such a manner. In support of this, Nova1 is phosphorylated *in vitro* and *in vivo* (Stefani et al., work in progress).

Neuron-specific splicing is likely to have many layers of control that will undoubtedly involve the regulated interplay between various tissue-specific and ubiquitous factors. In addition, the unique physiology of neurons allows them to modify these cellular processes even further. There have been reports that neuronal activity can alter the splicing pattern of various transcripts. For example, depolarization of pituitary neurons represses the inclusion of an exon in the BK potassium channel mRNA through the action of CaMKIV on an RNA element that is sufficient to confer repression control to a heterologous, constitutive exon (Xie and Black, 2001). Other transcripts that are regulated by activity-dependent splicing in the brain include the human tra-2 gene (Daoud et al., 1999) and the NMDA receptor NR1 subunit (Vezzani et al., 1995). To get a better understanding of how splicing works in mammalian cells (and specifically in neurons), and identify the role of the different cis- and trans-acting factors involved, there will need to be a concerted effort to identify these factors, examine their relative levels in different cell types, and analyze their function. Then, it will be feasible to elucidate the ways with which splicing is regulated, and how it is interconnected with other cellular processes such as signal transduction, transcription regulation and cell cycle control.

In summary, many regulatory processes such as signal transduction, neuronal synaptic activity and protein-protein interactions can control alternative splicing which in turn can result in the differential RNA processing of other, downstream RNA targets. This can be achieved if the alternative isoforms of the regulated gene product exhibit

different patterns of association and co-localization with other RBPs (including splicing factors) or other regulatory proteins (inclusing kinases). Examples include the recent description of different functional activities for the alternative spliced isoforms of the cyclin Ania-6 (Berke et al., 2001) and PTB (Wollerton et al., 2001) and have sparked interest in the co-localization patterns of splicing factors as a means for identifying functional interactions. The splicing of Ania-6 depends on the stimulation by various neurotransmitters and exhibits functional consequences *in vivo*, as the longer isoform of the transcript co-localizes, when translated, with nuclear speckles, the splicing factor SC35 and RNA pol II and could, thus, affect RNA processing in turn (Berke et al., 2001).

Since alternative splicing occurs in the nucleus, the search for trans-acting factors that might regulate it has focused on nuclear RNA-binding proteins. Reports that nuclear events have further consequences in the cytoplasmic regulation of the transcript have expanded this model and have refocused attention on the localization of splicing factors as a means of identifying protein-protein interactions and regulatory associations.

Nuclear localization patterns of RNA-binding proteins

As more RBPs are found to have multiple roles in the regulation of RNA metabolism and are believed to contribute to the coupling of sequential steps in the life of the mRNA transcript, it becomes increasingly important to examine their specific localization within the cell and its regulation thereof. Additionally, as the model of nuclear organization switches from a rigid compartmentalization to one of dynamic fluctuation, the processes that regulate this architecture are likely to be of major significance to the functionality of these proteins (Phair and Misteli, 2000). The mechanisms controlling protein localization

include specific interactions with other macromolecules, post-translational modification and processing of the actual RNA-binding protein and, lastly, methods based on cellular activity and protein function.

The nuclear localization of RBPs can be affected by their interactions with other macromolecules, including themselves (dimerization), other proteins, and RNA targets. Self-association has been described as a method for the regulation of nuclear localization in the case of p80-coilin (Hebert and Matera, 2000), PML (Ishov et al., 1999), SMN (Lorson et al., 1998), and Sam68 (Chen et al., 1999) proteins. Protein-protein interactions that affect the nuclear localization of RBPs have been described for many proteins that localize to specific sub-nuclear structures. For example, the interaction between p80-coilin and Nopp140 is crucial to the proper formation of coiled bodies (Isaac et al., 1998). The CTD domain of RNA pol II interacts with and targets SR proteins and snRNP splicing factors to sites of active transcription in the nucleus (Misteli and Spector, 1999). While both RNA polymerase II and splicing factors have distinct and not always overlapping localization patterns, their interaction suggests that splicing is dependent on transcription (perhaps occurring at the same location) and that a peptide domain is responsible for that connection.

