

2002

Nova Regulation of Alternative Splicing in the CNS

B Kate Dredge

Follow this and additional works at: http://digitalcommons.rockefeller.edu/student_theses_and_dissertations



Part of the [Life Sciences Commons](#)

Recommended Citation

Dredge, B Kate, "Nova Regulation of Alternative Splicing in the CNS" (2002). *Student Theses and Dissertations*. 333.
http://digitalcommons.rockefeller.edu/student_theses_and_dissertations/333

This Thesis is brought to you for free and open access by Digital Commons @ RU. It has been accepted for inclusion in Student Theses and Dissertations by an authorized administrator of Digital Commons @ RU. For more information, please contact mcsweej@mail.rockefeller.edu.



THE LIBRARY

Rockefeller University Library
1230 York Avenue
New York, NY 10021-6399



Nova regulation of alternative splicing in the CNS

A. thesis presented to the faculty of Rockefeller University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

by

B. Kate Dredge

April, 2002

Thesis Committee:

Nathaniel Heintz – Chair

Michael Young – Faculty Member

Douglas Black – Outside Examiner

Robert Darnell – Thesis Advisor

Acknowledgements

My utmost thanks go to my thesis advisor Bob Darnell for his unerring support of me. His patience, enthusiasm and respect for the members of his laboratory made for an enjoyable and productive work environment. I'd also like to thank all the members of the Darnell Lab, past and present. It was the good nature of the people in the lab, a reflection of Bob's leadership, that cemented my decision to make Bob's lab the home of my graduate work and enabled me to remain happy and productive over the years.

In particular I wish to thank Kirk Jensen and James Okano for their time, patience and sense of fun in helping me to get started in the lab. I especially owe much gratitude to Kirk for collaboration on the analysis of the Nova-1 knockout mice and microscopy work, as well as his constant guidance and technical help. I am also extremely grateful to have had Alexi Polydorides and Giovanni Stefani as peers during my tenure as a graduate student, and to have worked alongside Jennifer Darnell. I thank Ru Zhong for the generation and maintenance of the Nova-1 knockout mice; my thesis work would not have been possible without his efforts. I am also thankful to a number of people outside the lab who aided me with protocols, reagents and technical advice, especially Alex Gardiol who took the image of Nova foci in spinal cord and Dan Lavery for help with the SABRE technique.

I owe many thanks to my faculty advisory committee; Nat Heintz and Mike Young, for their support, guidance and constructive criticism throughout the course of this work. I'd also like to thank Doug Black for his enthusiastic participation as my outside examiner.

Finally, I would never have taken on, let alone completed this work without the loving support of my family and friends from both sides of the globe.

Table of Contents

Abstract.....	1
Chapter 1 – Introduction.....	2
Functions of RNA-binding proteins (RBPs).....	2
RNA editing.	2
Pre-mRNA splicing	4
Nuclear RNA export.....	6
RNA localization.....	7
Alternative splicing	9
Splicing can influence cytoplasmic fate	11
Alternative splicing in the nervous system.....	13
Splicing and complexity of gene products.....	16
Neurologic disease associated with aberrant splicing of mRNAs	18
Inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17).....	18
Spinal muscular atrophy (SMA)	19
Amyotrophic lateral sclerosis (ALS).....	21
Spinocerebellar ataxias (SCA1 and SCA8) and Myotonic Dystrophy (DM).....	23
Neurologic disease associated with immune targeting of neuron-specific RNA binding proteins	24
Paraneoplastic neurologic disorders (PNDs)	24

Paraneoplastic encephalomyelopathy / sensory neuropathy (PEM/SN) Hu family of RBPs	26
Paraneoplastic opsoclonus myoclonus ataxia (POMA) - Nova family of RBPs	28
Summary	29
Chapter 2 – Materials and Methods	30
DNA preparation and restriction digest analysis	30
Plasmid constructs	30
RNA preparation	31
RT-PCR	31
RNase protection assays	31
Primer Extension	31
Cell transfection	32
Western blot analysis.....	32
Fusion protein synthesis	33
Nitrocellulose Filter Binding Assays.....	33
Boundary Mapping.....	33
Immunofluorescence of cell cultures.....	34
Immunofluorescence of tissue sections	34
cDNA Arrays	35
Selective amplification via biotin- and restriction-mediated enrichment (SABRE). 35	
PCR Primers.....	36
Antibodies	37
Chapter 3: Defining Nova as a splicing factor <i>in vivo</i> – Analysis of Nova null mice	38

Introduction.....	38
In vitro analysis of Nova binding.....	38
Nova null mice	41
Results	43
Assay Development.....	43
GlyR α 2 Splicing in Nova-1 null mice.....	44
Specificity of Splicing Defects in Nova-1 null mice.....	45
Genotype and developmental profile of splicing changes.....	47
Nova-1 exon H Splicing in Nova-1 heterozygous and Nova-2 null mice	48
Discussion.....	49
Nova-1 regulates tissue specific alternative splicing of 3 distinct pre-mRNAs	49
Nova-1 mediated splicing regulation and neuronal dysfunction.....	51
Chapter 4 – Characterization of Nova action on Defined pre-mRNAs	63
Introduction.....	63
GABA _A R γ 2 alternative splicing.....	63
Nova-1 exon H alternative splicing.....	64
Results	65
Nova regulates GABA _A R γ 2 alternative splicing in cell lines	65
Defining the region of GABA _A R γ 2 RNA necessary for regulation by Nova-1	67
In vitro analysis of Nova binding to GABA _A R γ 2 RNA	73
Analysis of the regions of Nova-1 protein required for regulation of GABA _A R γ 2 alternative splicing.....	75
Autoregulation of Nova-1 alternative splicing.....	76

Discussion.....	79
Nova-dependent regulation of GABA _A R γ 2 E9 alternative splicing.....	79
Autoregulation of Nova-1 alternative splicing.....	82
Chapter 5 – Nova-1 subcellular localization	105
Introduction.....	105
Nucleolus	105
Cajal (coiled) bodies and gems (gemini of coiled bodies)	106
Nuclear speckles (interchromatin granule clusters) and perichromatin fibrils.	107
PML bodies.....	108
Results	109
Nova localizes to a novel subnuclear compartment.	109
Actinomycin-D disrupts Nova foci.	110
Transfection of a splicing target does not disrupt Nova foci.	111
Nova forms subnuclear foci in mouse spinal cord	112
Discussion.....	112
Chapter 6 – Differential gene expression screens.....	119
Introduction.....	119
Non-selective methods:	119
Selective Methods	121
Results	124
cDNA Arrays	124
SABRE (Selective amplification via biotin- and restriction-mediated enrichment)	
.....	126

Discussion.....	130
Chapter 7 Discussion	140
Physiological consequences of Nova-dependent splicing dysregulation.	140
Proposed mechanisms of Nova-dependent splicing regulation.	143
Summary.....	151
References	153

List of Figures

Figure 1: RT-PCR assays to assess GlyR α 2 E3A/E3B alternative splicing.	54
Figure 2: RNase protection assay controls.	55
Figure 3: Aberrant GlyR α 2 splicing in Nova-1 null mice	56
Figure 4: Specific abnormalities in alternative splicing of GlyR α 2 and GABA $_A$ R γ 2 transcripts in Nova-1 null mice.	57
Figure 5: Confirmation by RNase Protection Assay.....	58
Figure 6: Regional specificity of splicing defects.....	59
Figure 7: Changes in GlyR α 2 and GABA $_A$ R γ 2 Alternative Splicing as a Function of Gene Dosage and Development.	60
Figure 8: Nova-1 alternative splicing is altered in Nova-1 heterozygous mice.	61
Figure 9: Nova-1 alternative splicing is altered in Nova-2 null mice.....	62
Figure 10: GABA $_A$ R γ 2 minigene assay development.....	85
Figure 11: Nova-1, but not hnRNP-E1, enhances GABA $_A$ R γ 2L splicing in heterologous cell lines.	86
Figure 12: Sequence comparison of intronic regions surrounding GABA $_A$ R γ 2 exon 9 ...	87
Figure 13: Mutations within the surrounding introns do not interfere with Nova's ability to regulate GABA $_A$ R γ 2 exon 9 alternative splicing.	88
Figure 14: Generation of shortened minigenes by Exonuclease III digestion.....	89
Figure 15: Alternative splicing of a severely truncated GABA $_A$ R γ 2 minigene is still regulated by Nova-1: narrowing down the sequence elements required.	90

Figure 16: Sequence surrounding GABA _A Rγ2 E9 is not sufficient for regulation of alternative splicing by Nova-1 – sequence around E10 is necessary.	91
Figure 17: Intronic sequence upstream of GABA _A Rγ2 E10 is sufficient for regulation of alternative splicing by Nova-1.	92
Figure 18: Sequence comparison of intronic regions upstream of GABA _A Rγ2 E10.....	93
Figure 19: Mutagenesis of the YCAY repeats abrogates Nova-dependent regulation of chimeric minigene alternative splicing.....	94
Figure 20: Mutagenesis of the YCAY repeats close to E10 abrogates Nova-dependent regulation of GABA _A Rγ2 minigene alternative splicing.	95
Figure 21: Nova-1 binds with high affinity to GABA _A Rγ2 RNA <i>in vitro</i>	96
Figure 22: Mapping the boundaries of Nova-1 binding to GABA _A Rγ2 9C RNA.....	97
Figure 23: 24nt intronic sequence is sufficient for regulation of alternative splicing by Nova-1.	98
Figure 24: KH3 and the spacer region contribute to the function of Nova-1 in regulating alternative splicing.....	99
Figure 25: All 3 KH domains contribute to the function of Nova-1 in regulating alternative splicing.....	100
Figure 26: Nova-1 regulates alternative splicing of its own message in tissue culture cells.	101
Figure 27: Mutations within the downstream intron do not interfere with Nova's ability to regulate exon H alternative splicing.	102
Figure 28: Alternative splicing of a truncated Nova-1 minigene is still regulated by Nova- 1 protein: narrowing down the sequence elements required.....	103

Figure 29: Sequence within Nova-1 exon H is necessary and sufficient for autoregulation of alternative splicing.	104
Figure 30: Nova localizes in to a novel subnuclear compartment.	115
Figure 31: Actinomycin-D disrupts Nova-containing foci, cyclohexamide does not.....	116
Figure 32: Transfection of a splicing target does not disrupt Nova foci.	117
Figure 33: Nova forms subnuclear foci in mouse spinal cord.	118
Figure 34: cDNA expression arrays.....	133
Figure 35: Selection of tester homohybrid species by SABRE.....	135
Figure 36: Enrichment of cDNA fragments overexpressed in spinal cords of Nova-1 null mice.	136
Figure 37: RT-PCR confirmation of contactin associated protein 1 (Cntnap1, clone 1H36) upregulation in Nova-1 null mice.....	138
Figure 38: Several SABRE clones correspond to alternative mRNA isoforms.	139
Figure 39: Model for Nova regulation of alternative splicing.....	152

List of Tables

Table 1: Differentially expressed genes identified using Clontech cDNA arrays.	134
Table 2. Differentially expressed genes identified by SABRE selection.....	137

Abstract

Nova-1, an autoantigen in paraneoplastic opsoclonus myoclonus ataxia (POMA), a disorder associated with gynecologic cancer and CNS motor dysfunction, is a neuron-specific nuclear RNA binding protein. The work presented here confirms that Nova-1 acts as a regulator of alternative splicing *in vivo*. In addition to the known target of Nova-dependent splicing regulation, exon 3A of glycine receptor $\alpha 2$ subunit (GlyR $\alpha 2$), two new targets are identified, which are the alternative exon, E9 in the inhibitory neurotransmitter receptor subunit, GABA_AR $\gamma 2$, and the alternatively spliced exon H in Nova-1's own message. Nova acts to enhance alternative exon inclusion in GlyR $\alpha 2$ and GABA_AR $\gamma 2$ mRNAs and to repress alternative exon usage in its own message, thus acting as a dual function splicing regulator. Sequence elements necessary and sufficient for Nova-dependent splicing regulation of these two new targets were elucidated, and a model is presented whereby Nova differentially regulates exon inclusion in a manner dependent on the location of the Nova binding site within the pre-mRNA. Also, Nova proteins localize to distinct subnuclear foci, but fail to co-localize with a number of known proteins that occupy sub-nuclear structural domains. Unlike nuclear speckles, which contain a number of other splicing factors, Nova foci are disrupted by treatment of the cells with actinomycin-D, consistent with the hypothesis that the formation and maintenance of Nova foci is an active process that is transcription dependent. Finally, differential gene expression screens were undertaken, leading to the identification of a number of additional potential targets of Nova regulation.

Chapter 1 – Introduction

Functions of RNA-binding proteins (RBPs)

The process of eukaryotic gene expression involves an ordered and linked series of events within various subcellular compartments. RBPs act at multiple levels to regulate and diversify gene expression, and such functions are likely to be of particular importance to neurons. The regulation of neuronal differentiation and function is likely to depend on a large number of neuron specific genes; it has been estimated that 30-50% of all genes are specifically transcribed within the brain (He and Rosenfeld, 1991). Brain-specific gene expression is not only regulated by transcriptional controls (Ghosh et al., 1994; Lemke, 1993) but also, through the action of both ubiquitous and neuron-specific RBPs, post-transcriptional controls. The RBPs critical to these processes recognize a small subset of RNA targets in a sea of cellular RNAs. One question central to the understanding of gene expression is how do these proteins recognize their targets in order to subsequently mediate their regulation? This problem is well illustrated by considering RNA editing.

RNA editing.

Post-transcriptionally, two forms of processing result in changes in the coding capacity of the transcribed RNA at the sequence level: RNA splicing, the removal of intronic regions of pre-mRNA, and RNA editing. RNA editing can involve insertion or deletion of nucleotides from a pre-mRNA, a process which has been described in protozoa such as trypanosomes (Benne et al., 1986). Alternatively, nucleotides in a pre-

mRNA may be modified. In mammals, the two most common modifications that occur are adenosine-to-inosine (A-I) and cytidine to uracil (C-U) editing. C-U modification occurs in the pre-mRNA of apolipoprotein B (apoB) pre-mRNA by the action of the cytidine deaminase APOBEC1 (apoB mRNA-editing enzyme catalytic polypeptide 1)(Benne et al., 1986). This enzyme forms a complex with ACF (APOBEC complementation factor) which contains three single stranded RNA recognition motifs (RRMs) at the amino-terminus and a double stranded RNA binding domain at the carboxyl-terminus of the protein, and supplies the RNA binding function (Lellek et al., 2000; Mehta et al., 2000). The result of C-U modification in the apoB transcript is a change from the coding of a glutamate to a stop codon, resulting in a second, shorter apoB protein isoform. This process is further influenced by at least one other RNA binding protein, GRY-RBP (Gly-Arg-Tyr-rich RNA-binding protein) which inhibits RNA editing (Blanc et al., 2001). In addition, KSRP, a KH-domain containing protein involved in alternative splicing regulation copurifies with ACF, and can bind apoB RNA, however the functional consequences of these interactions are not yet known (Lellek et al., 2000).

A-I RNA editing is most prevalent in the central nervous system (Keegan et al., 2001). In this instance the enzymes responsible for the catalysis of the reaction, ADARs (adenosine deaminases acting on RNA), harbor two or three double-stranded RNA-binding domains in their amino-termini, obviating the need for an RNA-binding cofactor. RNA binding occurs to a double stranded region formed by base-pairing of the editing site to an editing site complementary sequence (ECS), often present in a downstream intron (Higuchi et al., 1993). The consequence of adenosine deamination is that the

modified base is then interpreted by the translation machinery as a guanosine. In some instances this results in a single amino-acid change in the resultant protein, for example an arginine to glutamine (R-Q) change in the glutamate receptor subunit GluR-B, which occurs in the channel-pore lining segment of the protein and drastically alters the Ca^{2+} permeability of the channel (Sommer et al., 1991). Alternatively, RNA editing can influence pre-RNA splicing, for example ADAR2 edits its own pre-mRNA creating a 3' splice-site which, when used, results in the addition of 47 nucleotides to the resultant mRNA and changes the open reading frame (Rueter et al., 1999).

It is clear from these examples that even single nucleotide changes can have profound biological effects. The specific recognition of sites to be modified is achieved through the action of one or more RBPs either working in concert with the enzymatic components of the complex, or in the case of ADARs, by also supplying the catalytic function within a separate domain of the protein. Specific recognition also depends on specificity within the RNA, both at the level of primary sequence and secondary structure.

Pre-mRNA splicing

Pre-mRNA splicing also involves the concerted interactions of a large number of protein factors, some of which are required to mediate the recognition of the RNA elements that define the splice sites. Newly synthesized pre-mRNA in the eukaryotic nucleus is recognized by the splicing apparatus which includes small nuclear RNAs (snRNAs) complexed with a multitude of proteins which form what have been termed small nuclear ribonucleoproteins (snRNPs). These snRNPs (U1, U2, U4, U5, and U6) associate with

the pre-mRNA in an orderly way and constitute the basal machinery that mediates splicing (reviewed in (Staley and Guthrie, 1998)).

While introns can vary substantially in size and sequence, they maintain several conserved motifs. In mammals, the 5' splice site (splice donor site) consensus sequence is **AG/GURAGU** ("/" denotes the **exon**-intron junction) and 3' splice site (splice acceptor site) consensus is **YAG/N**. The branchpoint (**YNYURAC**) and polypyrimidine tract lie close to the 3' end of the intron. These motifs are recognized by components of the splicing machinery during assembly of a large multicomponent ribonucleoprotein complex called the spliceosome. Initially, U1 snRNP binds to the 5' splice site, followed by the binding of SF1 (splicing factor 1, also called branch point binding protein BBP) to the branch point and U2 auxiliary factor (U2AF) to the polypyrimidine tract and 3' splice site. This complex is termed the early, or E, complex. U2 snRNP is recruited which binds to the branch point resulting in A complex formation. B complex is formed when U4/U6•U5 tri-snRNP is added to the complex. The spliceosome then undergoes extensive remodeling to become the catalytically active C complex. Two transesterification reactions follow; first, the branch point adenosine attacks the 5' splice site, cleaving it and resulting in lariat intermediate formation. Second, after further rearrangement of the complex, the 3' hydroxyl of the cleaved 5' exon attacks the 3' splice site, joining the two exons, and releasing the lariat.

The mechanism by which exons are differentiated from introns is both important and obscure, and the significance of the problem is magnified where exon splicing is regulated and may involve exons that are skipped (cassette exons), mutually exclusive or contain more than one splice sites (5' or 3'). The consensus sequences recognized by the

core splicing machinery are both short and degenerate and therefore cannot account for the exquisite recognition and accuracy achieved during pre-mRNA splicing. However, individual splice sites are not recognized independently, but involve interactions between the 5' and 3' splice sites and the factors that bind them. These interactions can occur across the intron (intron definition) or across the exon (exon definition) (reviewed in (Berget, 1995)), and usually involve a number of additional proteins, including RBPs.

Nuclear RNA export

After transcription and processing in the nucleus, mRNAs must be actively transported to the cytoplasm prior to their translation. RNA signals play a part in determining export; for example the presence of a 5' cap (and the concomitant cap-binding complex) stimulates export (Izaurrealde et al., 1992; Visa et al., 1996), whilst the presence of unspliced introns inhibits export. However, the most important mediator of mRNA export is the complement of RBPs bound to them. The majority of proteins and RNAs are transported via the nuclear pore complex (NPC) from the nucleus to the cytoplasm and require interaction with specific transport receptors. The best-studied of these receptors are the karyopherin receptors. These receptors mediate both nuclear export and import of proteins and RNAs. During export, the receptor plus cargo bind to RanGTP, followed by translocation to the cytoplasm. These complexes are then released in the cytoplasm by hydrolysis of RanGTP to RanGDP. Import occurs in essentially the reverse manner. Nuclear export of tRNA, rRNA and snRNAs all require karyopherin-type receptors. Shuttling hnRNP proteins such as hnRNP A1 have been suggested to play a role in mRNA export by acting as adapters between the RNP and transport machinery via karyopherin receptors.

An alternative pathway involves export of mRNAs through a different class of transport receptors, the Tap/NXF family. RNA export via this pathway can be stimulated by RNA splicing, in particular the recruitment of the mRNA export factor, Aly, during spliceosome assembly (Luo and Reed, 1999; Zhou et al., 2000). Aly, the mammalian homologue of yeast Yra1p contains a central RNA binding domain flanked by RGG rich regions. Aly is one component of a complex of proteins deposited 20-24 nucleotides upstream of exon-exon junctions during splicing (Le Hir et al., 2001). Aly interacts directly with Tap, a shuttling mRNA export factor that in turn associates with the NPC (Stutz et al., 2000) to mediate export. SR proteins, an essential component of the spliceosome, have also been implicated in mRNA export (Huang and Steitz, 2001). Conversely, non-shuttling hnRNP proteins have been suggested to play a role in the retention of partially spliced RNAs or the RNA byproducts of splicing, the excised introns within the nucleus (Reed and Magni, 2001). Thus RBPs play a crucial role in the regulation of RNA export from the nucleus.

RNA localization

RNA localization as a mechanism to regulate local protein concentrations through local protein synthesis has been documented in a wide variety of systems. For example, Ash1 mRNA is localized to the tip of the daughter cell in budding yeast, preventing mating type switching (Sil and Herskowitz, 1996). During development, morphogen gradients of some proteins are established by localization of the RNA, for example bicoid mRNA is localized to the anterior pole of *Drosophila* oocytes. What is common to all systems is the requirement of cis-acting RNA sequences, often located in the 3' untranslated region (UTR) of the message, and trans-acting protein factors that bind them. In fibroblasts,

actin mRNA is localized to the leading edge, a process requiring a “zip code” 3' UTR sequence to be bound by a protein named zipcode-binding protein 1 (ZBP-1)(Ross et al., 1997). ZBP-1 is an RNA binding protein containing four KH (hnRNP K homology) and one RRM (RNA recognition motif) RNA binding domains. RNA binding proteins are not only involved in directing the localization of the mRNA to a particular site, but also in anchoring it upon arrival. In the case of bicoid mRNA, Staufen, an RBP containing five double-stranded RNA binding domains anchors the RNA at the anterior pole of *Drosophila* oocytes (Ferrandon et al., 1994).

RNA localization also plays important roles in regulation of gene expression in neurons. Several mRNAs are localized to discrete subcellular regions in neurons. For example, microtubule associated protein MAP2 mRNA is found in dendrites (Garner et al., 1988), while tau mRNA is restricted to the proximal segment of axons in mature neurons (Litman et al., 1993). RNA localization in neurons has also been linked to local control of translation of mRNAs such as the α subunit of calcium/calmodulin-dependent protein kinase II (CAMKII α) in dendrites (Kang and Schuman, 1996; Martin et al., 1997). Furthermore, RNA localization and local synthesis of protein in dendrites have been coupled to synaptic activity in the case of Arc (activity-regulated cytoskeleton-associated) which is localized to the synapse in response to NMDA receptor activation (Steward and Worley, 2001).

Alternative splicing

Alternative splicing allows for different mRNAs, and thereby different proteins, to be produced from a single pre-mRNA by regulating the choice of exons to be spliced together. Regulation of alternative splicing is mediated through interactions of the splicing machinery with additional protein factors that enhance or repress the spliceosome. Understanding of this process comes largely from *Drosophila*, where protein factors that regulate splicing bind to specific RNA target elements present either within regulated exons or in adjacent intronic sequences.

One of the first known and best characterized examples of alternative splicing regulation via an intronic element is the regulation of the *transformer* (*tra*) pre-mRNA by sex lethal (Sxl) in *Drosophila* sex determination (reviewed in (Lopez, 1998)). The protein Sxl is expressed exclusively in females and blocks the more upstream of two competing 3' splice sites in the first intron of *tra* pre-mRNA. By switching splicing to the downstream 3' splice site, Sxl allows translation of functional Tra protein by exclusion of an in-frame stop codon. Sxl binds to a U-rich stretch of the polypyrimidine tract of the regulated (upstream) 3' splice site (Inoue et al., 1990), thus precluding binding of U2AF and favoring splicing to the downstream, weaker 3' splice site. *In vitro* experiments have confirmed that Sxl binds with approximately 100-fold higher affinity than U2AF⁶⁵ to the upstream polypyrimidine tract, and that Sxl binding excludes binding of U2AF⁶⁵ (Valcarcel et al., 1993). Further evidence for this mechanism of regulation comes from experiments both *in vitro* and *in vivo* where the RS (serine-arginine repeat) domain of U2AF⁶⁵, which mediates protein-protein interactions, was fused to Sxl. This fusion

transforms Sxl into an activator of the upstream 3' splice site (Granadino et al., 1997; Valcarcel et al., 1993).

Exonic splicing regulatory elements have also been documented in many instances. A well studied example of an exonic splicing enhancer (ESE) is female-specific inclusion of *doublesex* exon 4 in *Drosophila*. Exon 4 becomes the last exon in the transcript in females, and this splice choice results in the production of sex-specific transcription factors with different carboxyl-termini. Splicing to the 3' splice site adjacent to exon 4 requires a region of six 13-nucleotide repeats and a purine rich element (PRE) located approximately 300 nucleotides into the exon. Protein complexes form at each of these sequence elements, involving the binding of SR proteins: Tra-2 (transformer-2) and either RBP-1 (RNA-binding protein-1) at the repeats or dASF/SF2 (*Drosophila* alternative splicing factor/splicing factor 2) at the PRE. Specificity is introduced by Tra (transformer), which is expressed only in females and is crucial for stable complex formation at these sites (Lynch and Maniatis, 1996). Interestingly, the position of the ESE sequences relative to the 3' splice site is critical in maintaining the regulation of this splicing decision. If the enhancer is placed closer to the intron/exon junction, Tra is no longer required for stable complex formation and the subsequent splicing activation.

Alternative splicing of the *Drosophila* P-element transposase is mediated through an exonic splicing silencer (ESS). The expression of this transposase is inhibited in somatic cells by repression of intron 3 splicing. A complex of at least 4 proteins is involved which when bound to the regulatory sequence in the exon causes U1 snRNP assembly at a nonfunctional pseudo-5' splice site and thus sequesters it away from binding to the authentic 5' splice site. The *Drosophila* homologue of hnRNP A1 (hrp48)

and PSI (P-element somatic-cell-specific inhibitor), a KH-domain containing RNA binding protein, both contribute to this complex (Adams et al., 1996).

Similar mechanisms to those determined in *Drosophila* appear to be important in the regulation of alternative splicing in mammals. For example, PTB (polypyrimidine-tract binding protein) acts in a manner reminiscent of Sxl to inhibit 3' splice site selection. PTB proteins have been implicated in inhibition of 3' splice site selection in several pre-mRNAs including c-src, GABA_AR γ 2 and muscle α - and β - tropomyosins (Ashiya and Grabowski, 1997; Chan and Black, 1997; Mulligan et al., 1992), presumably through interference with binding of positive factors in this region. These examples will be discussed in more detail below.

Splicing can influence cytoplasmic fate

In addition to creating a diversity of protein products from a single gene, an under appreciated function of alternative splicing is to generate transcripts which are differentially regulated post-transcriptionally. In particular, splicing of non-coding regions of a transcript can result in incorporation of different signals which result in altered stability, polyadenylation, or nuclear export, and hence translation of the same protein. By extension of this paradigm, alternative splicing of non-coding regions could also influence mRNA localization within a cell since cis-acting sequences required for subcellular localization of a number of messages have been located in the 3'UTR of the mature transcripts (reviewed in (King et al., 1999; Oleynikov and Singer, 1998)). While specific examples linking alternative exon usage to control of cytoplasmic mRNA translation and/or localization have not been clearly described, it is interesting to note that MAP2, tau and CAMKII α all undergo alternative splicing.