The RS domains of SR proteins, which are thought to mediate interactions with other proteins (e.g. splicing factors), are sufficient for guiding proper sub-nuclear localization (Li and Bingham, 1991; Gama-Carvalho, 1997) and their overexpression can prove deleterious to it (Romac and Keene, 1995). Phosphorylation of the RS domain also promotes protein shuttling and can regulate the proper transport of bound mRNAs (Gilbert et al., 2001). Furthermore, interactions of SR proteins with other proteins

lacking RS domains are capable of targeting them to nuclear speckles (Patton et al., 1993; Hedley et al., 1995). Other domains implicated in targeting certain proteins to the nucleus have been shown to mediate an interaction with U2snRNP (Eilbracht and Schmidt-Zachmann, 2001).

Other functional domains of RBPs, including the RNA binding motifs, have been shown to be required for their specific staining pattern, again linking functional capacity to nuclear localization. For example, the localization of poly(A)-binding protein 2 (PABP2) and PTB-associated splicing factor (PSF) to sub-nuclear speckles depends on their capacity to bind RNA and is in contrast to other splicing factors whose localization in speckles increases in the absence of transcription (Calado and Carmo-Fonseca, 2000; Dye and Patton, 2001). In some human SR proteins, the RRM domains mediate nuclear localization, at least partly (Caceres et al., 1997). Finally, multiple RRM domains of the PTB protein are required for its localization to the PNC, suggesting that RNA-binding is necessary (Huang et al., 1997).

Post-translational modifications that influence protein localization mostly focus on the regulation of the phosphorylation state of the protein by kinases and phosphatases, perhaps as a result of cell cycle regulation. The nuclear localization of snRNP splicing factors and SR proteins has been shown to be influenced by phosphorylation (Gui et al., 1994; Colwill, et al., 1996; Misteli and Spector, 1996; Sleeman et al., 1998). The sub-nuclear localization of Sam68, a protein that mediates a connection between signal transduction pathways and RNA metabolism, also depends on phosphorylation (Hartmann et al., 1999). Coiled body formation has been suggested to be dependent on the phosphorylation level of p80-coilin (Lyon et al., 1997; Sleeman et al., 1998; Hebert

and Matera, 2000). Finally, the association of nuclear matrix components with active splicing complexes may also be regulated by phosphorylation (Chabot et al., 1995). The cytoplasmic accumulation of hnRNP K, depends on its phosphorylation and is necessary for its function in silencing mRNA translation (Habelhah et al., 2001). Another modification that has recently generated interest concerns the ubiquitin-like protein Sumo-1, as sumoylation of the PML protein has been hypothesized to regulate its localization in nuclear bodies (Muller et al., 1998, Zhong et al., 2000)

The effect of phosphorylation on the nuclear localization of RBPs suggests that the cellular machinery that achieves and regulates the localization pattern of a given protein is likely to have some universal features and can thus be highjacked by viral proteins. Indeed, the HIV protein Rev contains NLS and NES domains that allow it to shuttle, as well as a loop domain, located between two helices, that is required for proper phosphorylation of the protein by a cellular kinase (D'Agostino et al., 2000). The loop structure, together with the NES, is required for the proper sub-nuclear localization and association with splicing factors and hence for the functional activity of Rev in binding and trans-activating target viral RNAs.

The localization of RBPs can also be controlled by changes in cellular activity or as required by alterations in their function. For example, as nuclear RNA export is mostly mediated by RBPs, their localization in both the nucleus and the cytoplasm and their capacity to shuttle between the two becomes a requirement for proper function. In neurons, it has been reported that the dendritic targeting of certain messages (e.g. the CaMKIIα mRNA) depends on cis-acting elements in their 3' UTRs and is regulated by functional activity, namely neuronal depolarization (Mayford et al., 1996a; Mayford et

al., 1996b ; Mori et al., 2000). Furthermore, neurotrophin release induces the formation of an mRNP complex between β-actin mRNA and Zipcode-binding protein 1 (ZBP1). Transport of this complex to neurites is dependent on the 3' UTR of the mRNA and results in localized β-actin protein synthesis, which eventually affects growth cone motility (Zhang et al., 2001).

Localization-function coupling and RNA-binding proteins

By virtue of their diverse functions, varied localization patterns and ability to bind assorted RNA targets, RNA-binding proteins have the capacity to be in the center of the machinery that controls gene expression. This capability is manifested by the role of RBPs in linking the different stages of RNA metabolism in a manner that is efficient, economical and advantageous to the cell.

Transcription has been linked to splicing which, in some cases, is thought to occur concurrently (Misteli and Spector, 1999), and to the nucleocytoplasmic transport of RNAs (Pinol-Roma and Dreyfuss, 1991; Pinol-Roma and Dreyfuss, 1992). Splicing has also been shown to influence the nuclear export process (Kataoka et al, 2000; Le Hir et al, 2000a; Huang and Steitz, 2001) and the mRNA surveillance/non-sense mediated decay pathways (Kim et al., 2001a; Lykke-Andersen et al., 2001). In turn, nuclear export of RNA is linked, through the function of common RBPs, to mRNA processing, i.e. capping, 3' end cleavage and polyadenylation (Brodsky and Silver, 2000; Daneholt, 2001). Finally, the nuclear export process influences subsequent cytoplasmic localization and efficient translation (Choi et al., 2000; McKendrick et al., 2001).

A requirement for the ability of RBPs to efficiently serve as linkage factors between different steps in RNA metabolism and thus become crucial regulators of gene expression and cellular activity, is that they perform more than one function on target RNA molecules. This is underscored by the presence of multiple domains within the structure of RBPs and by the versatility of specific domains in mediating more than one biochemical reaction. For example, different regions of the carboxy-terminal domain of RNA polymerase II can independently regulate capping, splicing, 3' end cleavage, and polyadenylation (Fong and Bentley, 2001). The members of the Hu/Elav family have been suggested to play a role in mRNA splicing (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001), stability (Myer et al., 1997; reviewed in Brennan and Steitz, 2001), and translation control (Jain et al., 1997) and could possibly mediate all of the above on a single RNA target.

Reports show that commitment to a particular splicing pattern occurs very rapidly after transcription and might even be functionally coupled to it (Roberts et al., 1998). This is also supported by data that the perinucleolar compartment (PNC), where the PTB splicing repressor has been shown to localize, may be an active site of transcription (Matera et al., 1995; Huang, et al., 1998). Besides its role in splicing repression, hnRNP I/PTB has been implicated in the regulation of cap-independent translation (Gosert etal., 2000), cytoplasmic mRNA localization (Cote et al., 1999), stability (Irwin et al., 1997) and efficient polyadenylation (Lou et al., 1996; Moreira et al., 1998). In any case, specific multifunctional RBPs, such as PTB, are at the forefront of coupling events in the lifecycle of RNA molecules in a way that allows for effective regulation and firm control.

The RS domain of two Drosophila SR proteins is necessary for splicing function and sufficient for nuclear localization, suggesting that these two processes are intricately linked (Li and Bingham, 1991; Hedley et al., 1995). The situation in humans is thought to be more complex however, as reports have demonstrated that domains of the SR proteins (including the RS and RRM domains) can have additive and redundant functions in guiding sub-nuclear localization and targeting to sites of active splicing (Caceres et al., 1997; Gama-Carvalho et al., 1997). Even components of the nuclear matrix that have been hypothesized to belong to the SR family of RBPs can be associated with active splicing complexes (Blencowe et al., 1994).

The importance of the proper cellular localization of RBPs for their function is highlighted by the phenotype exhibited when this process is perturbed. For example, spinal muscular atrophy is a disease that is thought to disrupt the interaction of SMN, the gene affected by the disorder, with another protein, resulting in the absence of SMN localization from nuclear bodies and, presumably, defective function in snRNP biogenesis (Gangwani et al., 2001). The incorrect accumulation of the CUG-Binding protein (perhaps due to altered phosphorylation patterns) and the resulting aberrant processing of target RNA transcripts in the nucleus has been hypothesized to be a culprit in the pathophysiology of myotonic dystrophy (Roberts et al., 1997; Philips et al., 1998).