There is also increasing evidence that nuclear splicing is coupled to cytoplasmic events through more than just specification of the mature mRNA sequence. Using experiments involving microinjection of *Xenopus* oocytes Matsumoto et al. (Matsumoto et al., 1998) demonstrated that splicing dramatically influences translation of the same sequence in the cytoplasm. Luo and Reed (Luo and Reed, 1999) showed that splicing alters association of a message into mRNP particles, and is required for efficient nuclear export of some messages. Le Hir et al. (Le Hir et al., 2000) used site-specifically modified RNAs to demonstrate that splicing influences the association of proteins with an RNA molecule.

Thus it appears that many RNA transcripts must go through a particular nuclear pathway involving splicing in order to attain the correct mRNP composition and thus be processed correctly in the cytoplasm. For example, proteins that regulate splicing may direct regulation of mature mRNA in the cytoplasm in a manner analogous to the way that the well characterized *Drosophila* alternative splicing factor, Sxl, represses translation of msl-2 (male-specific lethal 2) in females (Bashaw and Baker, 1997; Kelley et al., 1997). Sxl blocks splicing of an intron in the 5' UTR of msl-2 pre-mRNA via binding to high-affinity Sxl-binding sites within the intron. In the cytoplasm, Sxl is thought to remain bound to these intronic sites, and this association causes repression of Msl-2 translation (Gebauer et al., 1998). Thus the assembly of specific splicing complexes is likely to lead to regulation of cytoplasmic mRNA in ways that are just beginning to be explored.

Alternative splicing in the nervous system

Alternative splicing is an important mechanism for generating cellular complexity, resulting in a versatile repertoire of functionally different proteins within individual cells. The significance of alternative splicing is clearly evident in highly specialized cells such as neurons. For example, all of the major neurotransmitter receptors contain subunits that are alternatively spliced, which can alter receptor localization, ligand binding, signal transduction and electrophysiological properties (Conn and Pin, 1997; Ehlers et al., 1995; Lin et al., 1997; Macdonald, 1995; Picetti et al., 1997; Stamm et al., 1994). Moreover, aberrant splicing of mRNAs encoding proteins critical for proper functioning of neurons has been associated with a number of neurological diseases some of which will be discussed in detail below.

Since the discovery that the transcript encoding the thyroid hormone calcitonin is alternatively spliced in neurons to generate the neuropeptide transmitter CGRP (Amara et al., 1982), speculation exists that there are specific systems that regulate splicing in neurons. This has been an elusive area of discovery – most of what we know regarding the regulation of neuron specific protein isoforms generated by alternative splicing involves contributions from splicing factors that are ubiquitously expressed. Such factors no doubt play essential roles in neuronal splicing, but are not likely to explain the regulation of all splicing events unique to neurons. Study of neurologic disease has resulted in the identification of Nova-1 (Buckanovich et al., 1993), a protein involved in splicing regulation which is unique to neurons (Jensen et al., 2000a). This is the first demonstration of a neuron-specific alternative splicing factor in a mammalian system,

and has exciting implications for the discovery of such a neuron-specific system for regulation of alternative splicing.

Studies of neuron-specific splicing in transcripts such as c-src, GABA_A receptor $\gamma 2$ subunit, *N*-methyl-D-aspartate receptor NR1 subunit and clathrin light chain B mRNAs (Ashiya and Grabowski, 1997; Chan and Black, 1995; Chan and Black, 1997; Wang and Grabowski, 1996; Zhang et al., 1996; Zhang et al., 1999) have identified a number of cis-acting repressor elements that mediate the exclusion of the neuron-specific exons in other tissues. These repressor sequences are mostly polypyrimidine-rich and, when present as competitors *in vitro*, stimulate neuronal splicing patterns (Chan and Black, 1997; Zhang et al., 1999). These observations led to the suggestion that neuron-specific splicing is selectively repressed in non-neuronal tissues by trans-acting RNA-binding proteins and that in neuronal cells, this repression is relieved by the presence of positive regulators that compete for the same or overlapping binding sites on the pre-mRNA (Grabowski, 1998; Zhang et al., 1999).

Biochemical approaches have been used to identify trans-acting factors that bind such regulatory sequences adjacent to alternatively spliced neuronal exons, including hnRNP H, hnRNP F and KSRP (Chou et al., 1999; Min et al., 1997). In addition, the ubiquitous RNA binding protein, polypyrimidine tract-binding protein (PTB), was identified through a variety of *in vitro* approaches as a trans-acting factor involved in the repression of n-src and GABA_A receptor splicing (Ashiya and Grabowski, 1997; Chan and Black, 1997). The *in vitro* approaches used to identify trans-acting factors which bind to specific messages and affect neuron-specific splicing have provided important information on the contribution of generally expressed factors to this process. However,

these approaches have yet to identify tissue specific factors which explain the unique patterns of alternative splicing seen in the nervous system.

The physiology of neurons may allow them to modify trans-acting factors and thereby regulate alternative splicing in unique ways. Post-translational modifications of RNA-binding factors in response to stimuli such as neuronal activity may provide one means of regulating their function. For example, phosphorylation of the SR splicing proteins regulates their splicing enhancer or repressor function, and proteins that regulate this modification have been identified (Cao et al., 1997; Kanopka et al., 1998; Petersen-Mahrt et al., 1999; Xiao and Manley, 1997). Although there is no evidence to date for brain-specific post-translational modifications able to regulate splicing, neuronal activity itself can alter the splicing of a number of primary transcripts (Daoud et al., 1999; Vezzani et al., 1995; Xie and Black, 2001).

For example, excitation has been specifically linked to the splicing regulation of the NR1 subunit of NMDA ionotropic glutamate receptor. Alterations in the inclusion of exon 21 in the hippocampus of rats exposed to kindling electrical stimulation (Vezzani et al., 1995), and in the cortex of rats treated with the muscarinic receptor agonist pilocarpine (Daoud et al., 1999) have been reported. Exon 21 inclusion in turn alters subcellular distribution (Ehlers et al., 1995), cell surface expression (Okabe et al., 1999), and phosphorylation of NR1 by protein kinase C (PKC) (Tingley et al., 1993). One plausible mechanism for such a phenomenon could be activity dependent changes in protein phosphorylation. More recently Xie and Black (Xie and Black, 2001) showed that depolarization of GH₃ rat pituitary cells in culture represses splicing of the STREX exon in *Slo* pre-mRNA transcripts. The presence of this exon confers higher Ca²⁺ sensitivity on

the BK (Slo) potassium channel (Ramanathan et al., 1999; Shipston et al., 1999; Xie and McCobb, 1998). Repression of splicing to this exon is mediated through a CAMK IV-responsive RNA element (CaRRE) present at the 3' splice site of the STREX exon, and this element was shown to be sufficient for repression of splicing of a heterologous exon by CAMK IV (Xie and Black, 2001).

Splicing and complexity of gene products

The diversity of cell types and functions, the richness of cell-cell contacts and networks formed, and the ability to adapt to environmental cues imply that a complex set of mechanisms regulate gene expression in the brain. Alternative splicing provides a plausible means for generating diversity during development and plasticity of the nervous system. In some instances, for example the neurexins (see(Missler and Sudhof, 1998)for review) and the Drosophila axon guidance receptor, Dscam (Schmucker et al., 2000), hundreds to thousands of different splice forms are produced from a single gene, although it is not yet clear what proportion of these messages get translated into protein. The central question is how are such complex splicing decisions made? One possibility is that such splicing choices are stochastic, analogous to the generation of immunoglobulin diversity in the immune system. Interestingly, the organization of the genes encoding the cadherin-related neuronal receptors bears striking resemblance to that of immunoglobulin and T-cell receptors in the immune system. The genes are arranged in clusters and each contains a large number of “variable region” exons followed by three “constant region” exons (Kohmura et al., 1998; Sugino et al., 2000; Wu and Maniatis, 1999). Although no evidence of gene rearrangement akin to that of the immune system has been documented

in the brain, alternative splicing of these genes may underlie the generation of a multitude of protein products.

Alternatively, the choice of exon inclusion, even in complex transcripts, may be precisely regulated. Such control has the potential to generate both protein diversity and control of mRNA expression in a fashion that could respond to specific signals, providing a more sophisticated control of gene expression. The current model for the generation of diversity in this manner without the need for as many regulatory proteins as there are regulated events, is through combinatorial control (reviewed in (Smith and Valcarcel, 2000)). Alterations in the relative proportions of positive and negative factors within different cell types as well as the presence or absence of specific binding sites within the RNA targets would result in alterations in the composition of complexes which form to regulate individual splice sites. This ultimately would allow for splicing events to be regulated independently of each other, and for a particular splice junction to be regulated differently within different cells or at different times. In many ways this idea is mechanistically similar to models of the regulation of transcriptional control of gene expression (see (Hertel et al., 1997) for review). An attractive model for regulation in this manner would be for a small number of key factors to coordinately regulate a functionally similar set of RNA targets in a specific manner. Within the nervous system, this type of regulation is probably important in neural development and plasticity where concerted changes in numerous different messages are required in response to an environmental signal.

Neurologic disease associated with aberrant splicing of mRNAs

Inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17)

Inherited frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17) is characterized by behavioral, cognitive and motor disturbances with frontotemporal atrophy associated with gliosis and intraneuronal tau-containing deposits. Multiple mutations in tau, a neuronal protein which binds to microtubules and promotes their assembly and stability, have been found in FTDP-17 patients and, in some cases, these mutations lead to aberrations in regulated splicing (Hutton et al., 1998; Spillantini et al., 1998). At least seven missense and one deletion mutation in tau coding regions, most of which alter the ability of tau to promote microtubule assembly, have been associated with FTDP-17 (D'Souza et al., 1999; Hasegawa et al., 1998; Hong et al., 1998; Poorkaj et al., 1998). In some cases, these coding region mutations also stimulate heparin-induced assembly of tau into filaments (reviewed in (Goedert and Spillantini, 2000)).

At least five other mutations in the tau gene have also been found which exert subtle effects on exon utilization suggesting that aberrant regulation of alternative splicing may lead to FTDP-17 in these cases. Tau transcripts undergo alternative splicing in 3 of the 11 coding exons to generate 6 major protein isoforms, and these splicing events are highly regulated both spatially and developmentally (Goedert et al., 1989; Kosik et al., 1989). Exon 10 encodes a microtubule-binding repeat domain, and alternative splicing of this exon results in the production of protein with either 3 or 4

microtubule binding domains. Missense, silent and intronic tau mutations have been identified in FTDP-17 patients which are associated with two- to six-fold increases in exon 10 utilization *in vivo* (D'Souza et al., 1999; Hutton et al., 1998).

Exon trapping and minigene experiments in cultured cell lines have confirmed the effects of these mutations on the splicing of exon 10 (Grover et al., 1999; Hutton et al., 1998; Jiang et al., 2000). A widely held view is that many of these mutations disrupt the formation of a stem-loop present at the 3' boundary of exon 10. This secondary structure is thought to be necessary for the correct regulation of alternative splicing at this site through prevention of efficient interaction of U1 snRNA with the 5' splice site (Jiang et al., 2000). In addition, cis-acting sequence elements that are involved in the regulation of tau exon 10 alternative splicing include both a splicing enhancer and silencer within the exon 10 (D'Souza et al., 1999).

These findings, as well as an animal model in which mice overexpressing the shortest human tau isoform develop CNS pathology similar to FTDP-17 (Ishihara et al., 1999), strongly imply that mutations that disrupt splicing regulation lead to FTDP-17, most likely through generation of tau aggregates. Thus relatively subtle changes in the ratio of spliced products (two- to six-fold) can lead to neurodegenerative disease, highlighting the importance of systems that tightly regulate alternative splicing in the nervous system.

Spinal muscular atrophy (SMA)

SMA is a fatal neurodegenerative disease of childhood, characterized by progressive paralysis with muscular atrophy due to degeneration of lower motor neurons. Linkage mapping resulted in the identification of SMN (for survival of motor neurons), as the

disease gene in SMA (Brzustowicz et al., 1990; Lefebvre et al., 1995; Melki et al., 1990). SMN protein is expressed in all tissues in the body with particularly high levels detected in motor neurons. The protein is present in distinctive speckles termed gems within the nucleus of cells, as well as the cytoplasm (Liu and Dreyfuss, 1996).

SMN protein has been shown to form complexes with a number of spliceosomal small nuclear ribonucleoproteins (snRNPs), and analysis of a dominant negative form of the protein indicates that SMN is critical for snRNP assembly in the cytoplasm of somatic cells (Pellizzoni et al., 1998). SMN also appears to be involved in maintenance and/or regeneration of splicing-competent snRNP complexes *in vitro* (Pellizzoni et al., 1999). Mutant forms of SMN found in SMA patients show reduced self-oligomerization, the degree of which correlates with disease severity (Lorson et al., 1998), as well as reduced binding to a subset of snRNP proteins (Pellizzoni et al., 1999). However, there is no evidence that SMN plays a regulatory role itself in alternative splicing, but rather a role in maintaining the snRNP machinery in all cells.

Interestingly, two distinct SMN genes exist in humans that apparently encode identical proteins (Lefebvre et al., 1995). The telomeric copy of the gene, SMN1 (also known as SMN^T), is deleted (93% of cases), interrupted (5.6%) or mutated in nearly all SMA patients [Lefebvre, 1995 #442]. Homozygous deletions of the centromeric copy of the gene, SMN2 (SMN^C) do not result in pathology, and an intact SMN2 gene cannot compensate for the deletion of SMN1. This curious result has been explained by differential splicing of exon 7 (Lorson et al., 1999). Exon 7 is constitutively included during the normal processing of SMN1, however the predominant product of SMN2 excludes exon 7. Careful analysis of the five nucleotides that differ between SMN1 and

SMN2 has identified a silent C-to-T alteration within exon 7 which has been shown to mediate exon skipping, yielding predominantly SMN Δ 7 transcripts (Lorson et al., 1999). This alteration occurs within an exonic splicing enhancer (ESE) in SMN1 and abrogates binding of ASF/SF2 resulting in the inefficient inclusion of exon 7 in SMN2 (Cartegni and Krainer, 2002).

The majority of the mutations documented in SMN1 of SMA patients are either point mutations or small deletions in the consensus splice sites of introns 6 and 7 which result in exon 7 skipping to yield SMN Δ 7. Together, these studies and those of SMN2 indicate that SMA results from loss of function of SMN through defects in a constitutive splicing event. Several reasons why SMN Δ 7 cannot fully compensate for full length SMN protein have been postulated, including reduced self-oligomerization (Lorson et al., 1998), reduced binding to snRNP Sm proteins (Pellizzoni et al., 1999) and reduced stability (Lorson and Androphy, 2000) of SMN Δ 7. It is as yet unclear how defects in SMN result in a pathology that is so specific to α -motor neurons. It is likely that aberrations in SMN result in a general defect in snRNP recycling to which motor neurons are particularly sensitive. In this way, the role of SMN in motor neuron disease may be similar to the proposed role of defects in other genes involved in motor neuron disease such as the superoxide dismutase gene, SOD1 (see below), which is involved in regulating general metabolic responses to oxidative stress.

Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is an adult-onset, progressive neurological disorder characterized by degeneration of upper and lower motor neurons. While the mechanisms of ALS pathogenesis are not well understood, oxidative stress is thought to contribute to

motor neuron degeneration in a large proportion of ALS cases, either through mutations in SOD1 (found in 15-25% of familial ALS) (Brown, 1996; Siddique and Deng, 1996), or via glutamate-mediated excitotoxicity. A decrease in EAAT2 protein has been observed in approximately two thirds of patients with sporadic ALS (Rothstein et al., 1994; Rothstein et al., 1992; Shaw et al., 1995). EAAT2, which is expressed by astrocytes (Rothstein et al., 1994), is a glutamate transporter which pumps glutamate from the extracellular space into cells where it is metabolized. Motor neurons are particularly susceptible to excitotoxicity as a result of exposure to high levels of glutamate, and as such decreased EAAT2 levels may account for their selective vulnerability in ALS.

One study associated the presence of aberrant splice forms of EAAT2 with ALS suggesting that altered splicing and/or RNA metabolism could contribute to the decrease in EAAT2 protein seen in some ALS patients (Lin et al., 1998). However, subsequent studies by two independent groups failed to substantiate the claims that such defects are not found in normal or disease controls and are restricted to neuropathologically affected areas in ALS patients (Honig et al., 2000; Meyer et al., 1999). Both of these subsequent groups reported slight differences in the proportions of some of these aberrant splice forms in ALS versus control brains, some of which have been shown to act in a dominant negative fashion in transfection assays (Lin et al., 1998). Thus it is conceivable that slight changes in the proportion of protein isoforms may have profound effects on protein function and disease state, as is the case for tau in FTDP-17. However, the link between splicing regulation and disease is far more tenuous for ALS than for other examples discussed here and more work needs to be done to establish whether splicing regulation plays an important role in ALS.

Spinocerebellar ataxias (SCA1 and SCA8) and Myotonic Dystrophy (DM)

The spinocerebellar ataxias are autosomal dominant neurodegenerative disorders which result in often fatal incoordination of limb movement, speech and gait. The majority of these disorders (SCA types 1,2,3,6 and 7) are caused by expansions of CAG trinucleotide repeats. These are translated into long polyglutamine tracts which are thought to impart the corresponding protein with a toxic gain of function. SCA1 is caused by polyglutamine expansion in ataxin-1, a nuclear RBP of unknown function. Although the mechanism of pathogenesis of mutant ataxin-1 is unclear, a number of RNA binding proteins have been identified as disease modifiers in *Drosophila* suppression screens (Orr and Zoghbi, 2001), including couch potato and mub, a 3 KH domain-containing protein related to mammalian Nova.

Recently, a novel form of ataxia, SCA8, was reported to be associated with an untranslated CTG expansion (Koob et al., 1999). While the mechanism by which CTG expansion causes neurologic disease is not known, much information can be drawn from the model of myotonic dystrophy (DM), the first (and only other) disease reported to be caused by a non-coding CTG expansion.

DM is caused by CTG repeat expansion in the 3' UTR of DM protein kinase (DMPK). DMPK RNA containing the CUG repeats is retained in the nucleus (Davis et al., 1997; Taneja et al., 1995) and these repeats in heterologous RNA transcripts appear necessary and sufficient to cause a DM-like phenotype in mice (Mankodi et al., 2000). CUG repeats have been found to bind an RNA binding protein termed CUG-BP (Timchenko et al., 1996). One model of DM pathogenesis suggests that in cells

harboring CUG repeats, CUG-BP and perhaps other cellular factors are sequestered resulting in abnormal processing of unrelated target transcripts. Indeed, alternative splicing of human cardiac troponin T (cTNT) pre-mRNA is perturbed in cells expressing transcripts containing CUG repeats, including tissue from DM patients (Philips et al., 1998). By analogy, SCA8 may also be caused by sequestration of cellular factors resulting in dysregulation of splicing of unrelated transcripts. The difference in pathogenesis of the two diseases may relate to the different populations expressing transcripts with CUG repeats. SCA8 transcript is expressed in brain tissue, but is absent or present in much lower levels in other tissues (Koob et al., 1999), consistent with the neurological symptoms of the disease.

Neurologic disease associated with immune targeting of neuron-specific RNA binding proteins

Paraneoplastic neurologic disorders (PNDs)

Paraneoplastic neurologic disorders (PNDs) are rare immune-mediated disorders associated with ectopic expression of neuron-specific proteins in tumors (reviewed in (Darnell, 1996; Musunuru and Darnell, 2001)). Immune targeting of these antigens in the central nervous system is thought to result in the neurological symptoms displayed by PND patients. Patients harbor high titers of antibodies specific for these antigens in their sera, which has enabled the expression cloning of a number of proteins whose expression is entirely restricted to neurons. These antigens fall into four general categories based on their function;

(1) nerve terminal and vesicle-associated proteins including glutamic acid decarboxylase (GAD), a target antigen in stiff-man syndrome (SMS) (Solimena et al., 1988) which was one of the first proteins identified in this context. A variant of SMS that develops in patients with breast cancer has also been associated with paraneoplastic antibodies against amphiphysin, a protein that appears to play a role in synaptic vesicle endocytosis (David et al., 1996; De Camilli et al., 1993) A further member of this category is β -NAP, a novel adaptin-like vesicle coat protein identified using antiserum from a patient with cerebellar degeneration (Newman et al., 1995).

(2) putative neuronal signaling proteins such as recoverin, a protein involved in receptor signaling in the photoreceptor and associated with paraneoplastic retinal degeneration and small cell lung cancer (SCLCa) (Polans et al., 1991; Thirkill et al., 1992), and the leucine-zipper protein cdr2 which is expressed in Purkinje cells in the cerebellum and was cloned using Yo antisera (Dropcho et al., 1987; Fathallah-Shaykh et al., 1991; Sakai et al., 1990).

(3) neuromuscular junction proteins implicated in paraneoplastic disease include the α -subunit of the nicotinic AChR expressed in thymomas associated with myasthenia gravis (MG) (Gattenlohner et al., 1994; Kornstein et al., 1995).

(4) neuron-specific RNA-binding proteins (n-RBPs). This family will be discussed in detail below.

Paraneoplastic encephalomyelopathy / sensory neuropathy (PEM/SN)

- Hu family of RBPs

The paraneoplastic Hu syndrome encompasses a clinically diverse set of neurologic degenerative symptoms which may present as single or multifocal disorders (Dalmau et al., 1992b). Neurologic dysfunction is most often localized to dorsal root ganglia (~60% of patients), but may also be found in the cerebellum, brainstem, limbic-, motor- or autonomic nervous-system. Antisera from Hu patients recognize a nuclear and cytoplasmic antigen which is pan-neuronal, but not present in other tissues (Dalmau et al., 1992a; Graus et al., 1985; Okano and Darnell, 1997). The first mammalian Hu family member to be identified, via expression cloning using patient antiserum, was HuD (Szabo et al., 1991). Three additional family members have subsequently been identified in mice (HuA-HuC) (reviewed in (Brennan and Steitz, 2001; Keene, 1999)). HuB, C and D are neuron-specific and display unique patterns of spatial and temporal expression (Okano and Darnell, 1997). In addition, Hu transcripts undergo alternative splicing which contributes to the potential for variability in function and/or targets of this family of proteins.

All Hu proteins harbor three RNA recognition motifs (RRMs) of the type found in a number of RNA binding proteins, including several involved in RNA splicing (for example sex-lethal (Sxl), ASF/SF2 and polypyrimidine tract binding protein (PTB)). The best understood function of Hu proteins is a cytoplasmic role in the stabilization of messages encoding several cytokines, lymphokines and protooncoproteins via specific binding to AU-rich elements (AREs) in the 3'UTR (reviewed in (Keene, 1999), (Brennan and Steitz, 2001)). While there has been no demonstration that Hu proteins are involved

in splicing in mammals, HuD shows remarkable homology to the *Drosophila* proteins ELAV (embryonic lethal abnormal visual system) and the well characterized splicing factor Sxl (Szabo et al., 1991). ELAV is essential for neurogenesis (Robinow et al., 1988) and has been suggested to regulate alternative splicing of several transcripts in *Drosophila* (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001). Thus it remains an intriguing possibility that Hu proteins also play a role in splicing regulation in neurons in addition to the suggested roles in early development of postmitotic neurons (Okano and Darnell, 1997).

Hu RBPs also play an important role in the nuclear export of certain mRNAs. HuR (or HuA) has been shown to bind to specific sequences in the 3' UTRs of mRNAs such as *c-fos* and to be necessary for their nuclear export (Brennan et al., 2000; Gallouzi and Steitz, 2001). Shuttling of HuR between the nucleus and the cytoplasm is mediated by a novel shuttling domain, the HNS (Fan and Steitz, 1998a; Fan and Steitz, 1998b) and specific interactions with proteins including pp32 and APRIL (acidic protein rich in leucine) which in turn interact with the nuclear export factor CRM1 (chromosomal region maintenance protein 1)(Brennan et al., 2000).

Immune targeting of Hu proteins in paraneoplastic disorder has been associated with a diverse set of neurological symptoms, perhaps reflecting targeting of different Hu family members in different patients. While the exact mechanisms of pathogenesis are not yet clear, Hu disease antisera recognize a discrete target epitope in HuD (Manley et al., 1995), suggesting that immune targeting of Hu protein contributes to neurologic dysfunction.

Paraneoplastic opsoclonus myoclonus ataxia (POMA) - Nova family of RBPs

The Nova family of proteins was identified as the target antigens in paraneoplastic opsoclonus myoclonus ataxia (POMA). POMA patients exhibit a loss of inhibitory control of motor neurons in the spinal cord and brainstem. Two members of the Nova family of RNA binding proteins have been cloned. Nova-1 expression is entirely restricted to the diencephalon, brainstem and motor neurons of the ventral spinal cord, correlating with regions implicated in the disease phenotype (Buckanovich et al., 1993). Nova-2 expression is largely reciprocal to that of Nova-1. High levels of Nova-2 expression are observed in the cerebral cortex, hippocampus and dorsal spinal cord (Yang et al., 1998). A subset of POMA patients develop encephalopathy and dementia which may be the result of immune targeting of Nova-2 expressing cells.

The Nova proteins belong to a growing class of RNA binding proteins containing KH type (for hnRNP K homology) RNA binding domains. KH domain containing proteins have been shown to play roles in a number of aspects of RNA processing including mRNA stability and translational control (hnRNP K and hnRNP E1/E2 (Kiledjian et al., 1995; Ostareck et al., 1997)) and subcellular localization of RNAs to specific sites in the cell cytoplasm (ZBP-1, Vera (Deshler et al., 1998; Ross et al., 1997)). Fragile-X mental retardation syndrome is associated with a lack of or mutation in the KH domain containing protein FMR-1 (De Boulle et al., 1993; Pieretti et al., 1991). A number of KH containing proteins have also been implicated in RNA splicing, including KSRP (Min et al., 1997) and SF1 (Arning et al., 1996) in mammals, PSI (Siebel et al., 1995) in *Drosophila* and MER-1 in yeast (Engbrecht et al., 1991). Nova proteins are sequence-

specific RBPs which have been shown to recognize sequences present in introns adjacent to alternatively spliced exons in at least two pre-mRNAs (Buckanovich and Darnell, 1997), as will be discussed in detail in Chapter 3. This suggested that Nova proteins were candidates for mammalian tissue-specific splicing regulators.

Summary

The importance of RNA-binding proteins in the nervous system is highlighted by the existence of many human disorders where the function of a specific RBP is affected. Alternative splicing appears to be of particular importance in neurons both as a mechanism to generate the enormous complexity of proteins and networks in the brain, and as a mechanism to respond on a cellular basis to neurological activity. Alternative splicing is highly prevalent in the central nervous system, including the existence of a number of neuron-specific exons in widely expressed transcripts. Very little is understood about how alternative splicing is regulated in the mammalian brain and in particular how tissue specificity is achieved. A common theme in the study of alternative splicing regulation in other tissues and organisms is that regulation is often achieved through interaction of accessory factors with the core basal splicing machinery. This thesis is aimed at determining whether Nova-1 can act as a regulator of alternative splicing *in vivo*, thus becoming the first neuron-specific splicing factor to be identified in mammals, and to determine the targets and mechanisms by which Nova-1 regulates alternative splicing.

Chapter 2 – Materials and Methods

DNA preparation and restriction digest analysis

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

Plasmid constructs

The GABA_AR γ 2 minigene containing full-length introns was generated by long-range PCR using primers γ 2 GABA F and γ 2 GABA R on a subclone derived from mouse BAC608F8 (Research Genetics) and cloned into pCDNA3 (Invitrogen). Exonuclease III digests were performed according to the manufacturer's instructions (Amersham). The β -globin minigene was generated by PCR using primers glo-KpnF and glo-Rc using plasmid sp64-H β Δ 6 (a kind gift from A. Krainer) as template, and cloned into pCDNA3 (Invitrogen). Exon 2 was shortened by PCR. Heterologous minigene constructs were generated by PCR.

cDNAs encoding T7 tagged Nova-1 with and without exon H and T7 tagged hnRNP E1 were cloned into pCI-neo (Promega). All DNA constructs generated by PCR or site-directed mutagenesis were sequenced in their entirety. Truncated Nova-1 expression plasmids were generated from the full length construct including exon H by restriction digest with *Bbv*CI and *Sal*I (Δ KH3) or *Xcm*I and *Sal*I (Δ spKH3), blunt ending using pfuTurbo (Stratagene) and religation with T4 DNA ligase (New England Biolabs). Mutations of both minigene and Nova-1 expression constructs were made with the QuikChange Site-directed Mutagenesis Kit (Stratagene) and specifically designed primers (Operon).