Nova-brPTB protein interactions in alternative splicing and nuclear localization

The results presented in this thesis concern the functional interactions between two neuronal RNA-binding proteins, Nova and brPTB. The brPTB protein, a member of the

PTB sub-family of RBPs that includes PTB, matrin3 and hnRNP L, is specifically enriched in the brain at the RNA and protein level. It was identified and cloned by virtue of its interaction with the paraneoplastic onconeural antigen Nova, through a yeast-two-hybrid screen. This interaction was confirmed both *in vitro* and *in vivo* and was shown to play an important role in the regulation of alternative splicing of a couple of neuronal transcripts. Furthermore, brPTB and Nova were shown to co-localize in distinct sub-nuclear structures in neuronal nuclei.

Placed in the context of the function of RBPs in the control of RNA metabolism, as discussed in Chapter 1, the interaction between brPTB and Nova has numerous implications. It is important to note that these two RBPs contain different types of RNA-binding motifs and yet can interact on the protein level. While the exact domains responsible for this interaction were not identified, and the question of whether an RNA molecule intermediates this interaction *in vivo* was not completely resolved, it is nevertheless apparent from the assays measuring the effect on splicing regulation, that this interaction has biological significance. The brPTB protein inhibits the Nova-specific increase in alternative exon utilization in a manner that is contingent on its binding to the RNA transcript. This would imply that the act of RNA-binding is necessary for the effect of brPTB in splicing inhibition, as it has been shown for the effect of Nova in splicing activation (Jensen et al., 2000a).

The possible means by which brPTB could inhibit the action of Nova, have already been discussed (see Chapter 4, Discussion). However, in light of the localization studies presented earlier, it is feasible that the effect of the brPTB-Nova protein interaction in splicing regulation is related to the nuclear setting of the two proteins. A

model that could tie these results together is presented in Figure 31. Three functional states of the Nova protein have been described in this work. One concerns the splicing activation of certain exons by Nova proteins in both neuronal and non-neuronal cell lines as evident by previous work and experiments presented here (Jensen et al., 2000a; this work). In some cases, when brPTB is also present, the result of its inhibitory action on Nova is splicing inhibition (Polydorides et al., 2000; this work). Finally, brPTB and Nova co-localize in distinct nuclear bodies (this work).

It is proposed in this model that the three functional states of Nova protein are in an equilibrium relationship with each other, the relative balance of which depends on the particular cell type and possible co-factors. In neuronal cells, specific co-factors may exist that could enhance the co-localization of brPTB and Nova proteins in nuclear bodies, thus shifting the equilibrium from the splicing inhibition state. This would explain both the presence of nuclear bodies in N2As and the absence of inhibitory action by brPTB in the splicing assays performed in these cells (see Chapters 4 and 5, Results).

In non-neuronal cells, the absence of co-localization between brPTB and Nova (possibly due to the lack of the required co-factor) allows the equilibrium to be switched to the splicing inhibition state and explains the observed effect of brPTB in bringing the level of exon inclusion back to the pre-Nova baseline (see Chapter 4). Since Nova and, most likely brPTB as well, do not exist in 293T cells these results concern transfected proteins. This poses a problem, as the interaction between brPTB and Nova proteins in these non-neuronal cells still occurs yet no co-localization is observed. One explanation could be that the localization interaction would require many more molecules co-localizing to become evident by immunostaining methods, while perhaps the splicing

inhibition on an RNA target transcript, where single molecules of Nova and brPTB could be interacting, is easily observable by the more sensitive RT-PCR assay.

Hence, the debate whether the nuclear bodies occupied by co-localizing brPTB and Nova proteins in neurons are indeed active splicing sites or inactive storage areas can be somewhat resolved. The mechanism by which brPTB inhibits Nova in splicing could involve blocking the recruitment of general splicing activators (such as SR proteins) to the exon. Alternatively, the nuclear bodies observed in neuronal cells could be the manifestation of sequestration of Nova by brPTB away from the RNA, thus inhibiting splicing. However, this would be contrary to the result presented here that brPTB inhibition of splicing requires its RNA binding and the observation that transcription inhibition reduces the appearance of the Nova nuclear concentrations.

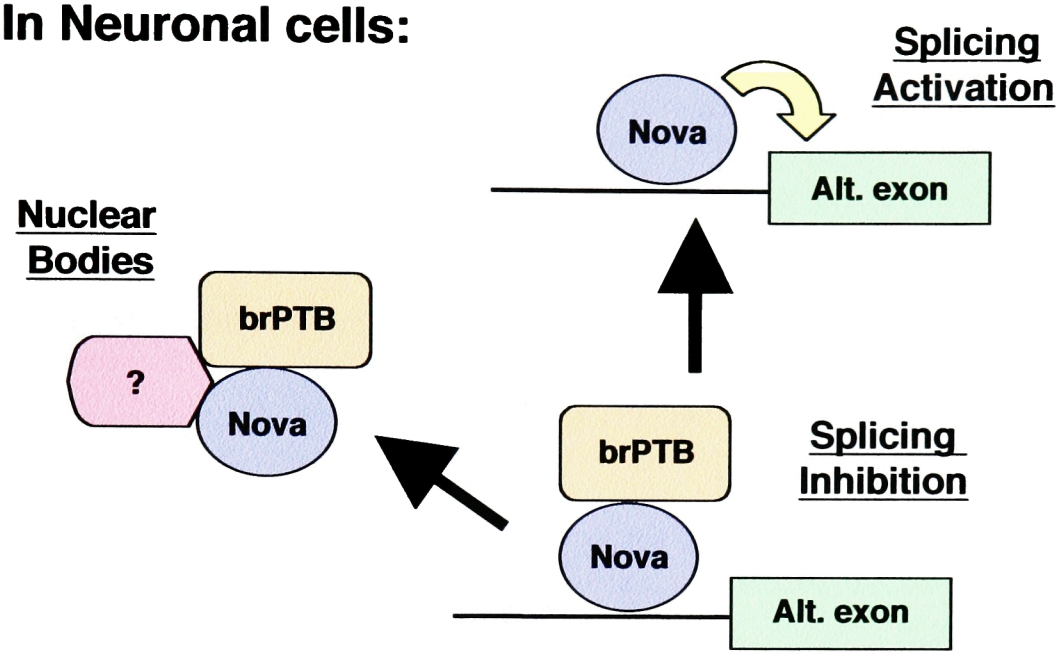
Summary

RNA-binding proteins play a significant part in the regulation of neuron-specific alternatively splicing. This function is intricately linked to their sub-nuclear localization, which can, in turn, influence their regulation. RBPs also couple splicing events to other steps in the metabolism of RNA molecules. A model where this hypothesis can be applied, involves the interaction between Nova and brPTB in terms of their roles in alternative splicing and nuclear localization in specific cell types. brPTB and Nova co-localize in neuronal cell nuclei where splicing assays fail to detect a repressive effect for brPTB in Nova-dependent alternative splicing. It is proposed that the explanation for this discrepancy lies in an intricate balance in the equilibrium between functional states for brPTB and Nova in the nucleus.

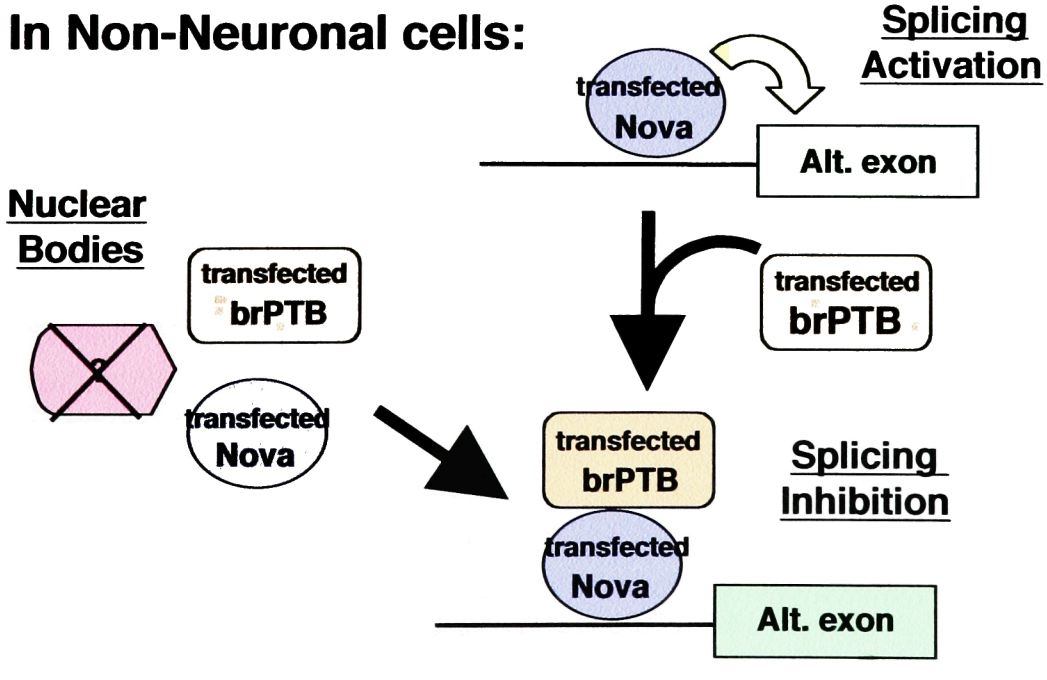
Figure 31. Schematic model of the Nova-brPTB functional interaction.