RNA preparation

Total RNA was extracted from mouse tissues using a modified guanidine-acid phenol protocol (Chomczynski and Sacchi, 1987). For SABRE, tissues were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction. RNA from tissue culture cells was purified using the RNeasy Mini kit system (Qiagen), including on the column DNaseI digestion.

RT-PCR

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

RNase protection assays

RNase protection assays were performed using the RPA II kit (Ambion) according to the manufacturer's instructions. For GlyR α 2 probes and control RNAs, E2-3A-4 and E2-3B-4 RT-PCR products were TA cloned into pGem-T Easy (Promega) using PCR primers E2F and E2R and AmpliTaq polymerase (Perkin Elmer). Plasmids were linearized by digest with NcoI or SpeI and *in vitro* transcribed using T7 or Sp6 RNA polymerase (New England Biolabs) for sense or antisense RNAs. Probes for GABA $_A$ R γ 2 and ClaB were *in vitro* transcribed using T7 RNA polymerase from gel-purified PCR products generated using primers gaba-rpa-F and -R and cla-rpa-F and -R. Transcription templates were confirmed by sequencing. Probes were labeled by incorporation of α - ^{32}P -CTP.

Primer Extension

Primer extension was performed using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. 2 μg total RNA and 1pmol γ - ^{32}P -ATP-labeled

primer EGFP-4 (5'-CGTCGCCGTCCAGCTCGACCAG) were used in a final reaction volume of 20 μ l.

Cell transfection

N2A and 293T cells were grown to 60% confluence in 6-well plates or chamber slides in 10% FBS/DMEM with penicillin/streptomycin. For in vivo splicing assays, 2.25 μ g of total DNA, comprised of 0.25 μ g of the appropriate minigene, and variable amounts of pNova-1 and empty pcNeo vector, were incubated with 95 μ l of DMEM and 5 μ l of Fugene6 (Roche) for 15 minutes at room temperature. The mixture was then added to the cells in a total volume of 2 ml 10% FBS-DMEM. After 40 hrs the cells were washed with 1xPBS, collected by scraping and halved for RNA extraction and protein extraction. Transfection in chamber slides was performed by adding a mix of 0.25 μ g of plasmid, 48 μ l of DMEM and 2 μ l of Fugene6 to cells in a total volume of 1 ml 10% FBS-DMEM. 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.

Western blot analysis

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

Fusion protein synthesis

Full-length recombinant T7/His tagged Nova1 fusion protein was produced and purified as previously described (Lewis et al., 1999). Full-length Nova-1 cDNA including alternative exon H was cloned into pET21a (Novagen) and transformed into BL21 competent cells, followed by standard IPTG-induction and purification by nickel-chelation chromatography. Nova fusion protein was assessed for purity by SDS-PAGE and Coomassie blue (BioRad) staining. The Nova-1 expression plasmid was made by A.D. Polydorides.

Nitrocellulose Filter Binding Assays

Labeled RNA (100–200 nmol preheated to 75°C and bench-cooled 5 min) was incubated with the indicated concentrations of protein. Protein-RNA mixes were incubated in a total volume of 50 μ L SBB (200 mM KOAc, 10 mM TrisOAc [pH 7.7], and 5 mM MgOAc), SBB 500 (500 mM KOAc, 10 mM TrisOAc [pH 7.7], and 5 mM MgOAc) or SBBhep (SBB + 1mg/ml heparin) for 10 min at room temperature. Binding solutions were passed through MF-membrane filters (0.45 HA, Millipore) and washed with 5 ml SBB (or SBB500). Data were plotted as percentage of total RNA bound versus log of the protein concentration, and Kds were determined with Kaleidograph software (Synergy Software). SB2 RNA was synthesized as previously described (Buckanovich and Darnell, 1997). 9C wild-type and mutant RNAs were transcribed using T7 RNA polymerase and gel-purified PCR templates generated using primers T7-GABA-9CF and xR' (wildtype, 159nt RNA) or i9AtoCR2 (mutant, 156nt RNA) and GABA_AR γ 2 minigenes as PCR templates. Glo RNA was transcribed using T7 RNA polymerase directly from minigene glo2A digested with BbsI, to yield a 175nt RNA.

Boundary Mapping

Boundary mapping was performed as described (Fitzwater and Polisky, 1996), with the following modifications. *In vitro*-transcribed 9C RNA (60 pmol) was 3' end-labeled with T4 RNA ligase (New England Biolabs) and [32P]pCp (NEN). 9C RNA (40 pmol) was 5' end-labeled with T4 polynucleotide kinase (New England Biolabs) and [-32P]ATP.

Following gel purification, RNA was digested with 2 μ l RNase T1 (GIBCO-BRL, 1180 units/ μ l) for ladder. Following alkaline hydrolysis, RNA was resuspended in 20 μ l DEPC H₂O and used in filter binding assays (SBBhep) with or without Nova-1 FP.

Immunofluorescence of cell cultures

For immunofluorescence of cell cultures, cells were plated onto chamber slides (Nalge Nunc), transfected where appropriate, and two days later rinsed with PBS, fixed (10 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 in the dark. Slides were then washed three times in PBG, once with DAPI in PBS, and once in PBS alone. Slides were left at room temperature to dry and then covered with mounting medium (Biomedex) and a coverslip (Fisher) before being sealed with nail polish. The slides were observed under multiple wavelength fluorescence from a mercury lamp (AttoArc), in an upright microscope (Zeiss AxioPlan). Pictures were taken with a digital camera (Hamamatsu Orca), using Openlab software (Improvision) and presented with Photoshop software (Adobe).

Immunofluorescence of tissue sections

Mice were deeply anesthetized with chloroform and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M PBS, pH 7.2. Spinal cords were removed and post-fixed overnight at 4°C in 4% PFA in PBS. Vibratome sections (50 μ m) were collected in cold PBS then incubated for 20 min in 50 mM NH₄Cl in PBS to quench the free aldehyde groups. After PBS rinses (3 x 10 min), sections were preincubated for 10 min in 0.1% Triton X-100 and 0.1% bovine gelatin in PBS. Primary antibodies were incubated in the same buffer overnight at 4°C. Sections were then rinsed in PBS (4 x 10 min) and incubated for 2 hr at room temperature with fluorescent secondary antibodies (in 0.1% bovine gelatin in PBS). After washes in PBS (4 x 10 min), sections were mounted on slides with Vectashield (Vector Laboratories) and observed by confocal microscopy

(Zeiss). Controls included the independent omission of each single major step of the immunocytochemistry protocol, one at a time.

cDNA Arrays

Mouse ATLASTM cDNA arrays (Clontech) were screened according to the manufacturer's protocol. Total RNA was extracted from the spinal cords of a P12 Nova-1 null mouse and a wild-type littermate or from the pooled hindbrains of 20 P0 Nova-1 null and 20 wild-type (litter matched) mice, followed by polyA⁺ purification using oligo-dT coupled magnetic beads according to the manufacturer's protocol (Dyna). 1.5 µg polyA⁺ RNA per filter was used to make radiolabeled ($\alpha^{32}\text{P}$ -dATP) probe.

Selective amplification via biotin- and restriction-mediated enrichment (SABRE)

Total RNA was extracted from the pooled spinal cords or hindbrains of 20 P0 Nova-1 null and 20 wild-type (litter matched) mice, followed by polyA⁺ purification using oligo-dT coupled magnetic beads according to the manufacturer's protocol (Dyna). 2.0 µg polyA⁺ RNA per sample was used for SABRE as described (Lavery et al., 1997) with the following modifications: Linker oligos: L1 5'-GGTCCATCCAACC-3', L2 5'-phosphate-GATCGGTTGGATGGACCGT-3'. After linker ligation, the reactions were loaded onto a 1% agarose minigel and cDNA pools ranging from 150 bp to 2 kb were isolated. Tester cDNA was PCR amplified with a 5'-biotinylated primer containing a *Bam*HI restriction site. Driver cDNA was PCR amplified with a non-biotinylated primer containing a mutated *Bam*HI restriction site. Tester primer, T1 5'-C(9)-biotin-CCAGGATCCAACCGATC-3'; Driver primer, D1 5'-GGTCCATCCAACCGATC-3'.

PERT hybridization was performed according to Miller and Riblet (Miller and Riblet, 1995). Following hybridization, single-stranded cDNA was digested with S1 nuclease and the whole reaction was mixed with 50 µl of Dynal M-280 streptavidin magnetic beads. After 2 h incubation at room temperature on a wheel, the beads were extracted using a magnetic concentrator and tester homohybrids were removed by *Bam*HI digestion. After five consecutive cycles of selection and amplification, the products were loaded on a sequencing gel and selectively amplified cDNA bands were cut out of the gel

and cloned into pGEM-Teasy (Promega). Following transformation of bacteria, plasmid inserts were sequenced from plasmid minipreps using T7 or M13R primers.

PCR Primers

The primers used were:

E2F 5'-AGCTTTCTGCAAAGACCATGAC

E4R 5'-GAAGATCTCCAAATCCAAGGAATCATCTGGG

α 2glyR E3A 5'-CATGGTGGTTTCTGTGACTGATC

α 2glyR E3B 5'-CATTGTAGTTTCTGCTATTGACCCAAAG

α 2glyR E4 5'-TCCAAATCCAAGGAATCATCTGGG

Actin F 5'-GTGGGCGCTCTAGGCACCA

Actin R 5'-CCCCCTGAACCCTAAGGCCAACCG

mGluR1F 5'-CCTGGGGTGCATGTTTACTCC

mGluR1R 5'-AGGCCGTCTCGTTGGTCTTCA

ICH-1 F 5'-GTCTCATCTTCATCAACTCC

ICH-1 R 5'-ATGCTAACTGTCCAAGTCTA

ClaB F 5'-ACCGAACAGGAGTGGCGGGAG

ClaB R 5'-GGGGTCTCCTCCTTGGATTCT

γ 2 GABA F 5'-GTATGGCACCCCTGCATTATTTTGTC

γ 2 GABA R 5'-TTGAATGGTTGCTGATCTGGGACG

ChAT F 5'-ATGCCTATCCTGGAAAAGGTCCC

ChAT R 5'-AGTGCTCCGAGCAAAGATCACAG

src-F 5'-CCAAGCTCTTCGGAGGCTTCAACTC

src-R 5'-CACATAGTTGCTGGGGATGTAACCG

agrin F 5'-GGGATAGTTGAGAAGTCAGTGGGGG

agrin R 5'-CGAAGCCAGCGGTTGGTGTG

gaba-rpa-F 5' GAAATTAATACGACTCACTATAGGGAGTTGAATGGTTGCTG
ATCTGGGACG

gaba-rpa-R 5' TCCCGCTCGTCGTCTGGTATGGCACCCCTGCATTATTTTGTC

cla-rpa-F 5' GAAATTAATACGACTCACTATAGGGAGGGGGTCTCCTCC
TTGGATTCT

cla-rpa-R	5'- TCCCGCTCGTCGTCTGACCGAACAGGAGTGGCGGGAG
glo E1F	5'- CTGAGGAGAAGTCTGCCGTTACTG
glo E3R	5'- CAGCACACAGACCAGCACGTTG
T7-GABA-9CF	5'- GAAATTAATACGACTCACTATAGGGAGCAGCTGCA CTGCTTAAGCGC
xR'	5'- TTTTGCTAGCCACAATGTCATCAATGAGATGATG
i9C-A R2	5'- GTACTTCCACAATGTCATCAATTAGATTATTAATCTGAAAA TTAGTATTCAGAAAATTGCTT
glo-KpnF	5'- ATGGTACCTGACTCCTGAGGAGAAG
glo-Rc	5'- CCACTTTCTGATAGGCAGCC

Antibodies

The following primary antibodies were used throughout this work:

human POMA patients' Ri serum (1:500, IF). (Buckanovich et al., 1993).

mouse monoclonal HRP-conjugated anti-T7 (1:5,000, W). Novagen.

mouse monoclonal anti-T7 (1:2,000, IF). Novagen.

mouse monoclonal anti-MAP2 (1:100, IF). Sigma.

rabbit polyclonal anti-coilin p80 (1:100, IF). R288, generously provided by Dr. Gall.

rabbit polyclonal anti-Nopp140 (1:1000, IF). RF12, generously provided by Dr. Meier.

mouse monoclonal anti-SIP-1 (1:100, IF) generously provided by Dr. Dreyfuss.

mouse monoclonal anti-Sm (1:100, IF). Y12, generously provided by Dr. Gall.

mouse monoclonal anti-SC35 (1:100, IF) generously provided by Dr. Gall.

mouse monoclonal anti-CTD domain of RNA polII (1:100, IF). 8WG16, Santa Cruz.

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 0.1 µg/ml to visualize cell nuclei.

Chapter 3: Defining Nova as a splicing factor *in vivo* – Analysis of Nova null mice

Introduction

In vitro analysis of Nova binding

Differing approaches based on RNA selection have led to the demonstration that Nova proteins act as sequence specific RNA-binding proteins. Through *in vitro* RNA selection, a method whereby a random pool of RNA targets is subjected to selection through binding to recombinant Nova-1 protein, a consensus binding sequence has been elucidated which consists of a stem-loop RNA containing (UCAUY)₃ in the loop region (Buckanovich and Darnell, 1997). This binding is sequence specific as mutagenesis of this core to (UCUUY)₃ abolishes RNA binding. Furthermore, mutagenesis and binding studies showed that the third KH domain of Nova-1 is necessary and sufficient for high affinity RNA binding to this sequence. Similar studies with Nova-2 yielded a consensus of GAGUCAU in a stem-loop, and whilst both proteins can bind the selected targets of the other *in vitro*, subtle differences in the affinities to these RNAs were observed (Yang et al., 1998). These results imply that Nova-1 and Nova-2 preferentially bind similar, perhaps overlapping, but not identical sets of RNA targets.

Studies using the third Nova KH domains in isolation revealed high affinity targets containing a single UCAY element, again within a hairpin loop context (Jensen et

al., 2000b), and allowed solution of the X-ray structure of a Nova KH3/RNA co-crystal. The crystal structure reveals that the 20 nucleotide RNA is in the predicted stem-loop conformation, and that the protein grips the core UCAY of the RNA in a “molecular vice” formed by a cleft between the variable and invariant protein loops of the Nova KH3 domain (Lewis et al., 2000). Within the RNA, a segment of the single stranded region, AUCAC, accounts for the vast majority of RNA-protein contacts within the co-crystal. The 5' A residue interacts with both N- and C-terminal amino acids in the protein via non-specific stacking interactions. The CA dinucleotide forms contacts with the protein to create pseudo-Watson-Crick base pairs and predicts that these residues are absolutely necessary for binding to occur. Both the first and last positions of the UCAY tetranucleotide could be filled by either pyrimidine according to the crystal structure, but would not be compatible with a larger purine in these positions due to steric constraints.

These predictions agree precisely with Nova KH3 domain/RNA binding studies in which RNA mutations still conforming to the consensus HYCAY (where H denotes A, C or U, Y denotes C or U) are tolerated (Jensen et al., 2000b). The stem region of the RNA appears to be important in constraining the conformation of the loop, retaining the UCAY core within the single-stranded loop.

The information gleaned from these selection studies was used to search several databases including one of neuron-specific alternatively spliced exons (Stamm et al., 1994). These searches identified two potential *in vivo* Nova binding sites based on RNA sequence. One is present in the inhibitory neurotransmitter receptor GlyR α 2 subunit pre-mRNA, 80bp upstream of an alternatively spliced exon, E3A, which is spliced in a mutually exclusive manner with a downstream exon, E3B (Kuhse et al.,

1991). The functional significance of the two resulting forms of the protein remains unknown, however these exons encode part of the extracellular domain of the receptor and thus may be involved in ligand binding (Kuhse et al., 1991). The second putative Nova binding site is located in the Nova-1 pre-mRNA itself, 8 bp downstream of exon H, also an alternatively spliced exon. This exon contains a number of potential sites for phosphorylation by serine/threonine protein kinases. Exon H may also alter protein turnover since it contains amino acid residues reminiscent of PEST sequences (enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (Rogers et al., 1986)) which are thought to lead to rapid intracellular degradation of the proteins containing them, including nuclear proteins (Chevaillier, 1993).

In vitro binding assays demonstrated that Nova is capable of binding RNAs corresponding to these regions with high affinity, and that this binding is both sequence specific (mutations of the UCAU repeats abrogate binding) and can be blocked specifically by POMA disease antisera, which suggests that these antibodies may be responsible for the development of neurological symptoms in POMA patients through abrogation of Nova-1 RNA-binding activity *in vivo* (Buckanovich et al., 1996). Moreover, native Nova protein in mouse brain lysates coimmunoprecipitates with GlyR α 2 and Nova-1 pre-mRNAs (Buckanovich and Darnell, 1997) and can be UV-cross-linked to an RNA encoding a region of GlyR α 2 intronic sequence, but not a corresponding RNA in which the UCAU repeats have been mutated to UAAU (Jensen et al., 2000a). These results demonstrate that these interactions are likely to occur *in vivo* also, and the proximity of the putative binding sites to alternatively spliced exons in both

cases suggested that Nova-1 may play a role in the regulation of alternative splicing *in vivo*.

Minigene constructs were generated to test whether Nova-1 is able to directly act on GlyR α 2 pre-mRNA to regulate alternative splicing of E3A and E3B. Cotransfection with a Nova-1 expression plasmid caused a dose-dependent increase in E3A inclusion relative to E3B in three separate cell lines (Jensen et al., 2000a). Mutation of the Nova binding element in this context abolished the increase in E3A inclusion upon the addition of Nova-1, and paradoxically caused an increase in E3B inclusion relative to E3A. One possible explanation for this observation is that the UAAU mutation allowed detection of a cryptic Nova-1 binding site able to mediate action on E3B splicing. Taken together, these experiments demonstrate that Nova-1 is able to act directly to enhance the inclusion of GlyR α 2 E3A in cell lines and that this action is dependent on the integrity of the intronic UCAU Nova binding site.

Nova null mice

Nova-1 null mice were generated by targeted disruption following extensive mapping of the Nova-1 gene. The first two coding exons of Nova-1, including the initiating methionine, were replaced by an IRES- τ -lacZ cassette resulting in the generation of mice completely deficient in Nova-1 expression. At the phenotypic level these mice appear normal at birth, but grow much less than their littermates and display progressive motor weakness culminating in death on average by postnatal day 7-10. Heterozygous mice harboring only one copy of the Nova-1 gene are phenotypically normal. Subsequently these mice were bred into a CD1 genetic background resulting in mice able to survive on

average 2-3 weeks after birth. These mice also display a severe phenotype, being markedly smaller than their wild type littermates after as little as 3-4 days post-birth, as well as displaying symptoms of motor impairment such as motor weakness and action-induced tremulousness as well as atrophy in the hind limbs, which are most pronounced by approximately P7-P10. As a result, these mice are unable to walk well, dragging their hind legs and spontaneously falling to the side, however there appear to be no sensory deficits.

Gross structure and histology appears normal in Nova-1 null mice at birth, however signs of neuronal degeneration become evident during the first post-natal week. A four-fold increase in the number of pyknotic cells in the ventral spinal cord is seen in null mice compared to wild-type littermates at P5 (Jensen et al., 2000a). Neuronal degeneration is particularly evident in brainstem motor nuclei and in motor neurons of the spinal cord, which correlates with regions of Nova-1 expression. There is no evidence for such neuronal degeneration within the cortex, hippocampus, or sensory neurons of the brainstem or spinal cord. These pyknotic cells corresponded to cells undergoing apoptotic cell death, as assessed by TUNEL staining. Thus Nova-1 is essential for survival but not development of subcortical motor neurons.

Nova-2 null mice have subsequently been generated and these mice display an overt phenotype remarkably similar to that of Nova-1 nulls. Nova-2 null mice are also born phenotypically identical to their littermates, but fail to grow as well, appearing markedly smaller by approximately P5. Nova-2 is also essential for viability, however in a similar mixed genetic background, Nova-2 null mice survive slightly longer than their Nova-1 null counterparts, an average of 14-17 days post-birth. Crosses into a number of

stable genetic backgrounds are in progress (M. Ruggiu, personal communication). Again, heterozygous mice appear identical to their wild-type littermates, and null mice display difficulty walking or righting themselves, however in contrast to Nova-1 nulls, Nova-2 null mice do not display action-induced tremors.

To assess the function of Nova proteins *in vivo*, proposed targets of Nova-1 alternative splicing regulation were examined in Nova-1 null and heterozygous mice. These results demonstrate that Nova-1 regulates neuron-specific alternative splicing of GlyR α 2 *in vivo*, in a manner consistent with previous biochemical and cell culture analyses. A survey of neuronal exon usage in Nova-1 null mice revealed a defect in inclusion of the GABA γ 2L cassette exon and thus led to the identification of an additional Nova-1 target. Finally, Nova-1 alternative splicing was analyzed in Nova-1 heterozygous and Nova-2 null mice on the basis of previous sequence analysis, and was also found to be regulated *in vivo* by Nova proteins.

Results

Assay Development

Figure 1A shows a schematic representation of GlyR α 2 alternative splicing and two complementary methods that were developed to assess GlyR α 2 splicing in Nova-1 null mice. First, RNA was RT-PCR amplified using primers specific to GlyR α 2 exon 2 (E2) and GlyR α 2 exon 4 (E4). Reaction products were then digested with restriction enzymes that recognize sites either in E3A (SspI) or E3B (BspMI, not shown) (with >98%

specificity, Figure 1B). In a second method, E3A and E3B-specific primers were used under reaction conditions in which each primer showed absolute specificity for their respective exon (Figure 1C). Quantitation of splicing using linear PCR reaction conditions gave highly reproducible results over a range of reaction conditions (input RNA, PCR cycle number) and between animals.

In a third set of experiments, RNase protection assays were performed. Since GlyR α 2 exons E3A and E3B are so similar (identical in 55 of 68 nucleotides), control experiments were again performed to ensure that the assay conditions used enabled absolute discrimination between the two spliced products. Figure 2A shows a schematic representation of the probes used. Figure 2B shows that in an experiment where *in vitro* transcribed RNAs were mixed in known ratios, absolute discrimination is achieved using either an RPA probe specific for E3A- or E3B-containing messages.

GlyR α 2 Splicing in Nova-1 null mice

Preliminary experiments demonstrated that the ratio of E3A/E3B splicing was decreased in Nova-1 null mice relative to wild-type littermates (Figure 1B,C). To determine the reproducibility of these observations, splicing to E3A and E3B was examined in 8 Nova-1 null mice from 6 different litters, and the results compared to splicing in normal littermates. In every case, RNA from Nova-1 null mice showed a decrease in the utilization of E3A relative to E3B (Figure 3). These results are consistent with the transfection data discussed above, in which Nova-1 acts to enhance the utilization of GlyR α 2 E3A, while in the absence of Nova-1 *in vivo* there is a relative deficit in the ability of neurons to utilize the E3A splice site.

Specificity of Splicing Defects in Nova-1 null mice.

The specificity of the defect in GlyR α 2 splicing was examined by comparing the splicing efficiency of six previously described alternatively spliced neuronal transcripts (Figure 4). Splicing of five of these transcripts was unchanged in Nova-1 null mice relative to their littermates, including clathrin light chain B (ClaB), agrin, mGluR1, ICH-1 and n-src. However, alternative splicing of the GABA_A receptor γ 2 subunit was significantly altered in Nova-1 null mice. The GABA_AR γ 2 transcript is widely expressed in the brain, but the pre-mRNA is alternatively spliced to include a cassette exon, γ 2L, preferentially in brainstem and spinal neurons (Zhang et al., 1996). Examination of brainstem and spinal cord RNA revealed a two-fold difference in the E3A/E3B GlyR α 2 splicing ratio and nearly a three-fold difference in the γ 2L/ γ 2S GABA_AR γ 2 splicing ratio in wild-type versus Nova-1 null mice, averaged over 11 and 9 litters, respectively (Figure 4).

These observations were confirmed by RNase protection assays (Figure 5). By this method, the difference between GlyR α 2 and GABA_AR γ 2 splicing ratios was calculated to be approximately 1.4-fold and 1.9-fold, respectively. These results were less reliable than RT-PCR in quantitating the low abundance splice variants in a tissue sample of high complexity, as anticipated (Foley et al., 1993), which most likely accounts for the difference in values. It is clear from these experiments however that Nova-1 null mice show a deficit in inclusion of the GlyR α 2 E3A and GABA_AR γ 2 exon 9 alternatively spliced exons.

In addition to differences in splicing, the steady-state level of GlyR α 2 mRNA is altered in Nova-1 null mice. Approximately 2.5-fold higher levels of GlyR α 2 mRNA are

seen in the spinal cord of Nova-1 null mice compared to littermates (Figure 5C). This is somewhat surprising, especially given the death of Nova-1 expressing neurons seen by histological studies which would perhaps predict a decrease in the overall level of neuronal messages in Nova-1 null mice. Whilst the mechanism underlying this observation is not clear, one possible explanation could be that as a result of the absence of Nova-1, processing of the RNA subsequent to splicing is altered. Recent evidence suggests splicing in the nucleus is coupled to mRNA export, and can subsequently influence stability (especially nonsense-mediated decay) and translatability in the cytoplasm (reviewed in (Reed and Hurt, 2002)). Failure to interact with Nova-1 may cause retention of the spliced RNA in the nucleus or incorrect processing in the cytoplasm and, thus accumulation in null animals. No difference in steady state level is seen for the GABA_AR γ 2 (or α -B) messages, which argues against a global upregulation of transcription of inhibitory neurotransmitter receptors as a result of neuronal dysfunction. If indeed GlyR α 2 mRNA accumulates as a result of incorrect post-transcriptional processing in Nova-1 null mice, this would imply that GlyR α 2 and GABA_AR γ 2 RNAs are handled somewhat differently by these cells, since no change in GABA_AR γ 2 steady state mRNA level is observed.

Although the GlyR α 2 and GABA_AR γ 2 genes are expressed throughout the brain, Nova-1 expression is relatively absent in rostral structures including the forebrain and sensory thalamus (Buckanovich et al., 1996; Yang et al., 1998). If aberrant GlyR α 2 E3A and GABA_AR γ 2L splicing results directly from the lack of Nova-1 in neurons, splicing of these RNAs should be relatively normal in the forebrain and abnormal in the spinal cord of individual Nova-1 null mice. Specific abnormalities in GlyR α 2 and GABA_AR γ 2

splicing were found in the spinal cord and hindbrain but not the forebrain (Figure 6). In forebrain of wild-type versus null mice no differences in alternative splicing for GlyR α 2, GABA $_A$ R γ 2L, or clathrin-B mRNA, nor in steady state levels of actin or the spinal motor neuron marker ChAT were observed (Figure 6). These results indicate that Nova-1 mediates a specific effect on the alternative splicing of GlyR α 2 and GABA $_A$ R γ 2 receptor pre-mRNAs in a cell autonomous manner.

Genotype and developmental profile of splicing changes.

The level of Nova-1 protein varies in a dose-dependent manner in wild-type mice relative to Nova-1 heterozygous and null mice (Jensen et al., 2000a). To assess whether the splicing defects of GlyR α 2 and GABA $_A$ R γ 2 RNAs are dependent on the level of Nova-1 protein, the utilization of these exons in mice as a function of Nova-1 gene dosage was examined. The GABA $_A$ R γ 2L splicing defect varied from 1.5 fold in heterozygous mice to a 3 fold change in Nova-1 null littermates (Figure 7A-B). GlyR α 2 E3A utilization was only abnormal in Nova-1 null mice, suggesting differences in the degree to which the Nova-1 protein is rate limiting for proper splicing of GlyR α 2 and GABA $_A$ R γ 2 pre-mRNAs.

When alternative splicing was examined as a function of age, utilization of the GABA $_A$ R γ 2L increased in wild type mice during the first three weeks of post-natal development (Figure 7C), consistent with previous analyses (Wang and Grabowski, 1996; Zhang et al., 1996). A deficiency in exon utilization first becomes apparent at P7 in Nova-1 null mice (Figure 7C). In Nova-1 heterozygous mice, there was again an intermediate deficit in GABA $_A$ R γ 2 usage evident at P7. The GlyR α 2 E3A/E3B ratio was

unchanged in P0-P4 Nova-1 null mice (<10% difference across multiple littermates), and thereafter differed by approximately two-fold.

Nova-1 exon H Splicing in Nova-1 heterozygous and Nova-2 null mice

A putative Nova-1 binding element containing four YCAY motifs is present eight nucleotides downstream of Nova-1 exon H, which is conserved between mouse and human (Figure 8A). Nova-1 alternative splicing was assessed in spinal cords of Nova-1 heterozygous and wild-type littermate mice (Figure 8B,C). In all litters tested over the age of P12, exon H inclusion was decreased in wild-type mice compared to heterozygotes. This implies that in addition to mediating alternative exon inclusion as was seen for GlyR α 2 and GABA $_A$ R γ 2, Nova-1 can also act in a negative fashion to mediate alternative exon skipping. The magnitude of the effect, whilst small, is comparable to the difference seen in GABA $_A$ R γ 2 splicing between wild-type and Nova-1 heterozygous mice (Figure 8D).

Nova-1 exon H alternative splicing was also examined in Nova-2 null mice. In both spinal cord and midbrain/hindbrain preparations, Nova-1 exon H inclusion was decreased in a manner consistent with a dose-dependent effect of Nova-2 to decrease exon H inclusion. Regional differences in exon H inclusion were also observed, ranging from approximately 19% inclusion in midbrain/hindbrain to 88% in cerebellum. No difference was seen in exon H splicing between wild-type and Nova-2 null mice in the cerebellum, however any increase in exon H inclusion in the null mice would be difficult to detect given the high level of exon inclusion in wild-type cerebellum. Taken together, these results imply that Nova-1 can autoregulate alternative splicing of its own message, and that this splicing decision can also be regulated by Nova-2. If the effects on splicing

are a direct result of Nova binding to its own message, these results imply that Nova-1 and Nova-2 share at least one target in common, and that Nova-1 and Nova-2 are co-expressed in at least a subset of Nova-expressing cells. Regional differences exist in the level of exon H inclusion, perhaps indicative of differences in the amount of Nova proteins (perhaps relative to other splicing regulators) in neurons in different regions of the brain.

Discussion

Nova-1 regulates tissue specific alternative splicing of 3 distinct pre-mRNAs

Genetic approaches have been used to demonstrate that Nova-1 functions to regulate alternative splicing in neurons. Combined with previous biochemical approaches, this provides strong evidence that Nova-1 functions to regulate alternative splicing in neurons by binding pre-mRNA in a sequence-specific manner to activate exon inclusion. In co-transfection assays, GlyR α 2 pre-mRNAs harboring (UCAUY)₃ sequence show a Nova-1 dose-dependent increase in utilization of the alternatively spliced E3A, while mutant pre-mRNAs harboring the (UAAUY)₃ point mutant do not show this effect (Jensen et al., 2000a). Consistent with this, in Nova-1 null mice, there is a reciprocal two-fold decrease in the ratio of GlyR α 2 E3A relative to E3B splicing. These data provide converging lines of evidence that Nova-1 regulates GlyR α 2 splicing by directly binding to the pre-mRNA in neurons.

A second specific defect in alternative splicing in Nova-1 null mice was also identified by surveying a number of messages known to be alternatively spliced in the

brain, deficient inclusion of the GABA_Aγ2L exon. The defective exon utilization of γ2L and GlyRα2 E3A in Nova-1 null mice, suggests that Nova-1 most likely acts to activate GlyRα2 E3A and GABA_Aγ2L exon inclusion in neurons. An alternative, and not entirely exclusive possibility, is that Nova-1 activates splicing indirectly, by competing with a splicing inhibitor. Such an interpretation would be consistent with the observation that Nova action can be antagonized by a recently discovered brain-enriched variant of PTB (Polydorides et al., 2000). However, a strictly indirect action of Nova appears unlikely, since the action of Nova-1 to activate GlyRα2 E3A splicing in transfected cells is abolished by mutation in an intronic RNA element (Jensen et al., 2000a). Experiments designed to assess whether Nova-1 also regulates GABA_Aγ2L exon inclusion in a sequence-specific manner are reported in Chapter 4.

These studies also provide evidence that Nova proteins regulate alternative splicing of a third pre-mRNA. In contrast to the two previous examples, Nova null and heterozygous mice show a relative increase in alternative splicing to Nova-1 exon H compared to their wild-type littermates. Thus, it is proposed that high levels of Nova cause a decrease in the inclusion of exon H. Again, it is unclear from the analysis of these mice whether this regulation of alternative splicing is due to an indirect action of Nova, for example Nova may alter exon H inclusion by inhibiting a splicing factor required for efficient utilization of this exon. However, it is intriguing that a putative Nova-binding element is located only 8bp downstream of exon H in the adjacent intron. This points to a hypothesis whereby Nova-1 splicing regulation is also regulated by sequence specific binding of Nova protein to the pre-mRNA. The difference in the resulting effect (Nova mediates exclusion rather than inclusion of this exon) may relate to the position of the

Nova binding site relative to the exon involved. Chapter 4 begins to address some of these questions.

The identification of the glycine and GABA_A receptor pre-mRNAs as well as the Nova-1 pre-mRNA itself as Nova-1 targets raises the question of the extent to which Nova proteins are used to regulate alternative splicing in neurons. A cursory examination of transcripts known to be alternatively spliced in the brain was performed, the results of which revealed that absence of Nova-1 does not globally alter splicing regulation, thus it appears that Nova is involved in only a subset of splicing decisions that are made in a single cell. How this specificity is achieved is not entirely understood, however sequence specific RNA binding and perhaps specific protein-protein interactions are both likely to contribute. The mechanism by which Nova-1 regulates alternative splicing is also not well understood. Detailed analysis of the sequence elements required for Nova's action on the two additional targets (GABA_AR γ 2 and Nova-1 exon H) has been undertaken in an attempt to begin to understand the mechanism by which Nova-1 regulates alternative splicing by comparison with studies of GlyR α 2 E3A splicing.

Nova-1 mediated splicing regulation and neuronal dysfunction.

Nova-1 expression is not only specific to neurons, but to a subset of neurons in the hypothalamus, brainstem and spinal cord (Buckanovich et al., 1993; Buckanovich et al., 1996; Jensen et al., 2000a). Therefore the identification of Nova-1 as a splicing factor provides a mechanism by which individual neurons can differentially regulate splicing in a qualitatively distinct manner from other cell types. GABA_AR γ 2L is preferentially included in the hypothalamus, midbrain, brainstem and spinal cord (Zhang et al., 1996), and this correlates precisely with the pattern of Nova-1 expression, supporting our

finding of a role for Nova-1 in generating the γ 2L splice variant in vivo. This study also shows that Nova-1 exon H inclusion varies regionally within the brain. It remains to be seen whether this could also be explained in part by a correlation with Nova protein levels, or expression levels of other splicing factors in these regions.

Analysis of Nova-1 null mice has shown that Nova-1 is essential for neuronal viability in mature neurons in postnatal mice (Jensen et al., 2000a). The defect in neuronal viability suggests either a loss of function, such as a failure of mechanisms necessary to insure survival of mature neurons, or a gain of function, such as acquisition of a new toxic activity that mediates neuronal death in Nova-1 null neurons. For example, aberrant splicing of the α_{1A} calcium channel in mice is believed to result in a gain of function mutation which leads to a delayed onset neurologic disorder manifest by ataxia, motor seizures and absence seizures (Fletcher et al., 1996). In Nova-1 null mice, defects in inhibitory receptor splicing could lead to a gain of function such that glycine and/or GABA receptors become toxic, for example by creating imbalances between neuronal inhibition and excitation that lead to excitotoxic cell death.

Patients with POMA have symptoms that suggest abnormal inhibitory control over brainstem and spinal motor pathways (Luque et al., 1991). Moreover, mice with the spasmodic mutation (Ryan et al., 1994; Saul et al., 1994) and humans with hyperekplexia (Shiang et al., 1993) harbor defects in inhibitory glycine receptors and suffer from myoclonic syndromes that bear marked similarities to the motor symptoms evident in Nova-1 null mice and in POMA patients. Thus the defects in splicing of the inhibitory glycine and GABA receptors in Nova-1 null mice suggest a reasonable model for the mechanism of motor dysfunction in both the murine and human systems. In POMA,

immunologic targeting of Nova protein may lead to defects in inhibitory receptor function and thereby to the excess motor activity evident in the disorder.

Figure 1: RT-PCR assays to assess GlyR α 2 E3A/E3B alternative splicing.

- (A) **Schematic representation of GlyR α 2 E3A/E3B alternative splicing.** The primers described below are depicted as colored arrows, stars denote ^{32}P -labeled primers.
- (B) **I. Specificity of the restriction digest assay.** PCR of a cDNA encoding the mature E2-E3A-E4 mRNA or the E2-E3B-E4 mRNA using ^{32}P -labeled E2 primer and unlabeled E4 primer was digested with SspI. Phosphorimage analysis of the data revealed that >98% of the E2-E3A-E4 DNA was digested, while <2% of the E2-E3B-E4 DNA was digested. **II. RT-PCR analysis of GlyR α 2 E3A and E3B splicing utilization.** Spinal cord RNA from a P16 Nova-1 null mouse and a wild-type littermate was analyzed by RT-PCR using the same conditions as in (I.) followed by SspI restriction digestion and autoradiography.
- (C) **I. Specificity of the E3A/E3B primers assay.** cDNA clones encoding the mature E2-E3A mRNA or the E2-E3B mRNA were PCR amplified with ^{32}P -labeled E2 and either E3A- or E3B-specific, unlabeled primers. Analysis of this data on a phosphorimager revealed that E2-E3A or E2-E3B products could not be detected using the mismatched primer pair (E2-E3B or E2-E3A primers, respectively). **II. RT-PCR analysis of GlyR α 2 E3A and E3B splicing utilization.** Spinal cord RNA from a P18 Nova-1 null mouse and a wild-type littermate was analyzed by RT-PCR using ^{32}P -labeled E2 primer and unlabeled E3A or E3B primer followed by autoradiography.

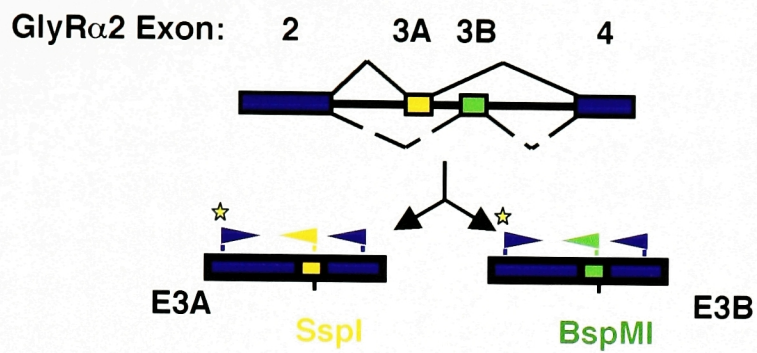
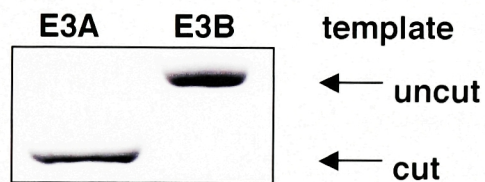
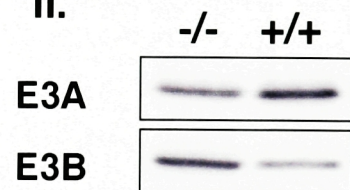
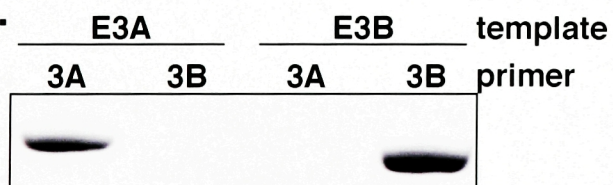
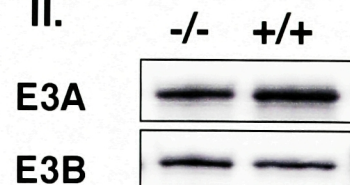
A**B****E2-E4 / SspI****I.****II.****C****E3A/E3B primers****I.****II.**

Figure 2: RNase protection assay controls.

- (A) Schematic representation of GlyR α 2 probes used for RNase protection assays.** Body-labeled antisense RNA probes complementary to E2-E3A-E4 mRNA or E2-E3B-E4 mRNA result in protected fragments of 288bp when hybridized to their own sense message, or 132nt when hybridized to the alternative splice form.
- (B) Probes differentiate between E3A and E3B.** Template RNA was *in vitro* transcribed from cDNA clones encoding the mature E2-E3A mRNA or the E2-E3B mRNA and mixed in precise ratios. These mixes were hybridized to body-labeled antisense RNA probes as described in (A), followed by RNase digestion and the results analyzed by denaturing PAGE and autoradiography.

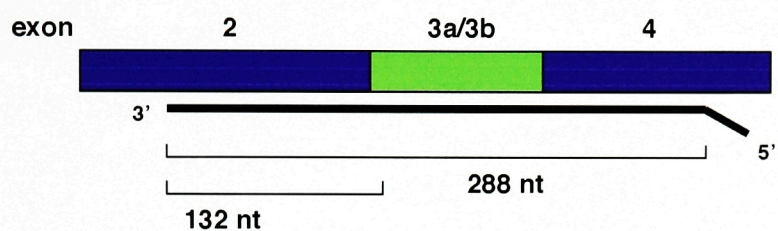
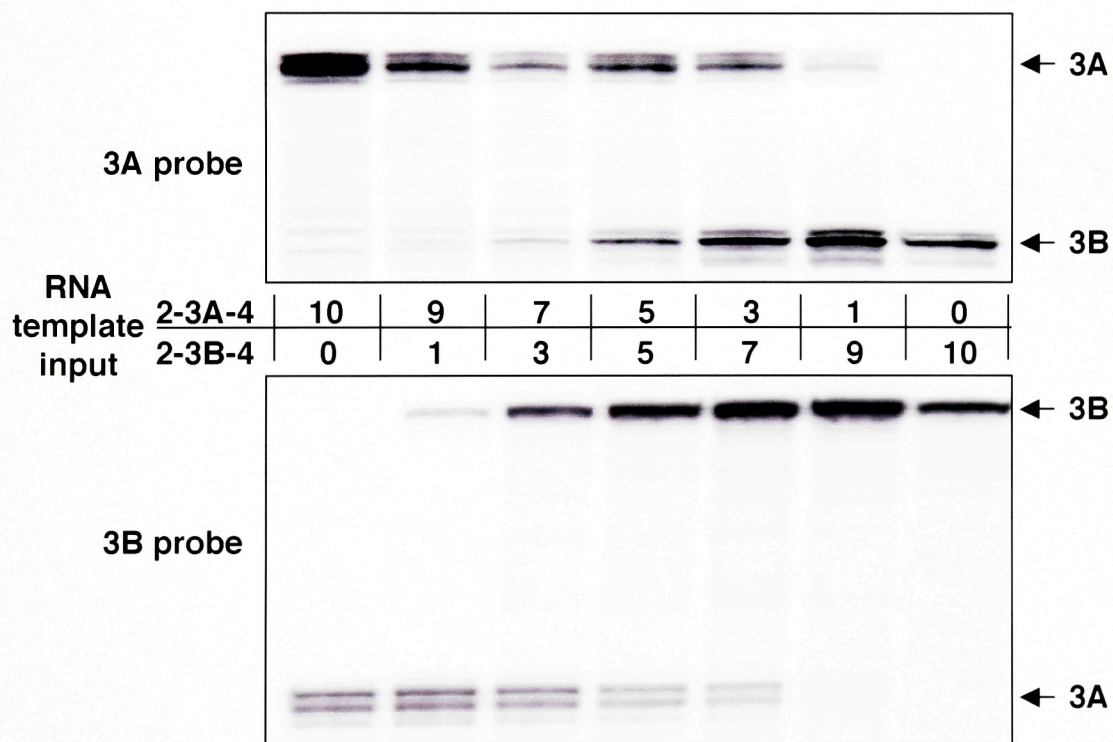
A**B**

Figure 3: Aberrant GlyR α 2 splicing in Nova-1 null mice

Reproducibility of the GlyR α 2 splicing defect in Nova-1 null mice. Spinal cord RNA from Nova-1 null mice and their littermates with indicated genotypes were analyzed as in Figure 1(C) for the presence of GlyR α 2 E3A and E3B isoforms. Data is presented as the ratio of E3A to E3B exon usage in each animal. PCR results were linear with respect to RNA input and cycle number. Actin RNA levels were measured in each sample by RT-PCR, and differed by ~ 5-10% between littermates.

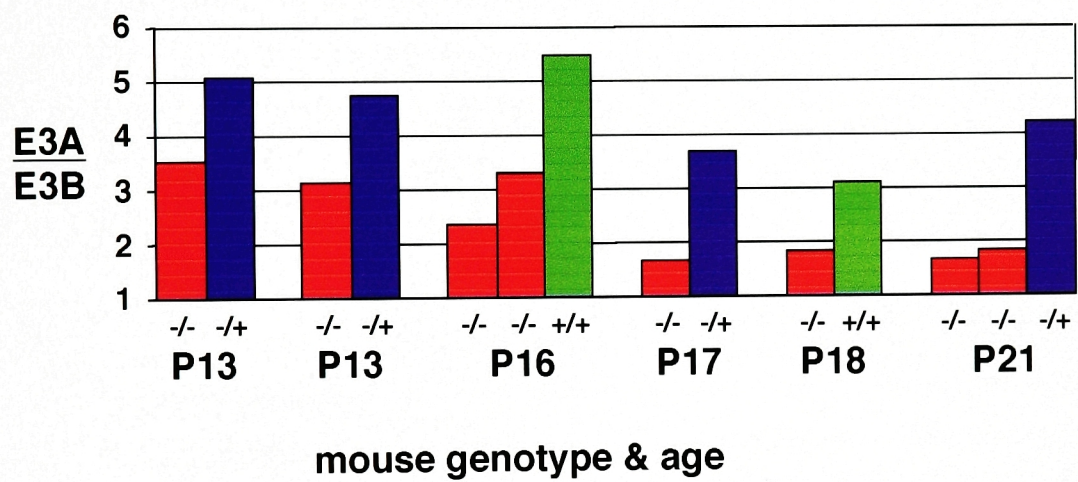
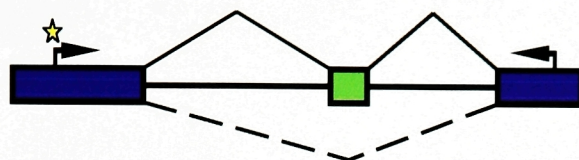


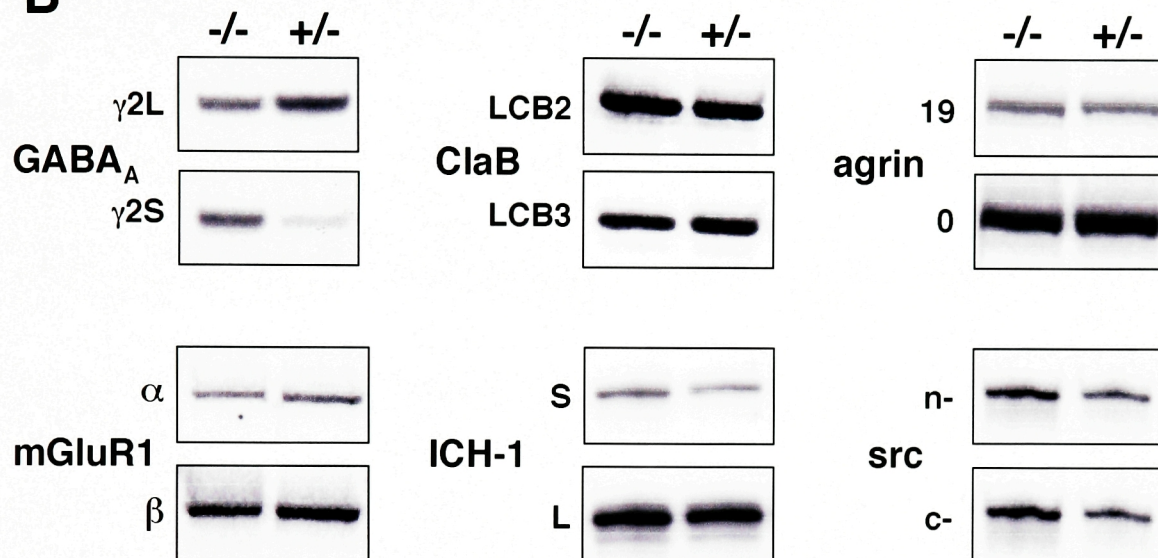
Figure 4: Specific abnormalities in alternative splicing of GlyR α 2 and GABA $_A$ R γ 2 transcripts in Nova-1 null mice.

- (A) **Schematic representation of the assay** used to assess alternative splicing of messages other than GlyR α 2. Primers to exons surrounding the alternatively spliced exon were used to RT-PCR both exon included and excluded forms of the messages. One primer was ^{32}P -labeled (star) and the products separated by denaturing PAGE and visualized by autoradiography.
- (B) **RT-PCR analysis of alternative splicing utilization.** RNA isolated from a P16 Nova-1 null mouse and a heterozygous littermate was analyzed by RT-PCR with primers specific to the indicated genes. All PCR reactions were performed in duplicate using minus-RT reactions (not shown). γ 2L includes and γ 2S excludes exon 9 in GABA $_A$ R γ 2 mRNA (Whiting et al., 1990). LCB2 includes and LCB3 lacks the neuron-specific EN exon in the clathrin light chain B (ClaB) mRNA (Wang and Grabowski, 1996). Agrin $_0$ lacks and agrin $_{19}$ includes both Z-site exons (Ferns et al., 1992). mGluR1 α lacks and mGluR1 β contains an additional exon in the metabotropic glutamate receptor mRNA (Hollmann and Heinemann, 1994). ICH-1S includes and ICH-1L lacks an additional exon in the IL-1 β converting enzyme mRNA (Wang et al., 1994). n-src mRNA includes a neuron-specific exon in the c-src message [Martinez, 1987 #632]
- (C) **Quantitation of the data presented in (B)** together with additional RNA samples (the number of litters examined is indicated by (n)). The ratio of long:short alternatively spliced products measured in wild-type animals relative to the respective ratio in the corresponding Nova-1 null littermate is presented. For GlyR α 2, the ratio of E3A/E3B in wild-type versus Nova-1 null littermate is depicted. The bars represent the average change in wild-type:null exon usage for the number of litters examined; error bars represent the standard deviation.

A



B



C

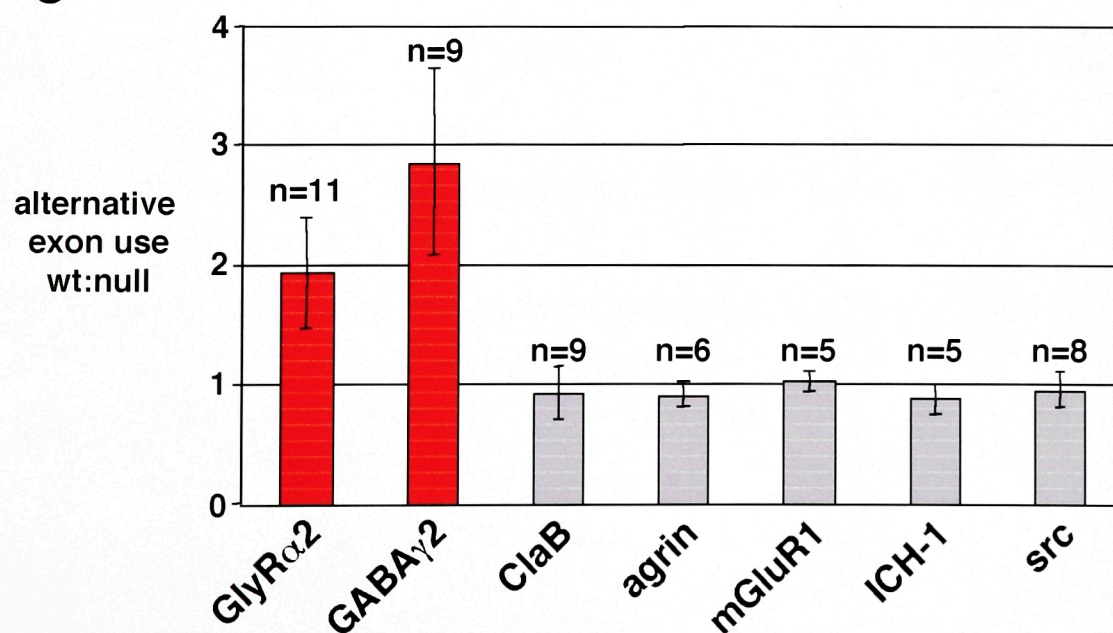


Figure 5: Confirmation by RNase Protection Assay.

- (A) **RPA analysis of alternative splicing utilization.** Schematic representation of the body-labeled antisense RNA probes used for RNase protection assays and the resulting product sizes used for quantitation are shown on the left. Spinal cord RNA isolated from P16 (GlyR α 2 analysis) and P13 (GABA $_A$ R γ 2 and Cla B) Nova-1 null mice and their littermates with indicated genotypes was analyzed by RNase protection assay. Autoradiographs are shown on the right.
- (B) **Quantitation of the data presented in (A)** together with additional RNA samples (the number of litters examined is indicated by (n)). Products were quantitated by phosphorimager and corrected for the expected number of labeled nucleotides (Cs). The ratio of long:short alternatively spliced products measured in control animals relative to the respective ratio in the corresponding Nova-1 null littermate is presented. For GlyR α 2, the ratio of E3A/E3B in control versus Nova-1 null littermate is depicted. The bars represent the average change in wild-type:null exon usage for the number of litters examined; error bars represent the standard deviation.
- (C) **RPA analysis of steady state mRNA levels.** Quantitation of the data presented in (A) together with additional RNA samples (the number of litters examined is indicated by (n)). Products were quantitated by phosphorimager and corrected for the expected number of labeled nucleotides (Cs). The total of both alternatively spliced products measured in control animals relative to the total mRNA level in the corresponding Nova-1 null littermate is presented. The bars represent the average change in wild-type:null mRNA level for the number of litters examined; error bars represent the standard deviation.