Model depicting the functional interactions between brPTB and Nova proteins. In neuronal cells, the equilibrium leans towards the formation of nuclear bodies, perhaps aided by a third, neuron-specific co-factor, and visible by immunocytochemical methods. This equilibrium state allows Nova to act in splicing activation as the addition of brPTB does not inhibit splicing in N2A cells. In non-neuronal cells, perhaps due to the absence of a co-factor, transfected Nova and brPTB proteins do not co-localize and the equilibrium is shifted, allowing brPTB to inhibit the action of Nova in splicing activation as seen in assays with 293T cells.

In Neuronal cells:



In Non-Neuronal cells:



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Appendix

YEAST TWO HYBRID SCREEN OF A MOUSE E11 TOTAL cDNA LIBRARY WITH THE NOVA2 SPACER REGION

N2 401	unknown
N2 402/422	novel, neuronal(?) RRM (=dbest clone on chr. 22)
N2 406	unknown
N2 407	KIAA 0127 gene
N2 409	AICAR formyltransferase / IMP cyclohydrolase (similar to rat; mouse homolog?)
N2 411	Human HLA-B-associated transcript 3 (BAT3) mRNA
N2 412	unknown
N2 413	tenascin
N2 414	M2 type pyruvate kinase
N2 416	unknown - domains homologous to desmoplakin/U2AF1-RS2/troponin
N2 417	T-cell tyrosine kinase
N2 418	beta-catenin
N2 421	beta-actin
N2 423	unknown
N2 426/473/484	YL2 / TAP / p32(SF2) / C1qBP
N2 428	Zac1 zinc finger protein
N2 429	elavG/elrA/HuA
N2 430	Kryn (SH3 domain binding protein)
N3 434	unknown (clone too short?)
N2 435	chicken p52/mouse h74 (SH3 domain containing protein)
N2 436	NADH Ubiquinone Oxidoreductase
N2 442	unknown
N2 443	unknown
N2 444	murine retrovirus ERV-L (gag, pol, dUTPase genes)
N2 447	unknown (62 bp insert)
N2 449	agrin precursor
N2 452	KIAA01812 gene mRNA, uncharacterized
N2 455	unknown
N2 456	unknown
N2 460	unknown
N2 462	laminin receptor
N2 464	delta proteasome subunit
N2 465	unknown
N2 466	unknown
N2 469	human brain cDNA KIAA0426
N2 474	cdc25A
N2 475	tRNA-His gene / PI transfer protein
N2 476	unknown
N2 477/505	tankyrase
N2 482	laminin B1
N2 483	novel, contains domains similar to keratin, general vesicular transport protein.
N2 485	HNRNP arginine N-methyl transferase (similar to human, rat, mouse homolog?)
N2 489	cyclophilin (cyclosporin A binding protein)
N2 493	unknown
N2 496	unknown
N2 503	NEFA protein (leucine zipper DNA binding, EF hand homology)
N2 506	unknown
N2 507	stathmin (phosphoprotein-cell regulation)