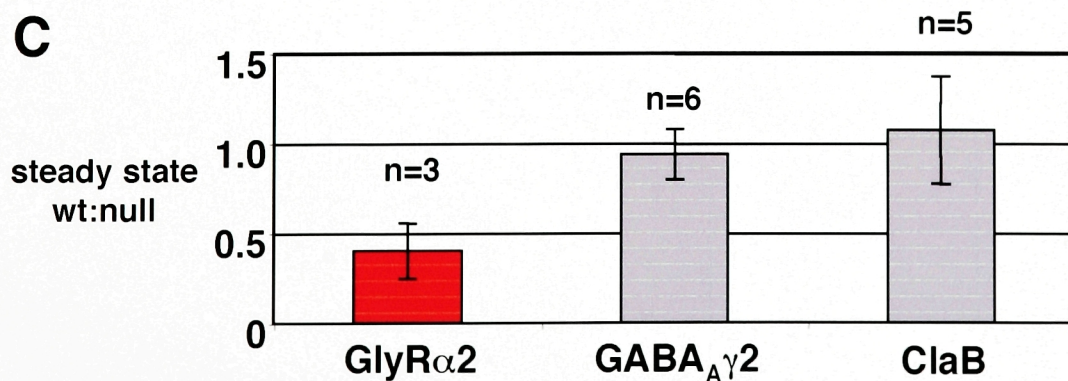
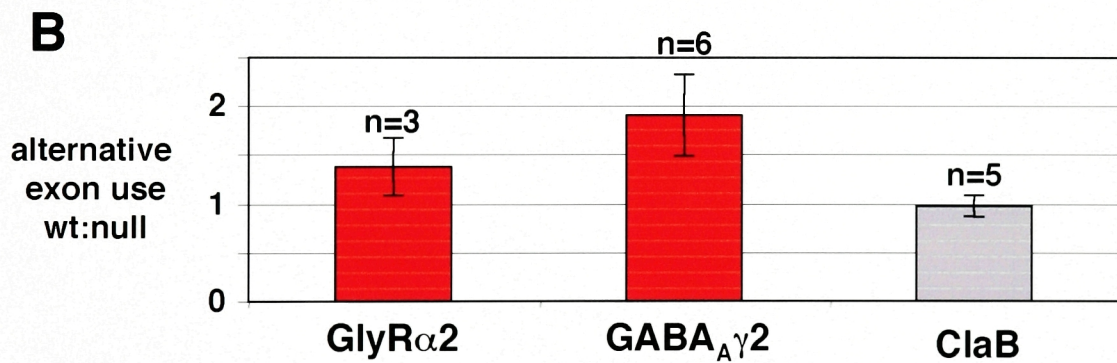
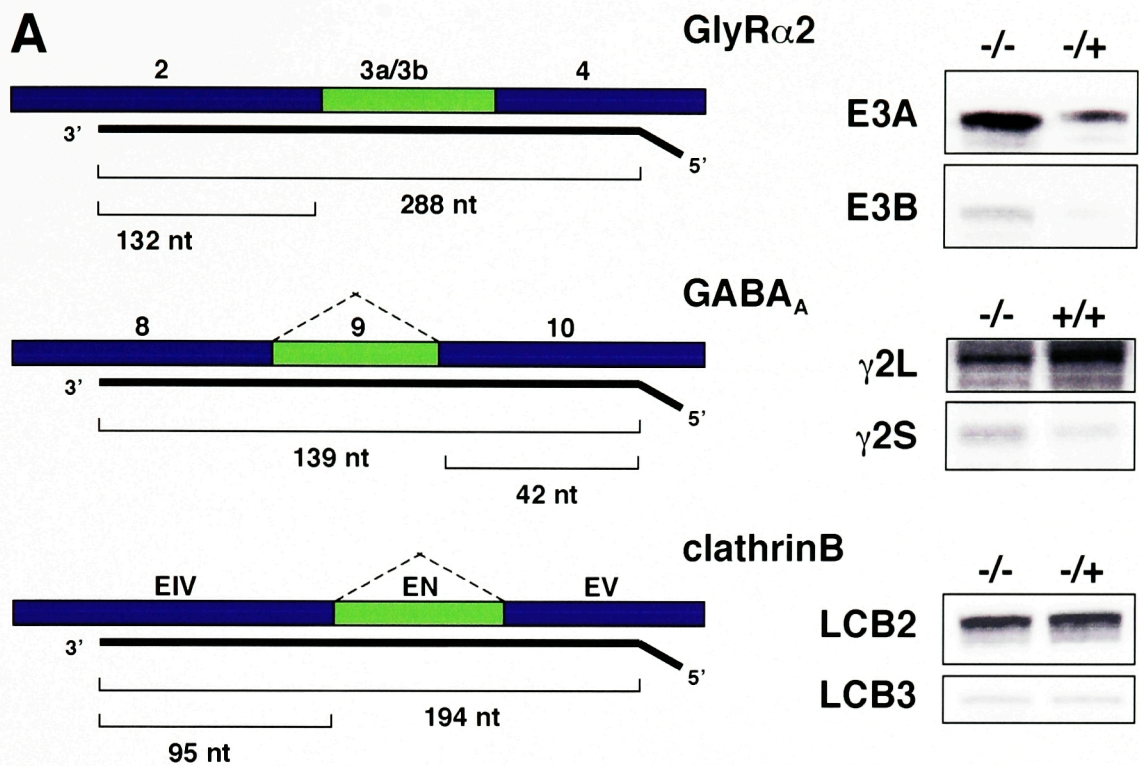


Figure 6: Regional specificity of splicing defects

GlyR α 2 and GABA $_A$ R γ 2 alternative splicing defects are restricted to Nova-1 expressing regions of brain in Nova-1 null mice. Spinal cord or forebrain RNA was isolated from a P18 (P7 for GABA $_A$ R γ 2) Nova-1 null mice and a wild-type littermate and analyzed for the indicated splicing products by RT-PCR. Data is presented as in Figure 3. Unlike the spinal cord, quantitation of phosphorimager data using RNA from forebrain revealed no differences in the ratio of GlyR α 2 E3A/E3B and γ 2L/ γ 2S GABA $_A$ R γ 2 exon usage between Nova-1 null animals and wild-type littermates. Clathrin B light chain alternative splicing showed no null:wild-type difference in either spinal cord or forebrain. Quantitation revealed no differences in the steady-state levels of RNA encoding the motor neuron marker choline acetyltransferase (ChAT) or actin in Nova-1 null spinal cord relative to wild type.

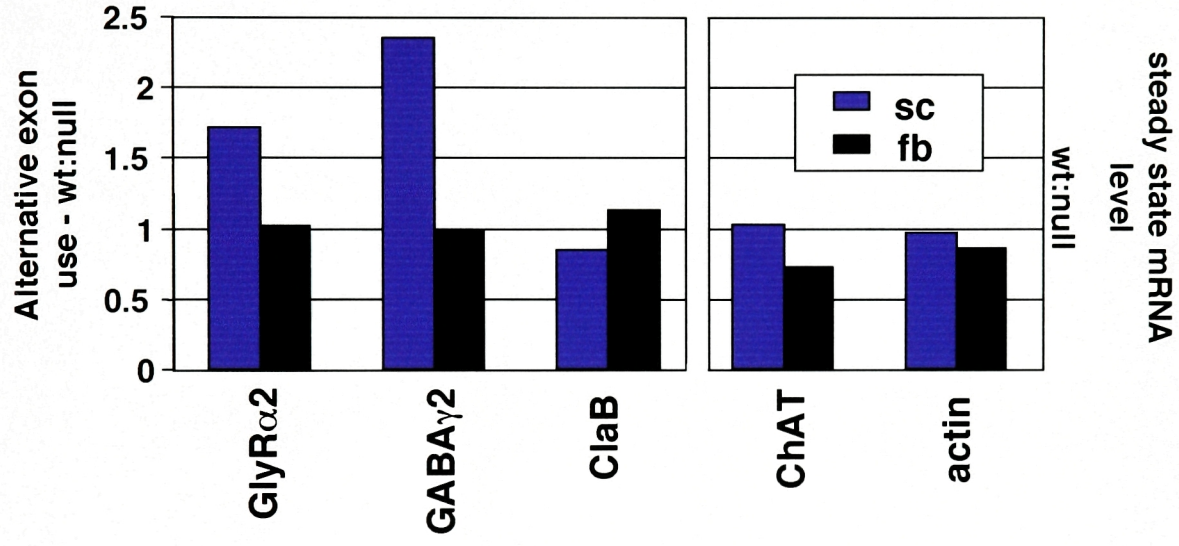


Figure 7: Changes in GlyR α 2 and GABA $_A$ R γ 2 Alternative Splicing as a Function of Gene Dosage and Development.

- (A) GABA $_A$ R γ 2, GlyR α 2, and c-src alternative exon usage in the spinal cords of a P16 Nova-1 null mouse and its heterozygous and wild type littermates were analyzed. There was a significant difference in alternative exon utilization in both the null and heterozygote mice for the GABA $_A$ R γ 2 message, whereas the GlyR α 2 E3A/E3B ratio is only disturbed in the null mouse. Alternatively spliced n-src mRNA is unchanged in all three genotypes.
- (B) Data obtained from (A) and the indicated number of additional litters is shown. GlyR α 2, GABA $_A$ R γ 2 and n-src alternative exon usage in Nova-1 null and heterozygote mice is plotted using the alternative exon ratio of the wild-type mouse set to 1. Bars represent the average of the normalized exon use ratio; error bars indicate the standard deviation.
- (C) Developmental change in the GABA $_A$ R γ 2L/ γ 2S exon use ratio. Points represent single measurements at the indicated developmental times.

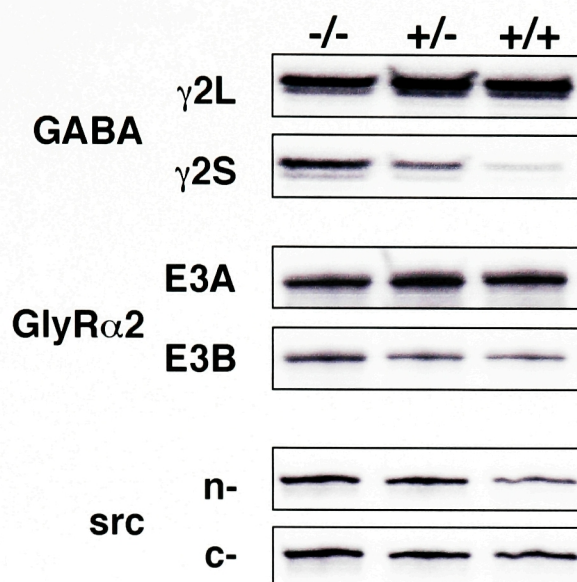
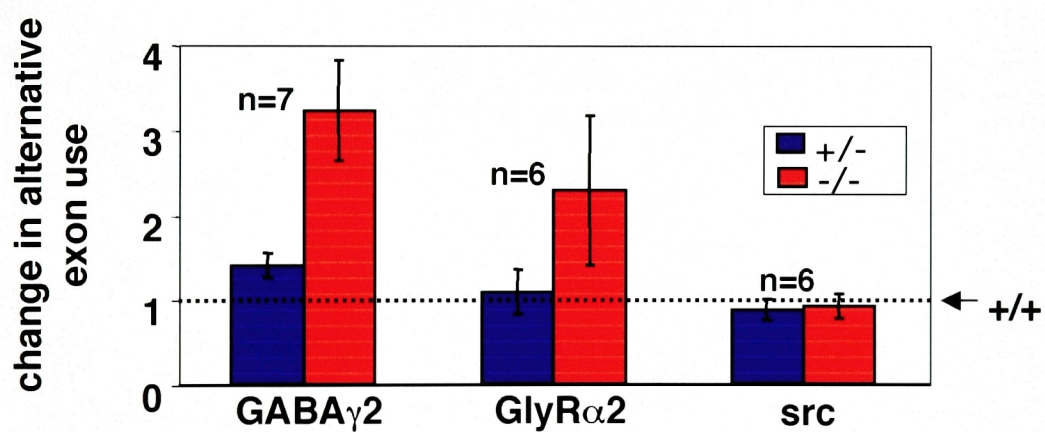
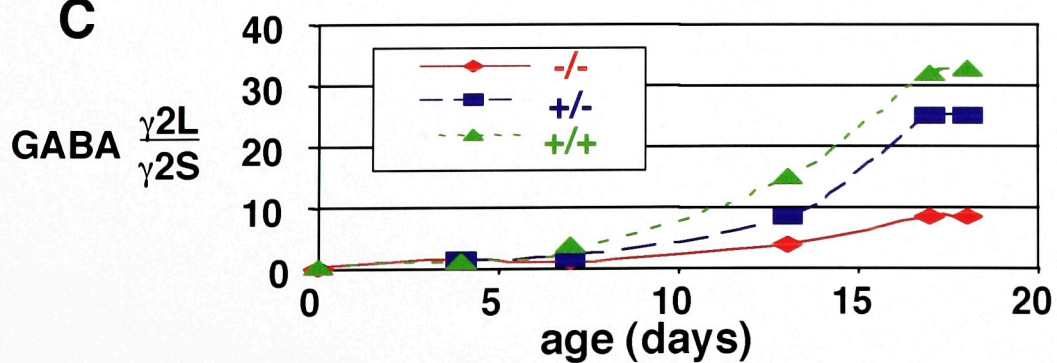
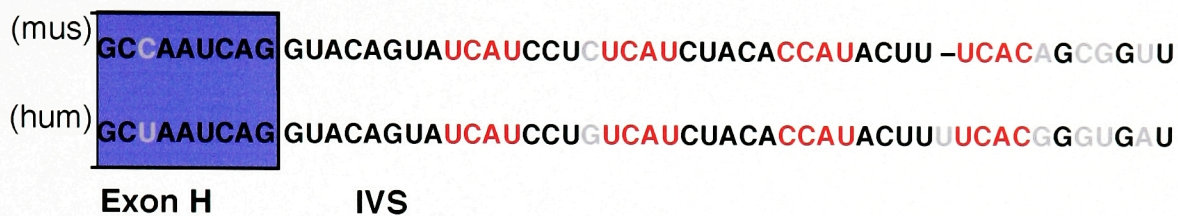
A**B****C**

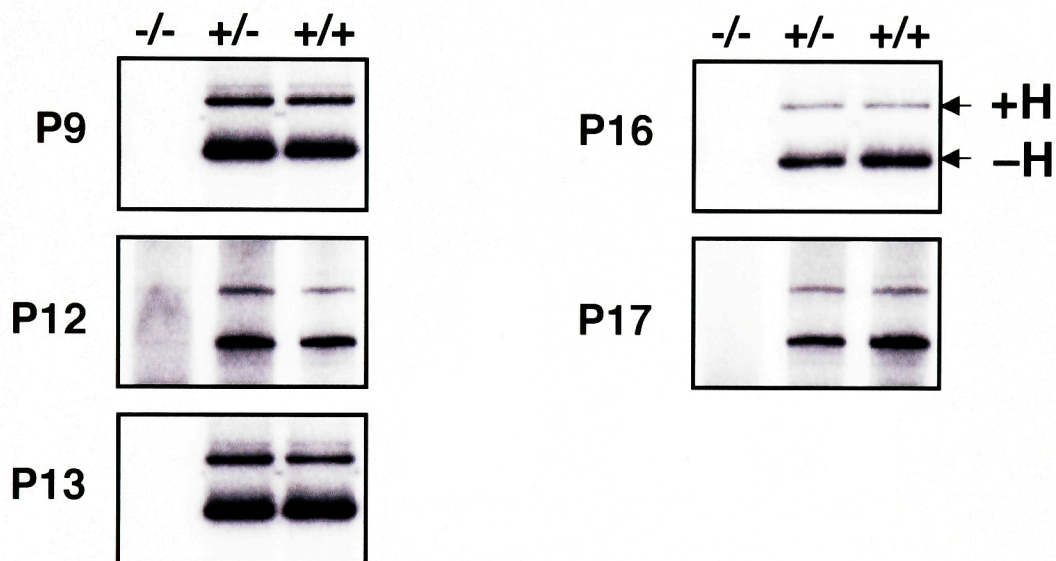
Figure 8: Nova-1 alternative splicing is altered in Nova-1 heterozygous mice.

- (A) **Alignment of Nova-1 genomic sequence downstream of exon H** from mouse (mus) and human (hum). Non-conserved nucleotides are depicted in gray, YCAY motifs in red.
- (B) **RT-PCR analysis of exon H utilization.** RNA isolated from Nova-1 null, heterozygous and wildtype littermates at the indicated ages was analyzed by RT-PCR with primers specific to exons surrounding exon H. Analysis of null mice confirms that these mice do not express Nova-1 mRNA, and confirms the specificity of the primers used.
- (C) **Quantitation of the data presented in (B).** Data is presented as the ratio of exon H included (+H) to excluded (–H) for each heterozygous and wildtype animal.
- (D) Data obtained in (C) from four litters aged P12 and over presented with data from Figure 7(B). Alternative exon usage in Nova-1 null and heterozygote mice is plotted using the alternative exon ratio of the wild-type mouse set to 1. Nova-1 data is presented as the fold increase in alternative exon inclusion in heterozygous compared to wildtype mice, all other data represents fold decrease. Bars represent the average of the normalized exon use ratio; error bars indicate the standard deviation.

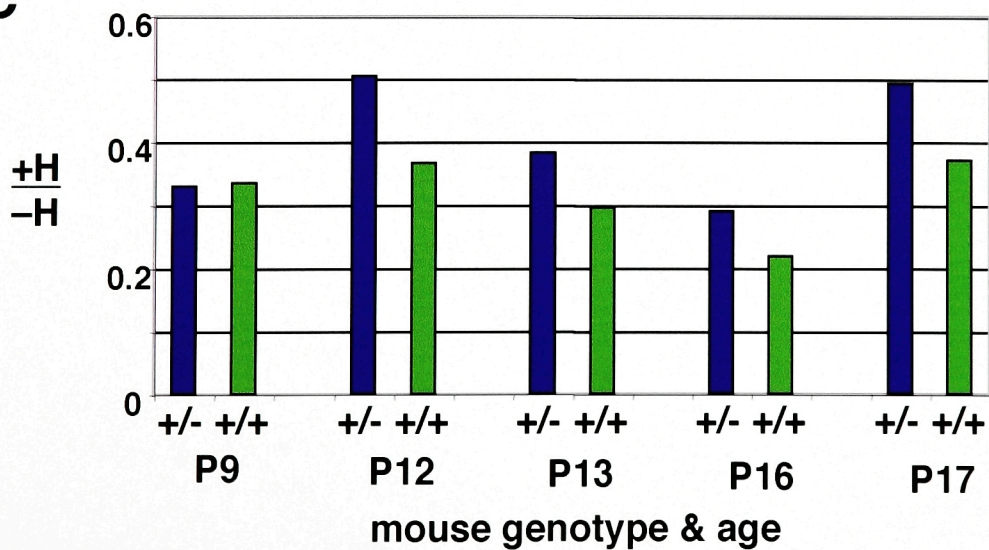
A



B



C



D

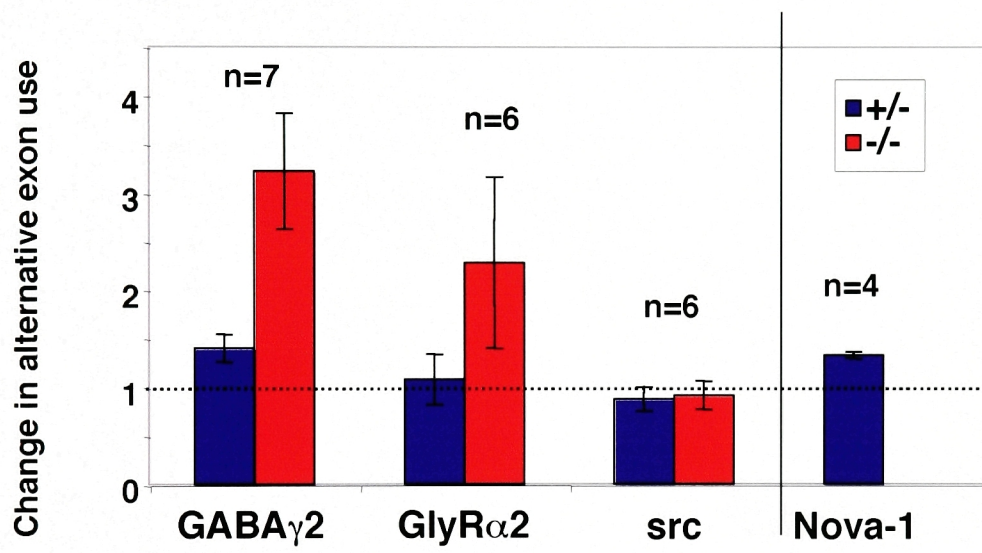
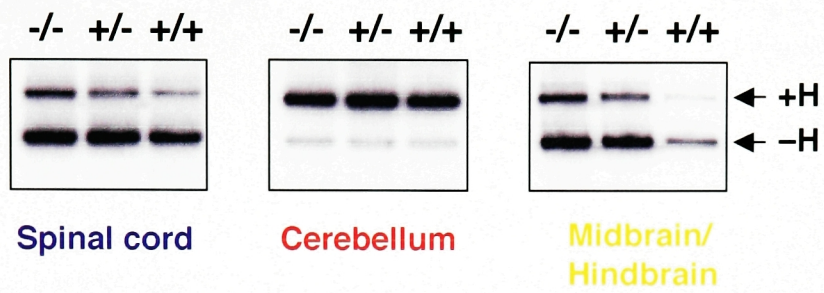
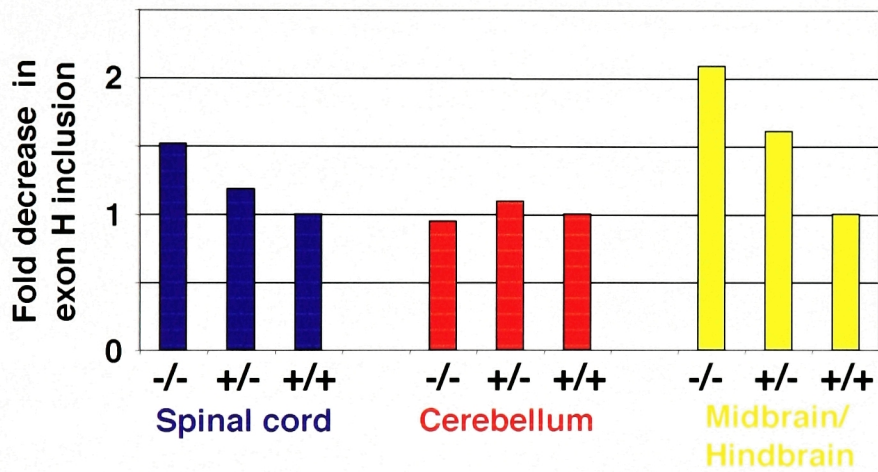
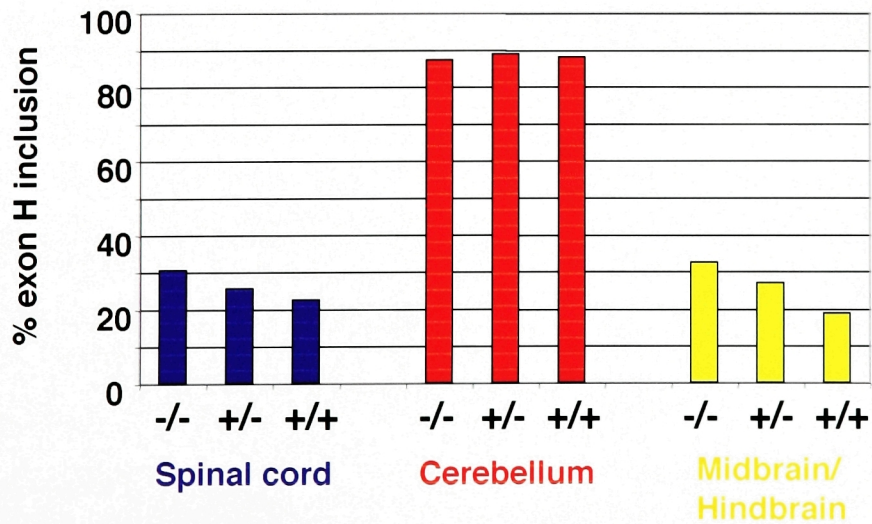


Figure 9: Nova-1 alternative splicing is altered in Nova-2 null mice.

- (A) **RT-PCR analysis of exon H utilization.** RNA isolated from the indicated tissues of P16 *Nova-2* null, heterozygous and wildtype littermates was analyzed by RT-PCR as in Figure 8(B).
- (B) **Quantitation of the data presented in (A).** Alternative exon H usage is plotted using the exon H included:excluded ratio of the wild-type mouse set to 1. Differences in exon H alternative splicing are seen in heterozygous and *Nova-2* null mice compared to wildtype littermates in RNA isolated from spinal cord and midbrain/hindbrain.
- (C) **Quantitation of the data presented in (A).** Exon H inclusion is plotted as a percentage of total spliced *Nova-1* mRNA. The level of exon H inclusion is similar in spinal cord and midbrain/hindbrain (23% and 19% in wildtype, respectively), but much greater in cerebellum (88% in wildtype).

A**B****C**

Chapter 4 – Characterization of Nova action on Defined pre-mRNAs

Introduction

GABA_AR γ 2 alternative splicing.

The GABA_A receptor is an important mediator of synaptic inhibition in the central nervous system. A number of studies have shown that alternative splicing of the γ 2 subunit of GABA_AR alters the pharmacological properties of GABA_A receptor channels (Krishek et al., 1994; Wafford et al., 1993). γ 2L and γ 2S forms of this subunit are generated by alternative splicing of a cassette exon, E9, inclusion of which adds an additional eight amino acids to an intracellular loop of the protein, and generates a site which can be phosphorylated by PKC (Krishek et al., 1994). Further evidence that this splice choice is functionally important comes from analysis of mice engineered to only express the short form of the GABA_AR γ 2 subunit, (γ 2S). γ 2L γ 2S^{-/-} mice display a higher level of anxiety than control mice as measured by both the number of entries onto the open arms of an elevated plus maze and the time spent on open arms (Homanics et al., 1999). In addition, these mice display increased sensitivity to benzodiazapines (Quinlan et al., 2000).

Splicing to GABA_AR γ 2 exon 9 is neuron specific, and the level of inclusion of this exon varies both regionally within the brain and in a developmentally regulated manner (Wang and Grabowski, 1996). Studies aimed at trying to understand the

mechanism behind regulation of this splicing decision have shown that nucleotides within the exon and the adjacent splice sites are essential for neuron-specific regulation as assessed by quantitation of the level of exon inclusion in a neuronal versus a non-neuronal cell line (Zhang et al., 1996). In addition, PTB has been shown to inhibit splicing to this exon, which has led to the hypothesis that splicing is regulated by a mechanism involving derepression of splicing at this site in neurons of the CNS (Ashiya and Grabowski, 1997; Zhang et al., 1999). This hypothesis implies a requirement for a tissue-specific factor which would alleviate the repression of splicing by PTB in the brain either through competition for RNA binding and/or through interactions with PTB which prevent its repressive action. The finding that Nova-1 null mice show a decrease in the level of inclusion of exon 9 suggests that Nova-1 likely plays a role in regulation of GABA_AR γ 2 alternative splicing, possibly through an interaction with PTB. In keeping with this hypothesis, Nova-1 has been shown to interact with a brain-enriched variant of PTB, brPTB in yeast-2-hybrid assays and this interaction antagonizes the ability of Nova-1 to enhance splicing to E3A of GlyR α 2 pre-mRNA (Polydorides et al., 2000). Alternatively, Nova-1 may act to enhance E9 splicing by sequence-specific binding to the RNA independent of any interaction with PTB.

Nova-1 exon H alternative splicing.

Nova-1 alternative splicing is by definition an additional example of neuron-specific splicing as this message is transcribed exclusively in neurons. Very little is known about either the function or regulation of alternative splicing of Nova-1 exon H. This exon contains a number of putative phosphorylation sites and preliminary evidence suggests that this region of the protein can be phosphorylated *in vivo* (G. Stefani and R.

Darnell, unpublished data). Analysis of Nova-1 heterozygous and Nova-2 null mice, as well as the existence of a putative Nova binding site in the adjacent intron provide tantalizing evidence that Nova proteins may autoregulate splicing of the Nova-1 pre-mRNA. If this is indeed the case, then Nova-1 is able to act as both a positive and negative regulator of alternative exon inclusion. SR proteins have been shown to act as both positive and negative regulators of alternative splicing depending on the position of the relevant binding site within the pre-mRNA (Kanopka et al., 1996). In addition, Tra-2 activates female-specific splicing of *dsx* RNA by enhancing splice site recognition through recruitment of general splicing factors (reviewed in (Lopez, 1998)) but represses splicing of the M1 intron within its own pre-mRNA (Mattox and Baker, 1991). Further evidence for a direct action of Nova on alternative splicing of both GABA_AR γ 2 and Nova-1 alternative splicing and information regarding the sequence requirements for such an action would help to elucidate the mechanism by which Nova-1 acts as a tissue specific regulator of alternative splicing *in vivo*.

Results

Nova regulates GABA_AR γ 2 alternative splicing in cell lines

To assess the sequence requirements necessary for Nova-1 action on GABA_AR γ 2 alternative splicing, a minigene construct was generated which contains the entire mouse genomic region surrounding the alternatively spliced exon, E9, and extends into the adjacent exons, E8 and E10. This was cloned into the mammalian expression construct pcDNA-3, under transcriptional control of the CMV promoter (see schematic, Figure 10 A). This construct was transiently transfected into N2A or 293T cells. Total RNA was

isolated 40 hours post-transfection and used for RT-PCR analysis of spliced products using PCR primers to E8 and E10. Figure 10 B,C show initial experiments performed in N2A cells which were used to optimize PCR conditions. Cycle number and input cDNA volumes demonstrated to be within the linear range of amplification in this experiment were used in subsequent studies (28 cycles, 3 μ l cDNA unless otherwise noted).

Cotransfection of a T7-tagged Nova-1 expression vector with the GABA_AR γ 2 minigene in mammalian cell culture recapitulated the splicing effects seen in Nova-1 null mice (Figure 11). Addition of Nova-1 plasmid caused a dose dependent increase in E9 inclusion, resulting in a roughly four-fold increase in the ratio of E9 included (γ 2L) to excluded (γ 2S) splice forms upon the addition of 2.0 μ g of Nova-1 plasmid in N2A cells (Figure 11A). Similar results were seen in a second cell line, 293T, where a five-fold increase in exon 9 utilization was observed (Figure 11B). Analysis of mock transfected cells showed that neither cell line expresses endogenous GABA_AR γ 2 mRNA (lanes M, Figure 11 A.I, B.I) meaning that any spliced products detected are derived from transfection of the minigene. Interestingly, the level of E9 inclusion without the addition of exogenous Nova-1 was substantially different between the two cell lines. The ratio of γ 2L/ γ 2S splice forms was approximately 10 fold higher in N2A cells than in 293Ts. This may reflect the fact that N2A cells express endogenous Nova proteins whereas 293T cells do not, although there are likely to be many other variables that also come into play.

Similar cotransfection experiments were also performed using a T7-tagged hnRNP-E1 mammalian expression vector. Addition of exogenous hnRNP-E1 caused a modest decrease in the utilization of GABA_AR γ 2 exon 9 (Figure 11 A, B). Comparable expression levels of the two proteins was confirmed by Western blot (Figure 11 A.II,

B.II). hnRNP-E1, like Nova, contains three KH-type RNA binding domains, the second two of which are separated by a spacer region. This result demonstrates that the action of Nova-1 in this assay cannot be replicated by another KH-domain protein, which is consistent with the hypothesis that sequence-specific interactions between Nova-1 and GABA_AR γ 2 RNA are necessary. As an additional control, fixed cells were stained with anti-T7-tag monoclonal antibody and visualized with Cy3-conjugated secondary antibody to confirm that both T7-tagged proteins are able to localize to the nucleus, and thus the result is not due simply to the fact that the exogenous hnRNP-E1 was spatially constrained (on a cellular level) from effecting splicing (Figure 11 C,D).

Defining the region of GABA_AR γ 2 RNA necessary for regulation by Nova-1

Given that Nova-1 regulates alternative splicing of GlyR α 2 pre-mRNA via binding to intronic YCAY repeats, the available genomic sequence surrounding GABA_AR γ 2 exon 9 from three organisms (human, mouse and rat) was examined in order to identify putative conserved Nova-1 binding sites. Within approximately 170 nt upstream and 100 nt downstream of exon 9 (Figure 12, yellow rectangle), only 2 YCAY motifs conserved between human and mouse were found (Figure 12, red underline). Within this region, two additional motifs were found in each species which were not conserved (Figure 12, purple underline).

The conserved YCAY motifs, plus a nearby (within 10nt) CA dinucleotide were mutated as indicated in Figure 12 in the context of the GABA_AR γ 2 minigene to test whether Nova-1 action to increase splicing to E9 requires these sequence elements. Figure 13A shows that the mutation within intron 8 has no effect on splicing of E9 with

or without the addition of exogenous Nova-1 in N2A cells. The mutation in intron 9, however, had a dramatic effect on splicing to E9, resulting in a decrease in the ratio of $\gamma 2L/\gamma 2S$ of approximately 15-fold in N2A cells (Figure 13A). Similar results were seen in 293T cells (Figure 13B). Again, mutation of intron 8 had no effect, but mutation of intron 9 reduced E9 inclusion approximately 5-fold, down to barely detectable levels. Despite this, addition of Nova-1 still leads to an increase in E9 inclusion in both cell lines (Figure 13A,B) of similar magnitude to the wild-type minigene. Thus this mutation likely disrupts an intronic splicing enhancer necessary for efficient utilization of E9, but is not at the site of Nova-1 action.

Since the introns surrounding GABA_AR $\gamma 2$ exon 9 are quite large (approximately 2.3kb and 1.4kb), identification of a suspected intronic Nova-1 binding site is difficult. To overcome this difficulty, exonuclease was used to generate a number of deletion minigene constructs with introns of varying lengths. These were then analyzed in the same manner as the full-length minigene in the hopes of identifying an even smaller region of sequence necessary for Nova-1's action on E9 splicing, and which would be more amenable to biochemical studies of Nova-1 binding *in vitro*, and to a more thorough mutagenesis approach. Figure 14A illustrates the approach used to generate such a library of constructs: the minigene was cut with restriction enzymes within either of the two introns. The introns were then shortened by bi-directional digestion using exonuclease III for varying lengths of time. A fraction of the products were resolved on agarose gels to confirm reduction in plasmid size (Figure 14B). The remaining products were recircularized and transformed into *E. coli*. Individual clones were screened by restriction digest to determine approximate insert size (Figure 14C), then sequenced.

A selection of the minigenes generated in this manner were then cotransfected into N2A cells with or without the addition of Nova-1 expression plasmid. Figure 14D shows the results of a number of these experiments. Although a large amount of variation was seen in both the level of E9 inclusion in the absence of Nova-1 addition, and the degree to which Nova-1 alters splicing, there did not appear to be a distinct cut-off point between constructs regulated by Nova, and those that were not. The only minigenes that displayed an increase in E9 inclusion of less than 2-fold upon Nova addition were those which did not splice correctly to E9, presumably because the branch-point adenosine in intron 8 was deleted in these cases (constructs D3 and D11). However, there was a drop (to < 3-fold) in Nova's effect upon deletion of sequences within 200bp upstream of E10 (Figure 14D, minigenes A1-30, A1-44, A1-43, A2-38). This region is rich in YCAY repeats as will be discussed below. Further attention was drawn to this region by the results of transfection of minigenes A1-20 and particularly A1-21 which show an increase in the magnitude of the Nova-1 effect.

A drop in Nova-1 effect was also seen upon deletion of sequences within 100bp upstream of E9 (compare constructs D7, D17, D12 to D4, Figure 14D). This region does not contain a single YCAY repeat in mouse (see Figure 12), but may contain a cryptic Nova binding site or another sequence necessary for the effect, but not directly bound by Nova.

To facilitate further investigation, especially in the region around E10, a truncated minigene was made by combining of constructs D12 and 2A2 (Figure 14D) which is depicted in Figure 15A (D12/2A2). In addition a minigene was constructed using the first 3 exons of the human β -globin gene and the entire intervening sequences ("glo", Figure

15A) with the aim of generating an unrelated construct not regulated by Nova-1 which could be used as a negative control and for the construction of chimeric minigenes. The central exon of this construct was uniformly included in the spliced product upon transfection into N2A or 293T cells (data not shown). It has been well established that the size of an exon can directly influence the efficiency of splicing to that exon (Berget, 1995; Dominski and Kole, 1991). In order to make a minigene more comparable to GABA_AR γ 2, the central region of β -globin exon 2 was deleted (“glo2A”, Figure 15A) to give a central exon size of 28nt.

Figure 15B shows that the truncated GABA_AR γ 2 minigene D12/2A2 behaved in a manner analogous to the full-length construct, showing increased E9 utilization upon the addition of Nova-1 and a lower level of basal E9 inclusion in 293T cells compared to N2A cells. Quantitation of the results revealed that addition of 2.0 μ g of pNova-1 resulted in an almost 6-fold increase in E9 inclusion in N2A cells, and a roughly 14-fold increase in 293T cells (Figure 15C), even more robust effects that were seen with the full-length construct. Minigene glo2A showed a similar level of alternative exon (E2A) inclusion to the GABA_AR γ 2 construct in N2A cells (Figure 15B,C), but was not regulated upon the addition of exogenous Nova-1 in either cell line.

To address which regions of GABA_AR γ 2 sequence remaining in the truncated minigene are necessary for the regulation of alternative splicing by Nova-1, chimeric constructs were generated incorporating regions of GABA_AR γ 2 and β -globin sequence (depicted in Figure 16A) and tested in cell transfection assays. The results displayed in Figure 16A and quantitated in Figure 16B show that the central 121nt region surrounding and including GABA_AR γ 2 E9 is not sufficient to mediate the level of Nova-dependent

splicing as seen with the GABA_AR γ 2 minigene truncate, either in the presence or absence of the upstream exon and intronic region (compare construct a. to b. and d., Figure 16B). Surprisingly, inclusion of an additional 43nt upstream of E9 (Figure 16, construct c.) did not restore the effect as may have been expected from comparison of constructs D4 and D12 in Figure 14C, making it unlikely that a cryptic Nova-1 binding site exists in this location.

Consistent with the observations made from analysis of the GABA_AR γ 2 deletion constructs (Figure 14D), the sequence element upstream and extending into the 5' end of the constitutively spliced exon E10 is necessary for the robust Nova-dependent effects on alternative splicing (compare constructs d. and e., Figure 16B). In order to further define the sequence sufficient for Nova-dependent regulation of GABA_AR γ 2 alternative splicing, additional chimeric constructs were generated and are illustrated in Figure 17A. Co-transfection of these constructs with Nova-1 demonstrated that the region upstream of and including exon 10 that was shown to be necessary for Nova-dependent splicing (Figure 16 construct e.) is also sufficient to mediate the effect (Figure 17, construct f.). Furthermore, the exon E10 included in the GABA_AR γ 2 minigenes is neither necessary nor sufficient for Nova-dependent splicing (Figure 17, construct g. and h.). These results were consistent in both cell lines tested: N2A (Figure 17B) and 293T cells (Figure 17C). Thus the 211nt intronic element situated immediately upstream of exon E10 in GABA_AR γ 2 pre-mRNA is sufficient to mediate Nova-dependent regulation of alternative splicing of a heterologous exon (Figure 17, construct g.).

This intronic element contains seven YCAY repeats (Figure 18, red underlines) and is highly conserved between mouse and human sequences; where there are

differences between the two species in the regions of these repeats, the variation is in which pyrimidine residue is used at either end of the motif, thus maintaining the consensus YCAY, or in one instance a repeat is shifted by two positions in human compared to mouse (purple underlines, Figure 18). Mutations were made in the first three (Figure 19A, denoted by green star), last four (red star), or all seven (green + red stars), mutating YCAY repeats to YAAY in the context of the chimeric minigene construct g (Figure 17A). Figure 19B-I shows that in N2A cells, mutation of the first three or last four YCAY motifs reduces the effect of Nova addition by approximately 2.4-fold, whilst mutation of all seven repeats abrogates it to a level similar to that seen for the β -globin negative control (-c, Figure 19). The effects of these mutation were recapitulated in 293T cells (Figure 19B-II). The results from Figure 19B are graphed again in Figure 19C as the change in ratio of long to short spliced forms (L/S) upon the addition of Nova-1, thus the basal level of E9 inclusion without the addition of exogenous Nova is set to 1. This form of presentation makes the effect of the mutations even more apparent. The results show that intact YCAY repeats are necessary for Nova-dependent regulation of alternative splicing in the context of the chimeric minigene.

Identical mutations were made in the full-length GABA_AR γ 2 minigene (Figure 20A) to determine whether the presence of these YCAY repeats is necessary for Nova-dependent regulation in this context. Co-transfection of these plasmids with pNova-1 into 293T cells yielded very similar results to those seen for the chimeric construct (Figure 20B, quantitation is shown in Figure 20C). Again mutation of the first three or last four YCAY repeats had a dramatic effect on Nova-dependent regulation, but did not completely abolish it. Mutation of all seven YCAY repeats resulted in a paradoxical

decrease in the inclusion of E9 upon the addition of Nova-1. These results indicate that these repeats are indeed vital for the regulation of GABA_AR γ 2 E9 alternative splicing by Nova-1.

In vitro analysis of Nova binding to GABA_AR γ 2 RNA

An RNA corresponding to the region of the GABA_AR γ 2 pre-mRNA harboring all seven YCAY motifs shown to be necessary for Nova regulation of alternative splicing was synthesized *in vitro* (RNA 9C, arrows Figure 18). Nitrocellulose filter binding assays were performed to determine whether purified Nova-1 fusion protein (NFP, Figure 21A. Fraction 2 was used) is capable of binding to this sequence with high affinity *in vitro*. These results show that full-length Nova-1 fusion protein is capable of binding to this sequence with affinity similar to that of binding to an RNA purified by RNA selection (SB2) under similar conditions to those used for the selection (1xSBB) (Buckanovich and Darnell, 1997) (Figure 21B-I). This binding is severely abrogated by the mutation of the YCAY repeats to YAAY (9Cmut, Figure 21), to a level similar to the binding of NFP to an irrelevant β -globin (glo) RNA of a similar length. Addition of heparin to the reaction both increases the binding affinity of the target RNAs to Nova-1 and decreases non-specific binding to the mutant RNA and β -globin RNAs (Figure 21B-II). This binding is severely though not entirely disrupted by high salt conditions (500mM KOAc) (Figure 21B-III).

To determine the minimal RNA sequence necessary for high affinity Nova-1 binding, the 5' and 3' boundaries of the RNA that bound to Nova-1 fusion protein were mapped (Figure 22). Full-length 9C RNA was labeled with ³²P at either the 5' or 3' end and subjected to mild alkaline hydrolysis. This RNA was then incubated with Nova-1

fusion protein and bound RNAs captured by filtration through nitrocellulose filters, eluted and analyzed by denaturing PAGE. The results suggest that a minimum of 4-5 YCAY repeats are necessary for binding of the RNA to Nova-1. Alternatively, the minimal sequence between the 2 boundaries may be all that is required for Nova-1 binding in this instance, although this has not been addressed directly.

To correlate the *in vitro* binding studies with the function of Nova-1 on the regulation of alternative splicing, additional minigenes were assayed in cell culture transfection assays which contained smaller GABA_AR γ 2 intronic elements of varying lengths (Figure 23A, lines are color coded to match the schematics shown in 23B). The results of cotransfection of these constructs with Nova-1 into 293T cells are shown in Figure 23B. Figure 23C displays the quantitation of these results and clearly indicates that constructs w and x containing only 114 and 68nt of sequence derived from GABA_AR γ 2, respectively are regulated to the same degree by Nova as the control, construct g. Reduction of this element to only 39, or even 24nt (constructs y and z, respectively, Figure 23) corresponding to the region bound by Nova-1 established by the boundary mapping experiment and a total of four YCAY motifs is sufficient to mediate alternative splicing regulation of a heterologous minigene construct by Nova-1, although the effect is slightly diminished. Taken together with the *in vitro* binding studies, these results are consistent with the hypothesis that Nova-1 binding is necessary and sufficient for regulation of alternative splicing in the context of a heterologous minigene.

Analysis of the regions of Nova-1 protein required for regulation of GABA_AR γ 2 alternative splicing.

Truncated versions of pNova-1 expression plasmid were generated to assess whether KH3 and the spacer region contribute to Nova-1 function in regulating GABA_AR γ 2 alternative splicing. Figure 24A illustrates the deletions that were made and the GABA_AR γ 2 minigene target, D12/2A2, that was used in the assay. Deletion of KH3 reduced the effect of Nova-1 on E9 splicing by approximately 2-fold in both N2A (Figure 24B) and 293T cells (Figure 24C), but does not completely abrogate the effect. This implies that KH3 likely contributes to the binding of Nova-1 to GABA_AR γ 2 RNA, but that other regions of the protein, presumably KH1 and KH2, also contribute to the binding. Deletion of the spacer region plus KH3 results in the production of a truncated protein with no effect on the alternative splicing of GABA_AR γ 2 E9 in N2A cells (Figure 24B). This shows that the spacer region is necessary for regulation of alternative splicing by Nova-1, possibly through interactions with other proteins. Attempts to express this protein in 293T cells resulted in very low or undetectable levels of exogenous protein expression, so these results were not included.

To address the role of exon H in GABA_AR γ 2 splicing and to ask which KH domains contribute to alternative splicing regulation, Nova-1 expression plasmids which lack exon H or harbor a single point mutation within one of the KH domains were used in cotransfection assays with a minigene target. Mutations were made in residues of the KH domains believed to be important for RNA binding from analysis of the Nova KH3/RNA co-crystal structure and comparisons to FMRP (Lewis et al., 2000). Mutations at these positions in KH1 and KH3 have been shown to greatly reduce binding of the isolated KH

domains to poly-rG *in vitro* (Buckanovich et al., 1996). Figure 25B shows that expression of the Nova-1 isoform lacking exon H had the same effect on GABA_AR γ 2 alternative splicing as Nova-1 including exon H. However, single point mutations in any of the KH domains resulted in a decrease in the effect of Nova-1 on E9 inclusion. Quantitation of these results (shown in Figure 25C) indicated that the magnitude of the decrease was roughly 2-fold. This is within the same range of effect as was seen upon truncation of the entire KH3 domain, and thus is consistent with the mutations resulting in complete abolition of RNA binding by that KH domain. Attempts to express a Nova-1 protein harboring all 3 KH mutations resulted in poor expression levels, so the results were not included in this study.

Autoregulation of Nova-1 alternative splicing

To investigate the role of Nova-1 on its own alternative splicing, two reporter plasmids were constructed harboring a Nova-1 minigene EGFP expression cassette (Figure 26A). The minigenes are composed of the first coding exon of Nova-1, exon E, which supplies an initiating methionine and a splice donor, and approximately 400bp of downstream intronic sequence. Exon E also contains the putative Nova-1 NLS. This fragment is then joined to a region of genomic DNA surrounding the alternatively spliced exon, exon H, which includes approximately 500bp of intronic sequence either side of the coding region. A 1bp insertion has been made in the 72bp exon such that inclusion of this exon will change the reading frame of downstream coding sequences. Finally the first 30bp of the terminal coding exon, exon I, along with 800bp of 5' intronic sequence has been fused to an EGFP reporter in either of two frames. The resulting plasmids are designed such that the EGFP portion of the coding sequence is in frame if exon H is included and

out of frame if exon H is spliced out in one instance, or vice versa for the other plasmid. These proteins will not contain any of the Nova-1 KH domains, and thus are not expected to contribute to RNA-binding function in these assays.

Figure 26B demonstrates that cotransfection of the minigene (designated pG-) which expresses functional EGFP when exon H is excluded from the spliced product with increasing amounts of pNova-1 results in increasing numbers of cells expressing detectable levels of EGFP protein. No change in EGFP expression was seen upon the cotransfection of exogenous Nova-1 with the parental EGFP expression plasmid (data not shown). Thus addition of exogenous Nova-1 causes a decrease in the level of exon H inclusion. This is consistent with the results shown in Chapter 3 (Figure 8s and 9) that Nova-1 heterozygous and Nova-2 null mice show an increase in the level of Nova-1 exon H inclusion compared to their wild-type littermates.

Mutations (YCA Y to YAC Y) were made in pG- to disrupt the putative Nova-1 binding site immediately downstream of exon H and are depicted in Figure 27A. In order to avoid issues of primer competition since primers to exonic regions within the minigene would also amplify the Nova-1 cDNA from the pNova-1 expression plasmid, spliced products were analyzed by primer extension using a ³²P-end labeled primer specific to EGFP, as well as by EGFP protein expression. Figure 27B shows that mutation of the YCA Y repeats downstream of exon H did not effect exon H splicing in the presence or absence of exogenous Nova-1. Quantitation of the results (Figure 27B) showed that the mutations decreased the basal level of exon H inclusion by about 2-fold, but that Nova-1 was still able to mediate a robust decrease in exon inclusion. The magnitude of the effect of exogenous Nova expression on exon H splicing is shown in Figure 27D by

presentation of the results after normalizing the basal level of exon H inclusion to 1, and indicates that the mutations had no effect on the ability of Nova-1 to regulate splicing of its own alternative exon. Thus if Nova acts to regulate alternative splicing of its own message by directly binding to the RNA, as has been shown to be the case for GlyR α 2 and GABA $_A$ R γ 2, binding is not likely to occur within this region.

An approach similar to that used to assess the sequences necessary for Nova-dependent regulation of GABA $_A$ R γ 2 alternative splicing was also undertaken to analyze Nova exon H splicing. Figure 28A shows the sequence of the entire region surrounding and including exon H that was present in the pG- minigene. Chimeric minigenes, depicted in Figure 28B, were constructed harboring exon H and various lengths (marked by arrows in Figure 28A) of the surrounding intronic sequences between human β -globin exons 1 and 3. Figure 28C clearly shows that chimeric minigenes containing exon H plus as little as 69 and 47nt of Nova-1 intronic sequence 5' and 3' respectively are still robustly regulated by cotransfection with Nova-1 in both N2A and 293T cells. This data was quantitated and is presented graphically in Figure 28D). Further chimeric minigenes (illustrated in Figure 29A) were constructed to assess whether this effect was mediated through intronic or exonic sequences. Figure 29B shows that exon H sequence alone is sufficient to mediate Nova-1's effect on alternative splicing to this exon in a heterologous context, and that the intronic regions have no effect on splicing of a heterologous central exon. Examination of the sequence revealed that exon H contains five YCAY motifs (in red, Figure 29A). Mutation of these as depicted in Figure 29A abrogates this Nova-dependent splicing to this exon (Figure 29). Thus Nova-1 likely binds to this exon in a manner consistent with previous studies (i.e. via YCAY motifs), however binding to this

region causes skipping of this alternative exon in contrast to the effects on GABA_AR γ 2 or GlyR α 2 alternative splicing.

Discussion

The results presented in this chapter provide further evidence that Nova-1 regulates the alternative splicing of at least two pre-mRNAs, those encoding GABA_AR γ 2 and Nova-1 itself, in a sequence specific manner. Minigenes were constructed which recapitulated the findings in Nova-1 null mice: Nova-1 enhances splicing to GABA_AR γ 2 E9 and represses splicing to Nova-1 exon H. Nova-1 has been shown to enhance inclusion E3A of GlyR α 2 by specific interaction with the pre-mRNA. By analogy, it was hypothesized that Nova-1 regulates alternative splicing of GABA_AR γ 2 by directly binding to the pre-mRNA in a sequence specific manner. Chimeric minigenes were then analyzed in order to define the sequences necessary for Nova-dependent regulation of alternative splicing of these messages.

Nova-dependent regulation of GABA_AR γ 2 E9 alternative splicing.

Consistent with a previous report comparing GABA_AR γ 2 E9 splicing in a neuronal versus non-neuronal cell line (Zhang et al., 1996), large regions of the introns surrounding E9 were dispensable for neuron-specific splicing of GABA_AR γ 2 minigenes (Figure 14D). This group also showed that particular nucleotides within and upstream of E9 were essential for neuron specific splicing in their system (Zhang et al., 1996) and that PTB plays a negative role in regulation of splicing to E9 via sequences located near the 3' splice site (Ashiya and Grabowski, 1997). It is clear that a number of sequence elements influence splicing of E9, not the least of which are the small exon size and relatively

weak 5' splice site. In contrast to the experiments discussed above, in the experiments shown here increased inclusion of E9 was mediated solely by the co-transfection of a single factor, Nova-1, into cultured cells. The goal here was to try to separate non-specific effects from specific effects of Nova-1 on splicing of GABA_AR γ 2 E9. The experiments shown in Figure 13 indicate that this is feasible. The mutation made in intron 9 caused a dramatic decrease in the basal level of E9 inclusion. Interestingly, this sequence resembles that of intronic enhancer elements found in a number of messages including fibronectin, fibroblast growth factor receptor 2 (FGF-R2) and src (Carstens et al., 1998; Huh and Hynes, 1994; Modafferi and Black, 1997). However, addition of Nova-1 was still able to mediate an increase in E9 incorporation of approximately the same magnitude as was achieved with the wild-type minigene (Figure 13).

The majority of splicing regulatory sequences that have been characterized lie within or close to the exon that they regulate (Smith and Valcarcel, 2000). Combined with the results discussed above, it was therefore somewhat surprising to find that the region surrounding and including E9 could be replaced with heterologous sequence containing a short exon not normally regulated by Nova, and retain Nova-dependent regulation (Figure 16). This regulation was then shown to require a 211nt stretch of sequence normally present approximately 1.3kb downstream of E9, adjacent to the next, ubiquitously included exon, E10.

Several lines of evidence were gathered consistent with a direct action of Nova-1 on this element via sequence specific RNA binding. First, this 211 nt segment contains 7 YCAY motifs (Figure 18) which are known to be the preferred RNA sequence for Nova KH domain binding (Buckanovich and Darnell, 1997; Jensen et al., 2000b; Lewis et al.,

2000). Second, mutation of these YCAY motifs to YAAY completely abrogated Nova's effect in the context of the chimeric minigene (Figure 19), and more importantly the full-length minigene (Figure 20), indicating that these motifs are necessary for Nova-dependent splicing regulation. Mutation of the first three, or more dramatically, the last four motifs reduces the Nova effect but does not abolish it. Third, Nova binds with high affinity to the wild-type but not the mutated sequence corresponding to this region (Figure 21). Boundary mapping experiments indicated that this binding abruptly drops if the sequence contains less than 4 UCAY repeats (Figure 22). Furthermore a 24nt element spanning the last four YCAY repeats, which appear to be the most important given the minigene mutagenesis and boundary mapping studies, is sufficient to mediate Nova-dependent E9 inclusion (Figure 23).

In addition, deletion of KH3 or mutation of any single KH domain diminishes the effect of Nova-1 on GABA_AR γ 2 E9 splicing to a similar degree, implying that each KH domain contributes to GABA_AR γ 2 binding to approximately the same degree (Figures 24 and 25). Taken together, these results suggest that alternative splicing regulation of GABA_AR γ 2 is mediated through the binding of multiple KH domains to multiple YCAY repeats within the RNA. It was recently demonstrated by X-ray crystallography that two tandem KH domains within a single protein can concurrently bind to a single nucleic acid molecule with only a short (5 nt) linker between the bound nucleotides (Braddock et al., 2002). This suggests that a single Nova molecule may bind up to three YCAY motifs within one RNA. In addition Nova proteins can multimerize as evidenced by yeast-2-hybrid and GST-pulldown assays (Polydorides et al., 2000) thus several Nova protein molecules may co-operatively bind an RNA target. Both of these mechanisms would

effectively increase the affinity of binding to an RNA target, and help to explain the requirement for several YCAY motifs in the GABA_AR_{γ2} pre-mRNA for regulation of alternative splicing by Nova.