N2 425 = 412
N2 437 = 490 = 499 = 426
N2 439 = 440 = 481 = 402
N2 448 = 442
N2 470 = 485
N2 487 = 409

MAPPING THE INTERACTION OF NOVA2 CONSTRUCTS
WITH NOVA2 SPACER YEAST TWO HYBRID POSITIVE CLONES

		CONTROL	FULL LENGTH	KH 1	SHORTER SPACER	SPACER	SPACER + KH3	KH 3
	Clone	VA3	N2 full	N2 1-90	N2 230-354	N2 230-407	N2 230-488	N2 354-488
CONTROL	TD1	+/-	-	-	+/-	-	-	-
novel (RRM domain)	N2 402	-	+	-	+++	+++	+++	+++
	N2 422	-	-	-	+	+++	++	+
tenascin	N2 413	-	-	-	+/-	-	-	-
β-catenin	N2 418	-	-	-	+/-	+	+/-	+/-
β-actin	N2 421	-	-	-	+/-	+	+/-	+/-
C1qBP/	N2 426	-	-	-	+/-	+++	++	+/-
YL2/	N2 473	-	-	-	+/-	++	+/-	+/-
p32(ASF/SF2)	N2 484	-	-	-	+/-	+++	+	-
Zac-1	N2 428	-	-	-	+/-	++	+	+
HuA	N2 429	-	-	-	-	+	+/-	+/-
Kryn	N2 430	-	-	-	+/-	+	+/-	+/-
p52/h74	N2 435	-	-	-	-	+	+/-	-
ERV-L	N2 444	-	-	-	+/-	+	++	++
agrin precursor	N2 449	-	-	-	+/-	+	+/-	+/-
40S / laminin receptor?	N2 462	-	-	+/-	+/-	+	+/-	-
cdc 25A	N2 474							
Tankyrase	N2 477	-	-	+/-	+/-	+++	+/-	-
	N2 505	-	-	-	+/-	+++	+	+/-
laminin B1	N2 482	-	-	+/-	+/-	+	+	+/-
novel (keratin, GVTP)	N2 483	-	-	+/-	++	+	+/-	+/-
HNRNP arg meth transf	N2 485	-	-	+/-	+/-	+	+	+/-
NEFA	N2 503	-	-	+/-	+/-	++	++	+/-

Representative data set of GlyR 3A/3B splicing in 293T cels after Nova and brPTB transfections

Imagequant data	
transfection	3A/3B
2A	2.47218696
3A	2.6790851
4A	2.26062506
5A	1.93808412
6A	6.47917793
7A	4.97800629
8A	4.52765968
9A	2.748543
2B	2.62031864
3B	2.30816183
4B	2.17085283
5B	2.76023519
6B	5.36348234
7B	5.60872296
8B	4.67037842
9B	3.0444791

Amount transfected		Ratios	
Nova	brPTB	3A/3B	st. dev
		2.5462528	0.10474492
	2	2.49362346	0.26228236
	4	2.21573894	0.06347855
	6	2.34915965	0.5813486
0.5		5.92133014	0.78891592
0.5	2	5.29336462	0.44598404
0.5	4	4.59901905	0.10091739
0.5	6	2.89651105	0.20925842

Representative data set of GlyR CT Mutant 3A/3B splicing in 293T cels after Nova and brPTB transfec

Imagequant data	
transfection	3A/3B
2A	3.00483025
3A	2.9125848
4A	2.97134264
5A	2.75875291
6A	4.98263266
7A	4.91065806
8A	5.28530484
9A	6.90792117
2B	2.5680186
3B	2.86706353
4B	2.80109139
5B	2.39978879
6B	4.99776777
7B	5.01396715
8B	5.21756318
9B	3.70011208

Amount transfected		Ratios	
Nova	brPTB	3A/3B	st. dev
		2.78642443	0.30887248
	2	2.88982416	0.0321884
	4	2.88621701	0.12038581
	6	2.57927085	0.25382596
0.5		4.99020021	0.01070214
0.5	2	4.96231261	0.07305056
0.5	4	5.25143401	0.04790059
0.5	6	5.30401663	2.26826356

Representative data set of GABA L/S splicing in 293T cels after Nova and brPTB transfections