Autoregulation of Nova-1 alternative splicing

The results presented here and in chapter 3 demonstrate that Nova proteins repress alternative splicing to Nova-1 exon H. A minigene was constructed in which splicing to the alternative exon creates a frame-shift in the resultant protein. By precise fusion to an EGFP reporter this allows the degree of splicing to be assessed by the level of fluorescence within these cells. Similar constructs have also been made incorporating EBFP or ERFP as the marker (not shown) so that the protein products of both splice forms may be visualized and distinguished in the same sample. This strategy could be extended for *in vivo* use, for example via incorporation into BAC transgenic animals. This would be very useful for assessing the regional distribution of alternatively spliced forms of a message within an organism, even at the single cell level. Similar constructs could also be used to extend the transfection studies reported here by analysis of splicing of mutated constructs within an *in vivo* setting.

This Nova-1 minigene was initially tested in tissue culture cells. As predicted, the expression of EGFP was consistent with repression of Nova-1 exon H inclusion upon the addition of exogenous Nova-1 protein (Figure 26). It was initially predicted that Nova-1 may autoregulate its own splicing by sequence specific interactions with a region rich in YCAY repeats immediately downstream of exon H (Buckanovich and Darnell, 1997). Figure 27 shows that this is not the case. Mutation of these repeats, whilst slightly altering the basal level of exon H inclusion, did not change the magnitude of the effect of

Nova-1 on its own splicing (figure 27). Moreover, presence of the intronic regions surrounding exon H did not result in Nova-dependent changes in splicing to a heterologous exon (Figure 29).

Nova is capable of binding to sequence corresponding to this intronic region *in vitro*, and this binding is abolished upon mutation of the YCAY repeats (Buckanovich and Darnell, 1997). It is not clear, however, whether Nova binds to this exact region *in vivo*, since the co-immunoprecipitation of Nova-1 RNA reported in (Buckanovich and Darnell, 1997) may have been the result of Nova binding to the nearby exonic sequence which is also rich in YCAYs. In fact, this exon is necessary and sufficient for Nova-dependent regulation of alternative splicing, as shown in Figure 29. This effect is completely abolished by mutation of the YCAY repeats within the exon.

These results indicate that the presence of YCAY repeats capable of binding to Nova-1 *in vitro* in the vicinity of an alternatively spliced exon is not sufficient for Nova-dependent regulation of splicing to that exon. Intervening or surrounding nucleotides within the YCAY-rich elements defined in GlyR α 2 and GABA_AR γ 2 introns and Nova-1 exon H may be necessary but not sufficient for Nova binding to the element in the context of a larger RNA molecule, perhaps through contributions to secondary structure, in ways that are not apparent *in vitro*. Alternatively, position relative to nearby intron/exon junctions, which was carefully conserved in the generation of chimeric GABA_AR γ 2 / β -globin minigenes, may be critical for either Nova binding or Nova effect on splicing. Indeed position of the Nova-binding element within the pre-mRNA may explain the opposite effect of Nova addition on splicing of GlyR α 2 and GABA_AR γ 2

exons compared with Nova-1 exon H. This model for the mechanism of Nova regulation of alternative splicing of these messages will be discussed in more detail in Chapter 7.

Figure 10: GABA_AR γ 2 minigene assay development.

- (A) Schematic representation of the GABA_AR γ 2 minigene containing the full mouse intronic regions surrounding and including exon 9 plus shortened exons 8 and 10 constructed for transient transfection assays. The primers used for RT-PCR analysis are depicted as arrows, the star denotes the ³²P-labeled primer.
- (B) RT-PCR was performed on RNA isolated from N2A cells transiently transfected with the minigene depicted in A. I. A PCR master mix was prepared and aliquotted into 9 identical 20 μ l reactions (template amount was equivalent to 3 μ l per 20 μ l reaction). 1 reaction tube was removed and stored on ice after every 2nd cycle starting at the completion of cycle 20. Products (4 μ l) were separated by denaturing PAGE. II. Quantitation of data from (I). PCR amplification is linear for both products throughout most of the range of cycle numbers tested (20-36). Note that for cycle 26, slightly less product was loaded on the gel due to a pipetting error, however the ratio of the 2 products remains the same. 28 PCR cycles (red arrow) were used for subsequent studies unless otherwise noted.
- (C) RT-PCR was performed on RNA isolated from N2A cells transiently transfected with the minigene depicted in A. I. A PCR master mix was prepared without template cDNA and aliquotted into 5 tubes containing from 1 to 5 μ l of template cDNA (made up to 5 μ l with H₂O; final reaction volume, 20 μ l). Reactions were cycled for 28 cycles and products (4 μ l) separated by denaturing PAGE. II. Quantitation of data from (I). PCR amplification is linear for both products over a range of cDNA input from 1 to 4 μ l. 3 μ l of template cDNA (red arrow) was used for subsequent studies unless otherwise noted.

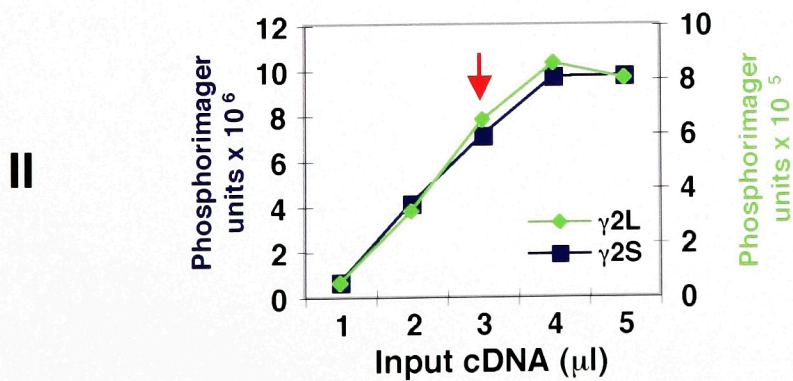
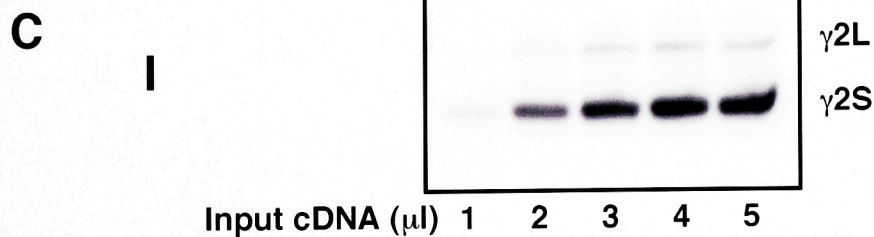
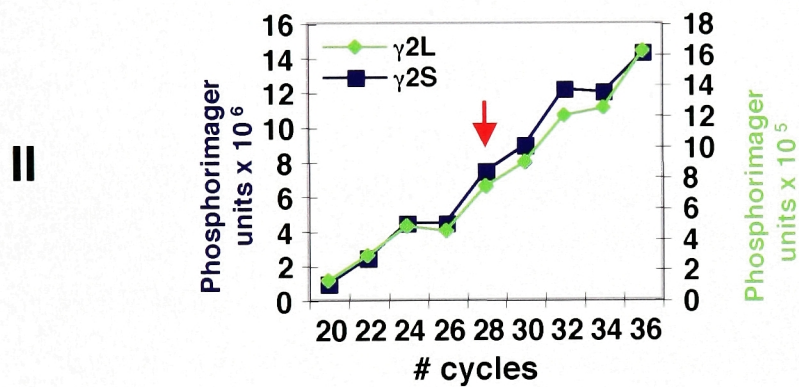
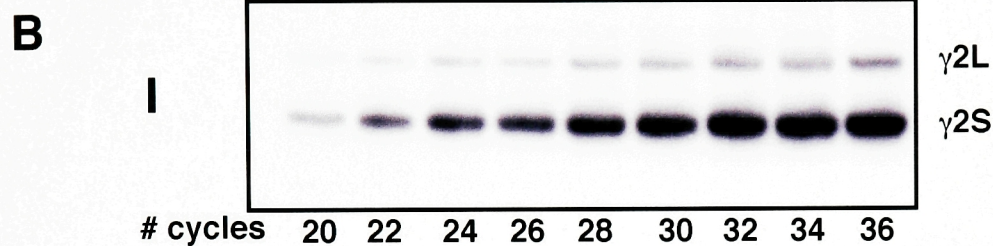
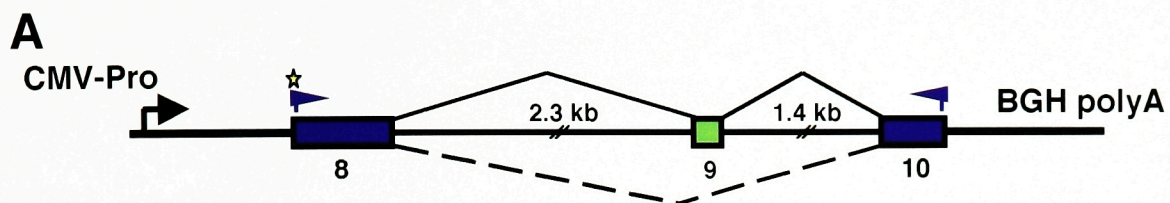
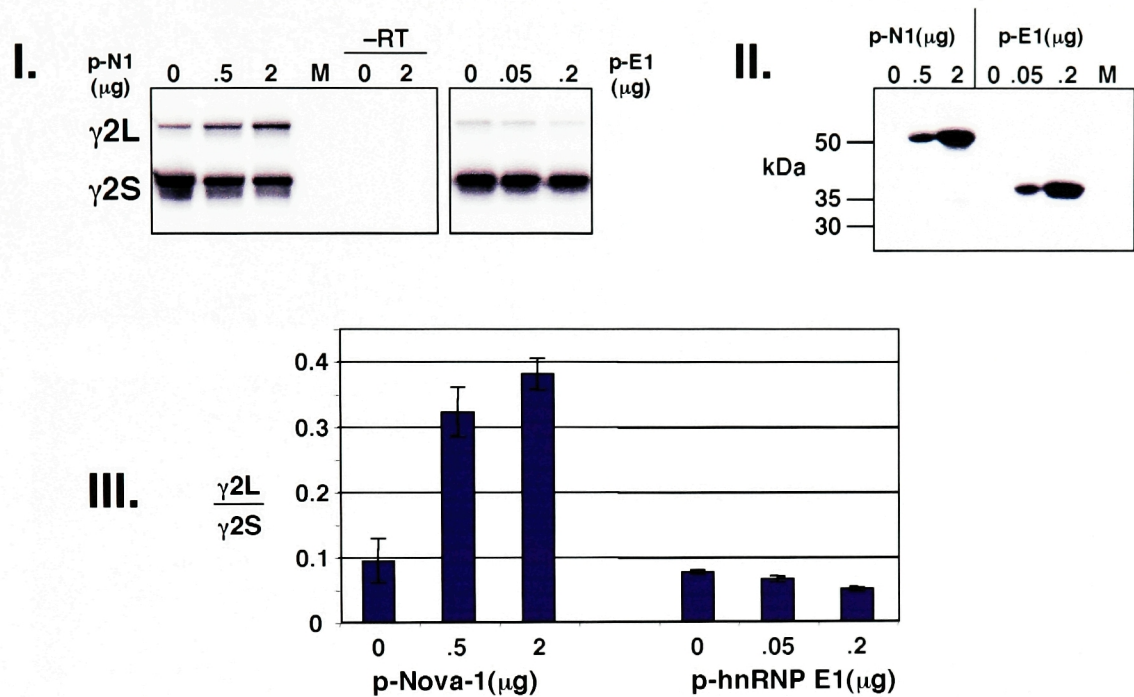


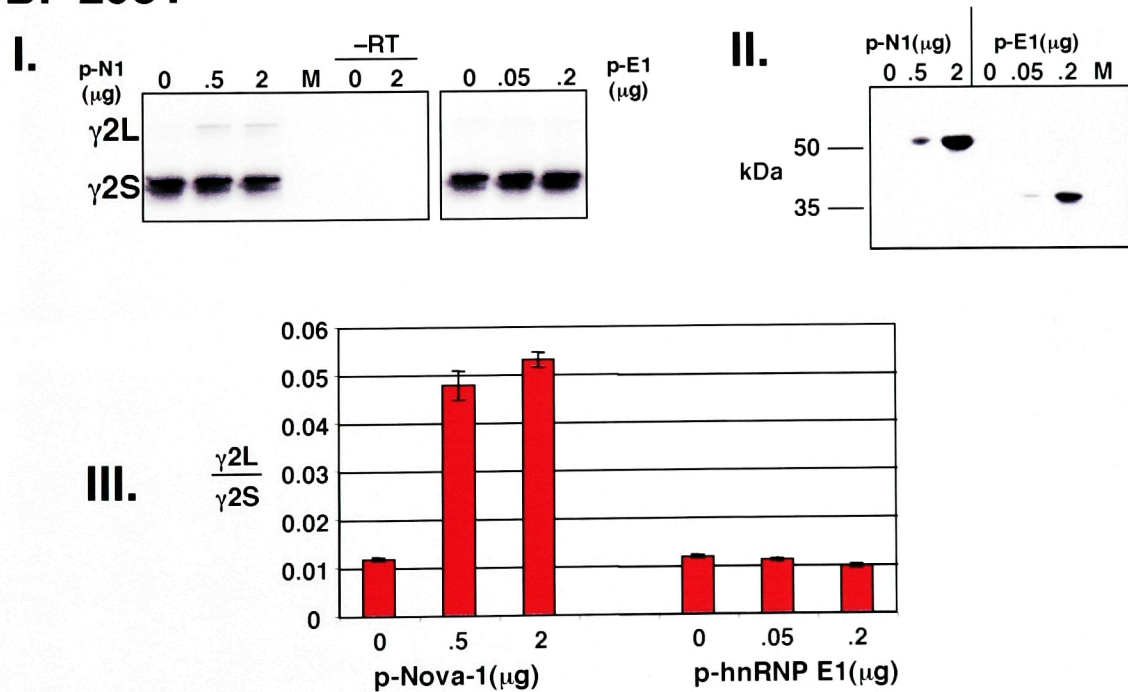
Figure 11: Nova-1, but not hnRNP-E1, enhances GABA_AR γ 2L splicing in heterologous cell lines.

- (A) **I.** RNA from N2A cells transiently transfected with the GABA_AR γ 2 minigene shown in Figure 10(A) and the indicated amounts of Nova-1 or hnRNP-E1 mammalian expression plasmid was analyzed by RT-PCR as in Figure 10. M= mock transfected cells. All PCR reactions were performed in duplicate using minus-RT reactions, representatives of which are shown (–RT). **II.** Western blot using anti-T7 tag antibody showing the titration of T7-tagged Nova-1 and hnRNP-1 protein levels after transfection. Plasmid concentrations were chosen which would lead to roughly equivalent levels of protein expression for the two T7-tagged proteins. **III.** Quantitation of the data presented in (A.I.) plus 2 additional independent transfections (n= 3). Data is presented as the ratio of γ 2L/ γ 2S products.
- (B) **I.** RNA from 293T cells transiently transfected with the GABA_AR γ 2 minigene and the indicated amounts of Nova-1 or hnRNP-E1 mammalian expression plasmid was analyzed by RT-PCR as in B. **II.** Western blot using anti-T7 tag antibody showing the titration of T7-tagged Nova-1 and hnRNP-1 protein levels after transfection. Plasmid concentrations were chosen which would lead to roughly equivalent levels of protein expression for the two T7-tagged proteins. **III.** Quantitation of the data presented in (B.I.) plus 2 additional independent transfections (n= 3). Data is presented as the average ratio of γ 2L/ γ 2S products; error bars represent standard deviation.
- (C) N2A cells were fixed post-transfection and stained with anti-T7-tag monoclonal antibody and visualized with Cy3-conjugated anti-mouse secondary antibody (red, first column). The cells were also stained for DAPI to visualize cell nuclei (blue, second column). The third column is a merged image of the first two. N1=cells transfected with pNova-1, E1= cells transfected with p-hnRNP-E1.
- (D) 293T cells fixed post-transfection and stained as per (C).

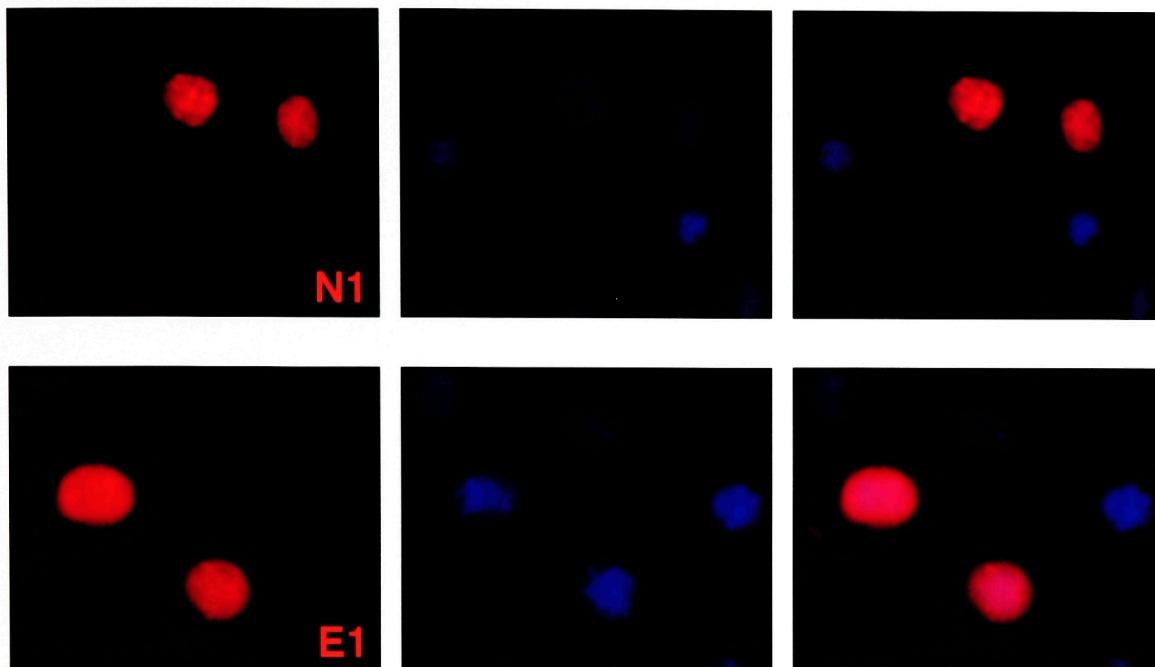
A. N2A



B. 293T



C. N2A



D. 293T

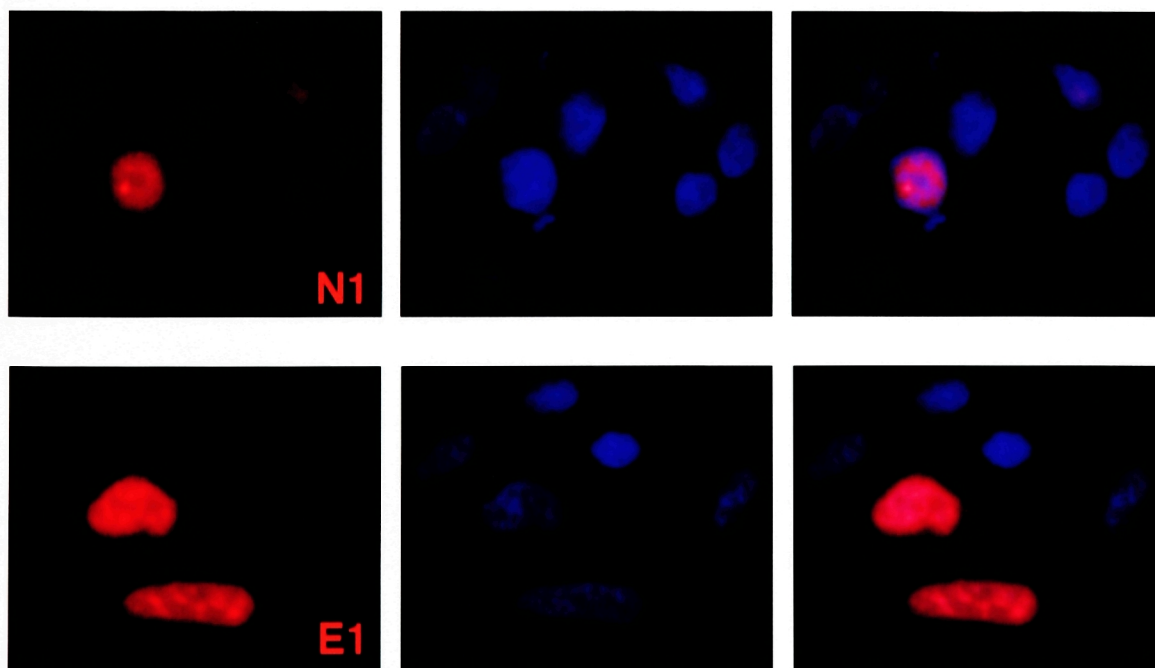


Figure 12: Sequence comparison of intronic regions surrounding GABA_AR γ 2 exon 9

Genomic sequence surrounding and encompassing GABA_AR γ 2 exon 9 (yellow box) from human (hum), mouse (mus) and rat was aligned by ClustalW alignment (MacVector). Conserved YCAY motifs are underlined in red, non-conserved YCAY motifs in purple. Mut8 and Mut9 represent mutations made in the mouse minigene within introns 8 and 9 respectively (see Figure 13).

mus AGA G T C C A ¹⁰ CCTTCTGTGTTTA ²⁰ G G C TTTCAG ³⁰ **AC**

hum AGA A C T C T CCTTCTGTGTTTA T A A TTTCAG

AG A C C C T T C T G T G T T T A T T T C A G

CTGC ⁴⁰ ⁵⁰ ⁶⁰

mus TTCAATTT T ACTTACTGTGTTTTCAAAATGT

hum TTCAATTT C ACTTACTGTGTTTTCAAAATGT

T T C A T T T T A C T T A C T G T G T T T T C A A A A T G T

mut 8

⁷⁰ ⁸⁰ ⁹⁰

mus ATTTTAAATTT G T C T T ATT TTGTTTCTCTTT

rat ATTTTAAATTT - - - ATC TTGTTCTCTTT

hum ATTTTAAATTT - - - AT C TTGT C TCTCT C

A T T T T T T A A T T T T A T T T G T T T C T C T T T

¹⁰⁰ ¹¹⁰ ¹²⁰

mus TCTCTCTTTT - TTTTCCCTTT T TCCTA TTTT T

rat TCTCTCT C TTTT C C TTTTCCCTTCT T CCTT A TTT A

hum T T T T T T TTT C A - TTTT T T TTCTCCTTTT -

T C T C T C T T T T T T T T C C T T C T C C T T T T T

¹³⁰ ¹⁴⁰ ¹⁵⁰

mus G T T T T ATTAAAAAATATGCAATTCTCTTT

rat A A A A A A A A A A A A C T A C G C A A T T C T C T T T

hum - - - ATTAAAAAACAATA T G C A A T T C T C T T T

A T T A A A A A A A T A T G C A A T T C T C T T T

Exon 9

¹⁶⁰ ¹⁷⁰ ¹⁸⁰

mus TCTGTCTACAAATCCAAAG CTTCTTCGGAT

rat TCTGTCTACAAATCCAAAG CTTCTTCGGAT

hum TCTGTCTACAAAC CCAAAG CTTCTTCGGAT

T C T G T C T A C A A A T C C A A A G C T T C T T C G G A T

¹⁹⁰ ²⁰⁰ ²¹⁰

mus GTTTTCTTCAAG GTATACTGTTTTTGGAA

rat GTTTTCTTCAAG GTATACTGTTTTTGGAA

hum GTTTTCTTCAAG GTATAA TGTTTTTGGAA

G T T T T C C T T C A A G G T A T A C T G T T T T T G G A A

CTGT AC ²²⁰ ²³⁰ ²⁴⁰

mus TGGAAATTTCACTGCATGC G ACTGCT G AAT

rat T G G G C A T T T C A C ACTGCT A AAT

hum T G G A A A T T - C A C T G C A T G C A ACTGCT A AAT

T G G A A A T T T C A C T G C A T G C A C T G C T A A T

mut 9

²⁵⁰ ²⁶⁰ ²⁷⁰

mus T - AACTATTAATGCTT C C C A T G G T G C TTT T A

rat T - AACTATTAATGCTT A C A T G G T G T T TTT A T

hum T A A C T A T T A A T G C T T C A T G G T G T T T T T

T A A C T A T T A A T G C T T C A T G G T G T T T T T

²⁸⁰ ²⁹⁰ ³⁰⁰

mus TT G T TTTT A ATGAGT G A A T ATTTAA A T A TC

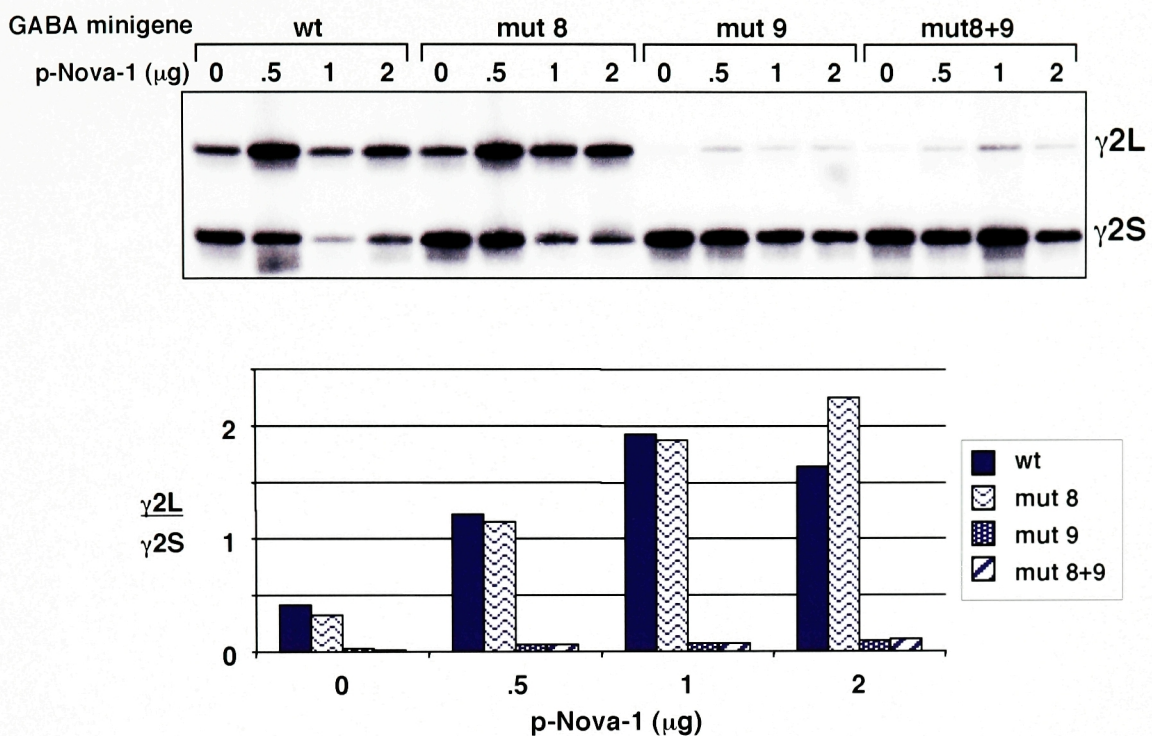
rat TT T G TTTT T ATGAGT A G A C ATTTAA G C A T TC

T T T T T T T A T G A G T A A A T T T A A A T C

Figure 13: Mutations within the surrounding introns do not interfere with Nova's ability to regulate GABA_AR γ 2 exon 9 alternative splicing.

The mutations depicted in Figure 12 were made in the GABA_AR γ 2 minigene, either individually or in combination and the resulting minigenes cotransfected with increasing amounts of pNova-1. Spliced products were measured by RT-PCR and phosphorimage analysis as in Figure 10, the data is presented as γ 2L/ γ 2S exon use ratio. The titration of transfected Nova-1 expression was monitored by western blot (not shown). (A) N2A, (B) 293T.