Imagequant data

transfection	ratio L/S	ratio L/S	Average ratio
2A	0.07018442	0.08088922	0.09247174
3A	0.07679634	0.06486322	0.08211882
4A	0.04589118	0.03766139	0.06148131
5A	0.04200696	0.03376851	0.06936156
6A	0.03785201	0.02807683	0.05112468
7A	0.03770101	0.02794043	0.05057753
8A	0.02776304	0.02497307	0.12632509
9A	0.04205606	0.03322085	0.06231841
2B	0.26571511	0.20089416	0.15713952
3B	0.29708633	0.19034669	0.1929067
4B	0.1855875	0.12331486	0.13945502
5B	0.18679193	0.10747677	0.13136766
6B	0.11622665	0.09152576	0.12114288
7B	0.12347042	0.08589394	0.1207896
8B	0.09630146	0.06896707	0.09309652
9B	0.0953829	0.06541665	0.08655169

Amount transfected

Nova	brPTB	L/S	st. dev
		0.07788729	0.00969303
	2	0.04836182	0.01405035
	4	0.03887875	0.01025274
	6	0.05277609	0.06570757
0.5		0.21734809	0.05276328
0.5	2	0.14566562	0.03312425
0.5	4	0.10984154	0.01663161
0.5	6	0.08428605	0.01371855

Representative data set of GABA GA2 Mutant L/S splicing in 293T cels after Nova and brPTB transfec

Imagequant data

transfection	ratio L/S
2A	0.04827491
3A	0.04284727
4A	0.02864832
5A	0.02707616
6A	0.02449787
7A	0.0249269
8A	0.02855375
9A	0.03288998
2B	0.11777077
3B	0.12823414
4B	0.08082518
5B	0.07634674
6B	0.0677589
7B	0.06399482
8B	0.05385417
9B	0.04747199

Amount transfected

Nova	brPTB	L/S	st. dev
		0.04556109	0.00383792
	2	0.02786224	0.00111169
	4	0.02471239	0.00030337
	6	0.03072187	0.00306618
0.5		0.12300246	0.00739872
0.5	2	0.07858596	0.00316674
0.5	4	0.06587686	0.00266161
0.5	6	0.05066308	0.00451288

Representative data set of GlyR 3A/3B splicing in N2A cels after Nova and brPTB transfections

Imagequant data		
transfection	3A/3B	3A/3B
2A	3.6877927	3.17930866
3A	4.302909	4.13476132
4A	5.42919164	5.05255542
5A	5.67742295	5.30093037
6A	6.48093565	5.91559202
7A	6.37372371	5.61386356
8A	5.99337766	5.60354741
9A	5.86104886	5.22499281
2B	7.97082414	6.23304603
3B	8.13739086	6.41826742
4B	8.25060437	6.94302171
5B	7.96573163	7.02109044
6B	8.74927638	6.4638677
7B	8.62804129	6.80258716
8B	8.31307775	6.30757323
9B	8.23935339	6.44061215

Amount transfected		Ratios	
Nova	brPTB	3A/3B	st. dev
	-	3.82619292	0.50335096
	2	5.36502509	0.26041966
	4	6.09602873	0.40427239
	6	5.67074169	0.33838731
0.5	-	7.18988211	1.00308986
0.5	2	7.54511204	0.66124924
0.5	4	7.66094313	1.19575815
0.5	6	7.32515413	1.09994527

Representative data set of GABA L/S splicing in N2A cels after Nova and brPTB transfections

Imagequant data

transfection	ratio L/S	ratio L/S
2A	0.81638181	0.76935679
3A	0.80348118	0.77464409
4A	0.59795864	0.85182671
5A	0.57122484	0.65319332
6A	0.50078961	0.6949757
7A	0.70576492	0.69464224
8A	0.49093681	0.52147169
9A	0.61369694	
2B	2.04846909	3.19284373
3B	1.64791842	3.15108504
4B	1.07981609	
5B	2.17325973	3.00035425
6B	2.20441923	2.72062352
7B	2.03290745	2.28523589
8B	2.02714557	2.61842648
9B	2.08181761	2.31050789

Amount transfected

Nova	brPTB	L/S	st. dev
		0.79096597	0.02262709
	2	0.66855088	0.12686149
	4	0.64904312	0.09897061
	6	0.54203515	0.0639113
0.5		2.51007907	0.78176318
0.5	2	2.58680699	0.58484414
0.5	4	2.31079652	0.29277408
0.5	6	2.25947439	0.26894215