A. N2A



B. 293T

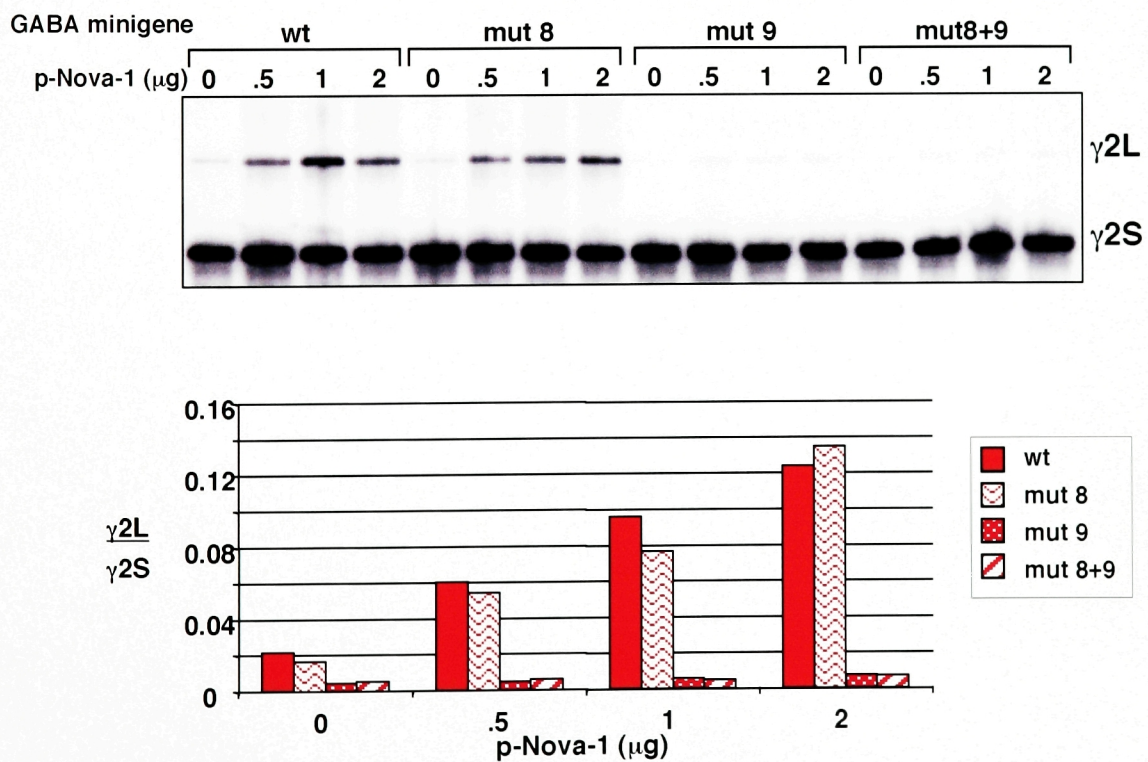


Figure 14: Generation of shortened minigenes by Exonuclease III digestion.

(A) Schematic representation of the approach taken to produce GABA_AR γ 2 minigene deletion constructs. To shorten intron 8; the full length GABA_AR γ 2 minigene was digested with unique restriction enzymes (Pac1 + Xcm1, followed by blunt-ending with pfu polymerase to remove 3' overhangs), then incubated with Exonuclease III for varying lengths of time (aliquots were removed at 10 second intervals).

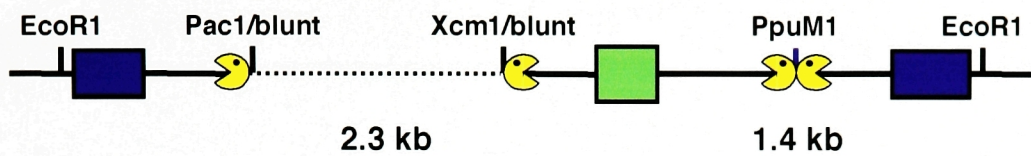
To shorten intron 9; a clone with blunted Pac1/Xcm1 sites religated was digested with PpuM1 (now unique), followed by incubation with Exonuclease III (PpuM1 generates 5' overhangs, therefore blunting was not required in this case)

(B) A fraction of the ExoIII digested plasmid from each time point was run on a 1% agarose gel. The remaining reaction was re-circularized with T4 DNA ligase.

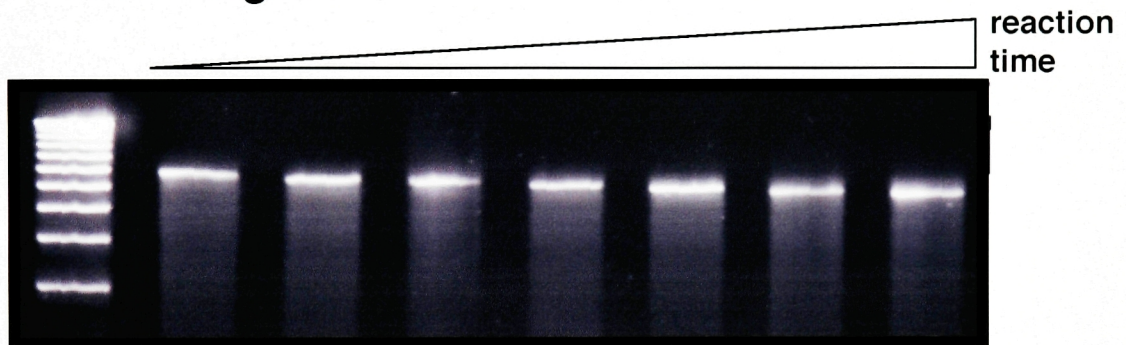
(C) Clones were digested with EcoR1 to estimate insert size, then sequenced.

(D) Survey of ExoIII generated minigene constructs. 0.25 μ g of minigene was cotransfected with 1.0 μ g of pNova-1 or empty vector into N2A cells grown in 6-well plates. Cells were harvested 40 hours post-transfection and analyzed by RT-PCR as in Figure 10. The average ratio of γ 2L/ γ 2S products over the number of trials (n) is shown on the left \pm standard variation (or deviation from the mean for n=2). The average increase in this ratio upon cotransfection of Nova-1 is listed in the next column. Minigenes are shown as schematic representations (not to scale), with the length of intronic sequence (in bp) remaining either side of the deletion point noted above.

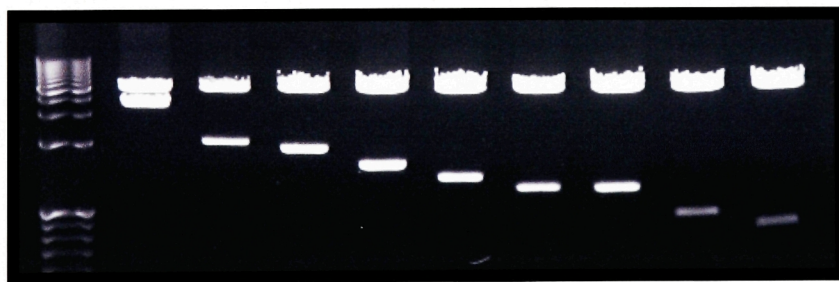
A



B Exo III digest

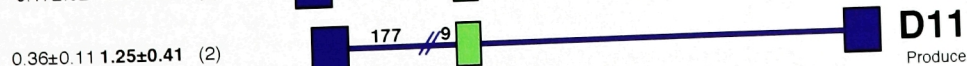
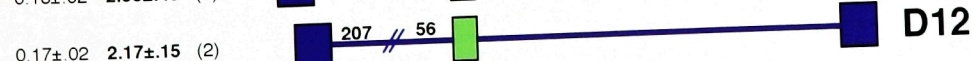
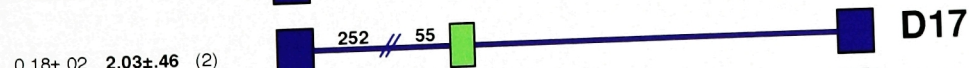
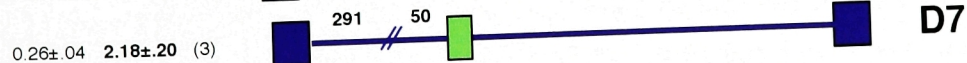
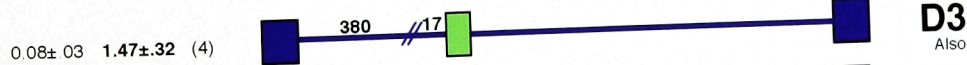
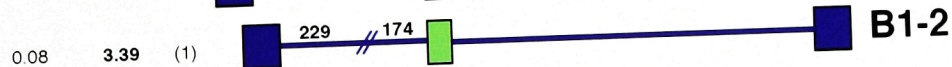
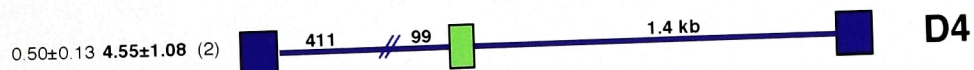
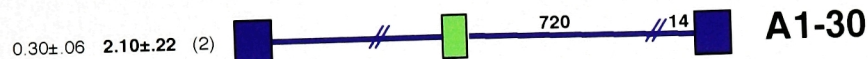
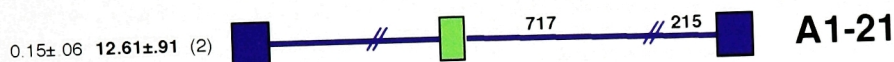
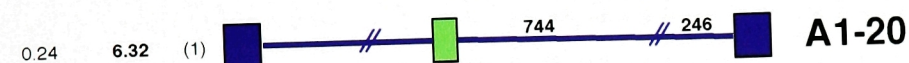
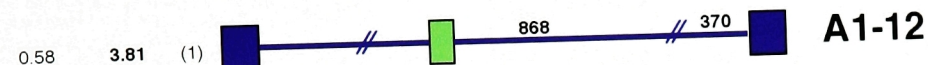
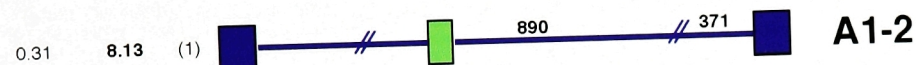
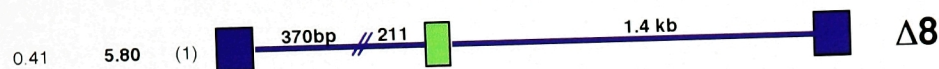


C *Eco*RI digest



D

Ave L/S \pm st.dev
Ave \uparrow \pm st.dev



Also produces aberrant product
(2nd 3'ss)

Produces aberrant product (retained
intron)

Figure 15: Alternative splicing of a severely truncated GABA_AR γ 2 minigene is still regulated by Nova-1: narrowing down the sequence elements required.

- (A) Schematic representation of minigene constructs generated for this study. A truncated GABA_AR γ 2 minigene was generated by fusing constructs D12 (5' of E9) and 2A2 (3' and including E9). A human B-globin minigene was constructed using the first 3 exons of the human gene and the entire intervening sequences. Subsequently the central region of B-globin exon 2 was deleted producing a version which is alternatively spliced in cell culture transfection assays. The sizes of the exonic and intronic sequences contained in the minigenes are noted above (in bp). B-globin exon 2A retains 10pb 5' and 18 bp 3' of the deletion site.
- (B) Minigenes were cotransfected with increasing amounts of pNova-1 and spliced products measured by RT-PCR and phosphorimage analysis as in Figure 10. The titration of transfected Nova-1 expression was monitored by western blot using α -T7tag antibody (lower panel).
- (C) Quantitation of the data presented in (B) plus additional experiments (n=4 for D12/2A2, n=5 for glo2A). The data is presented as γ 2L/ γ 2S exon use ratio.

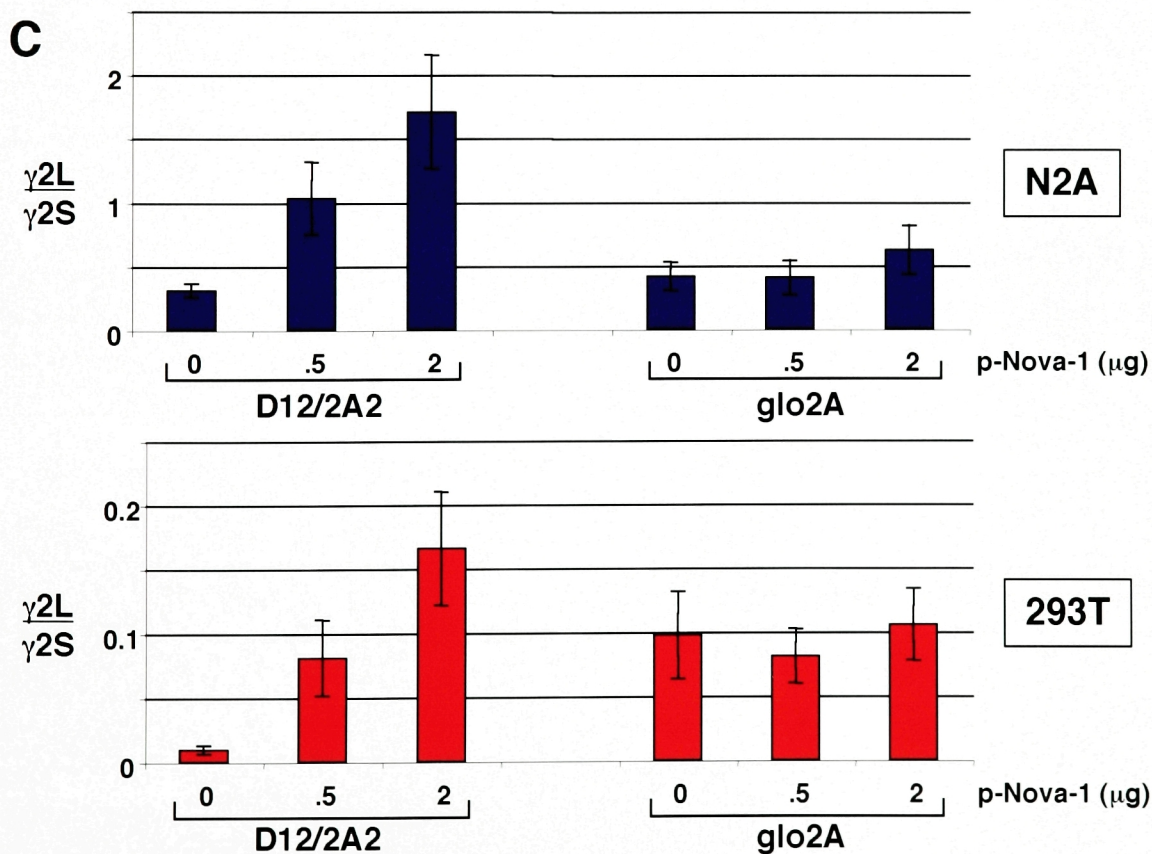
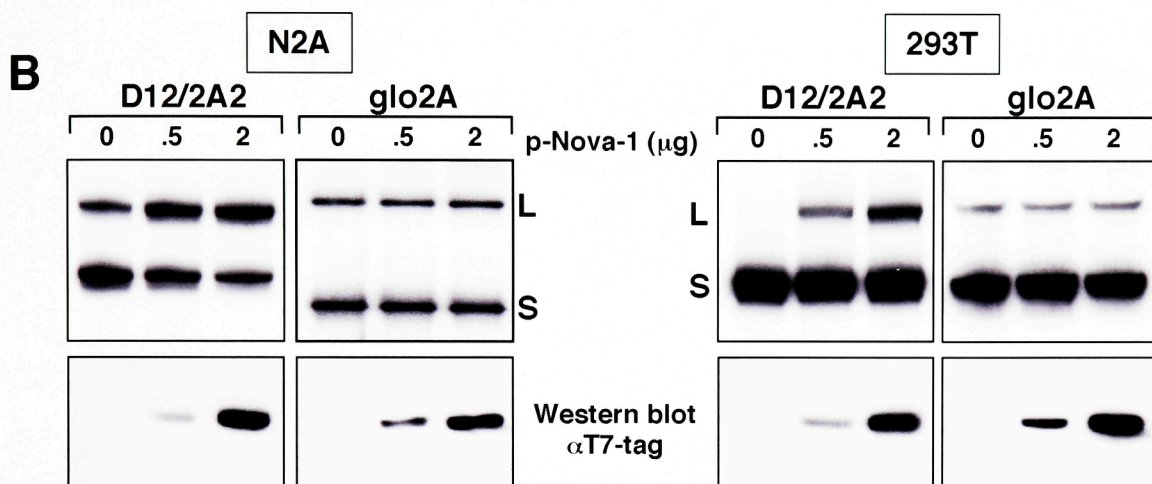
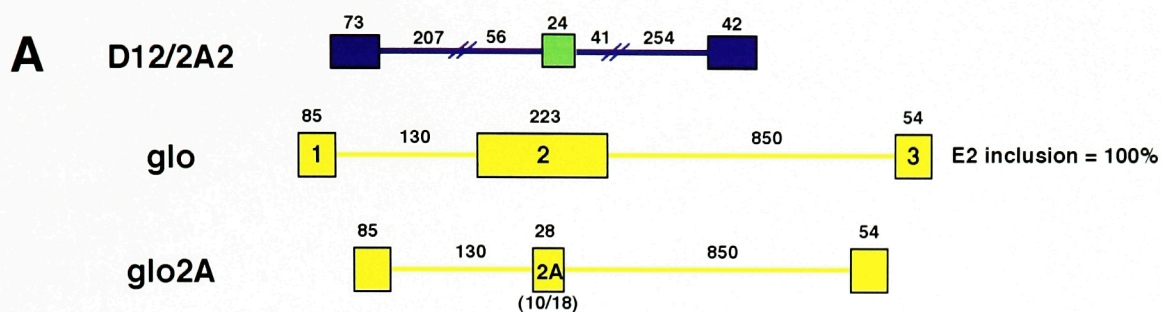
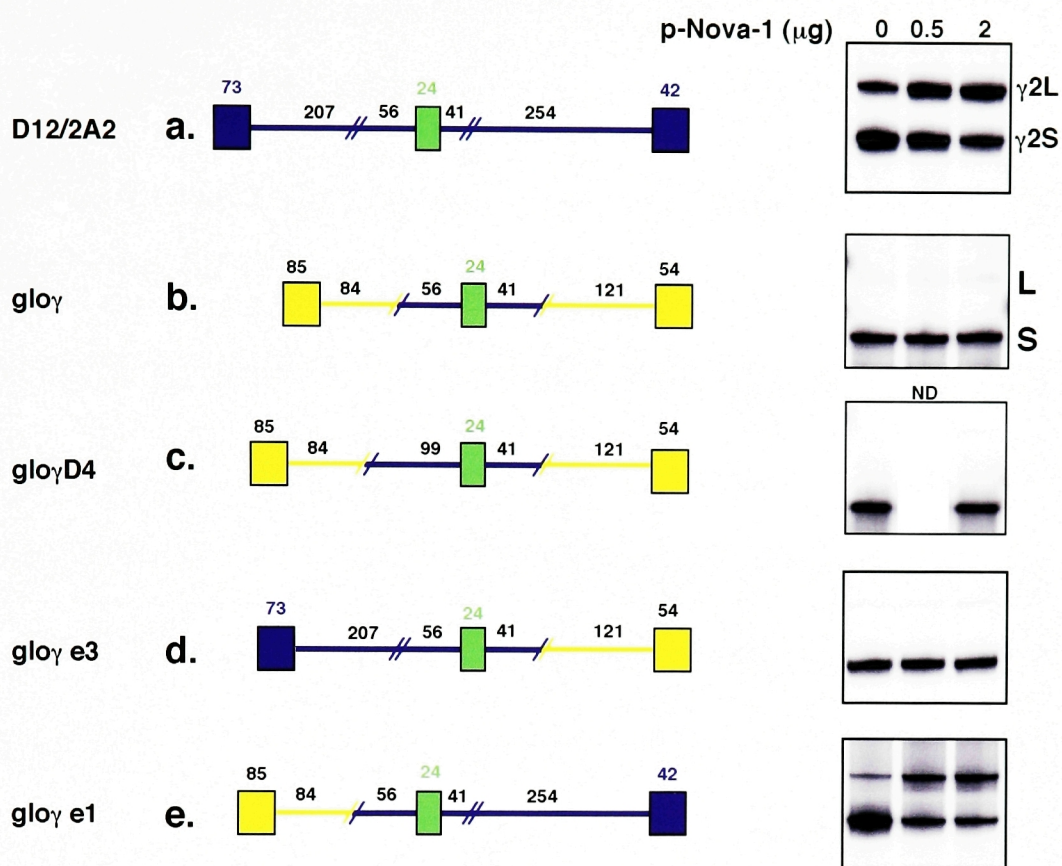


Figure 16: Sequence surrounding GABA_AR γ 2 E9 is not sufficient for regulation of alternative splicing by Nova-1 – sequence around E10 is necessary.

- (A) Schematic representation of minigene constructs generated for this study. Chimeric constructs were made by ligating portions of the GABA_AR γ 2 (blue and green) and B-globin (yellow) constructs shown in Figure 15. gloyD4 (construct c.) was derived from the ExoIII deletion construct D4 (Figure 14C). Minigenes were cotransfected with increasing amounts of pNova-1 and spliced products measured by RT-PCR using primers specific for the outside exons and phosphorimage analysis as in Figure 10 (right panel). The titration of transfected Nova-1 expression was monitored by western blot (not shown).
- (B) Quantitation of the data presented in (A). Since the baseline level of alternative exon inclusion varies widely between these constructs, the data is presented as the change in γ 2L/ γ 2S exon use ratio upon addition of Nova-1 (i.e. the ratio of γ 2L/ γ 2S with no added Nova is normalized to 1).

A



B

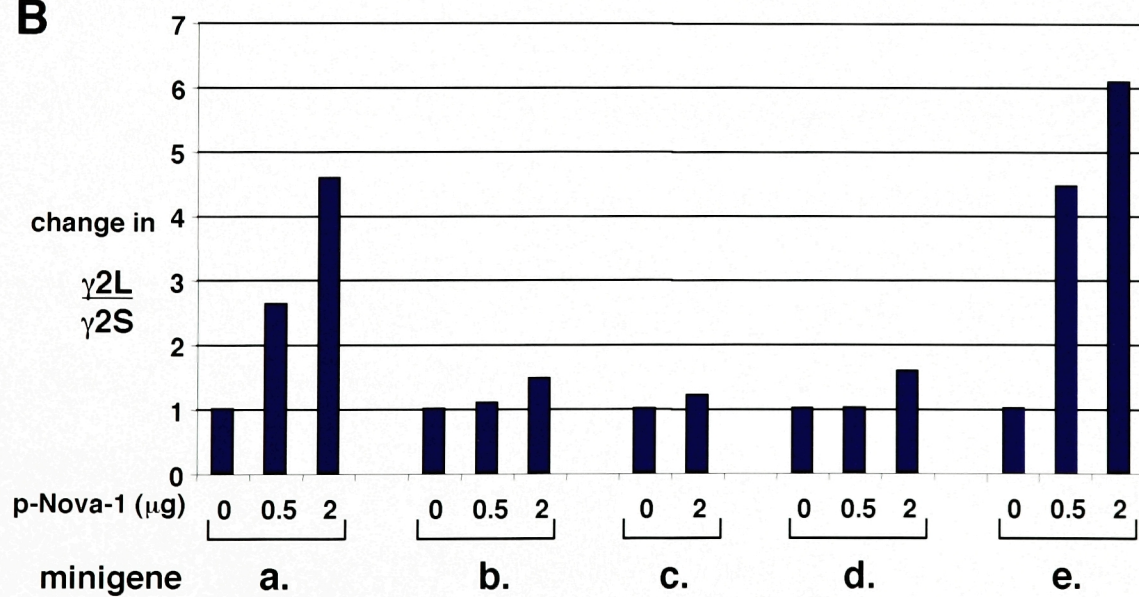


Figure 17: Intronic sequence upstream of GABA_AR γ 2 E10 is sufficient for regulation of alternative splicing by Nova-1.

- (A) The chimeric minigenes depicted on the left were cotransfected with increasing amounts of pNova-1 and spliced products measured by RT-PCR and phosphorimage analysis as in Figure 10 (right panels). The titration of transfected Nova-1 expression was monitored by western blot (not shown).
- (B) Quantitation of the N2A data presented in (A). The data is presented as the γ 2L/ γ 2S exon use ratio.
- (C) Quantitation of the 293T data presented in (A). The data is presented as the γ 2L/ γ 2S exon use ratio.

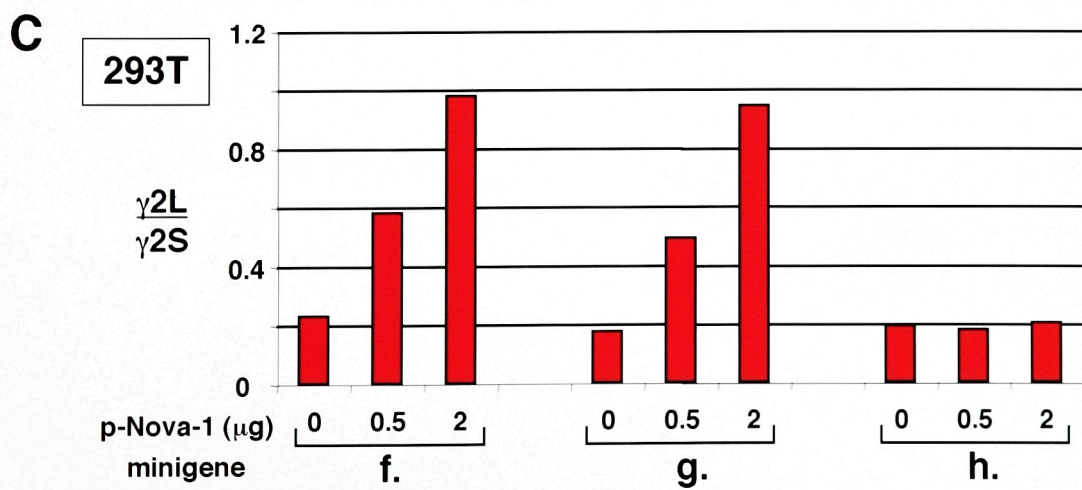
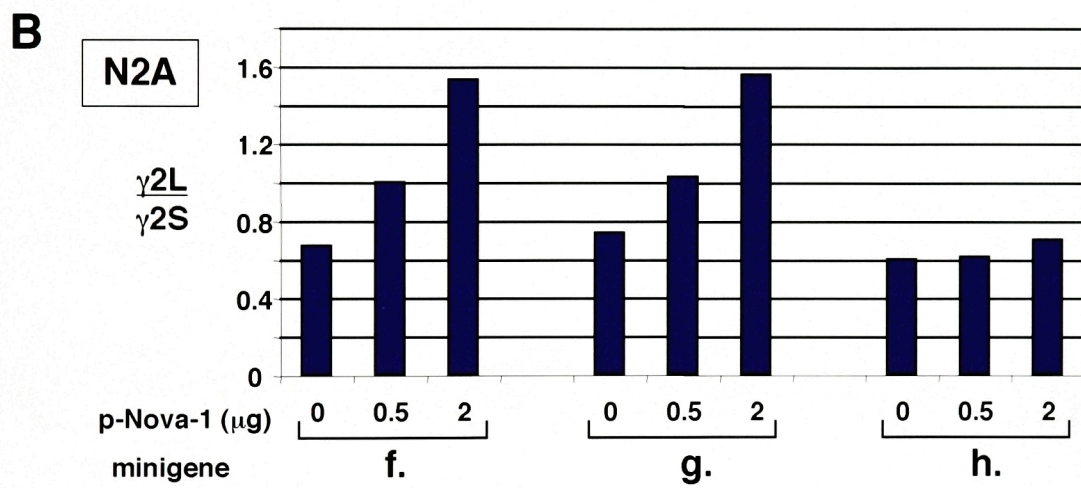
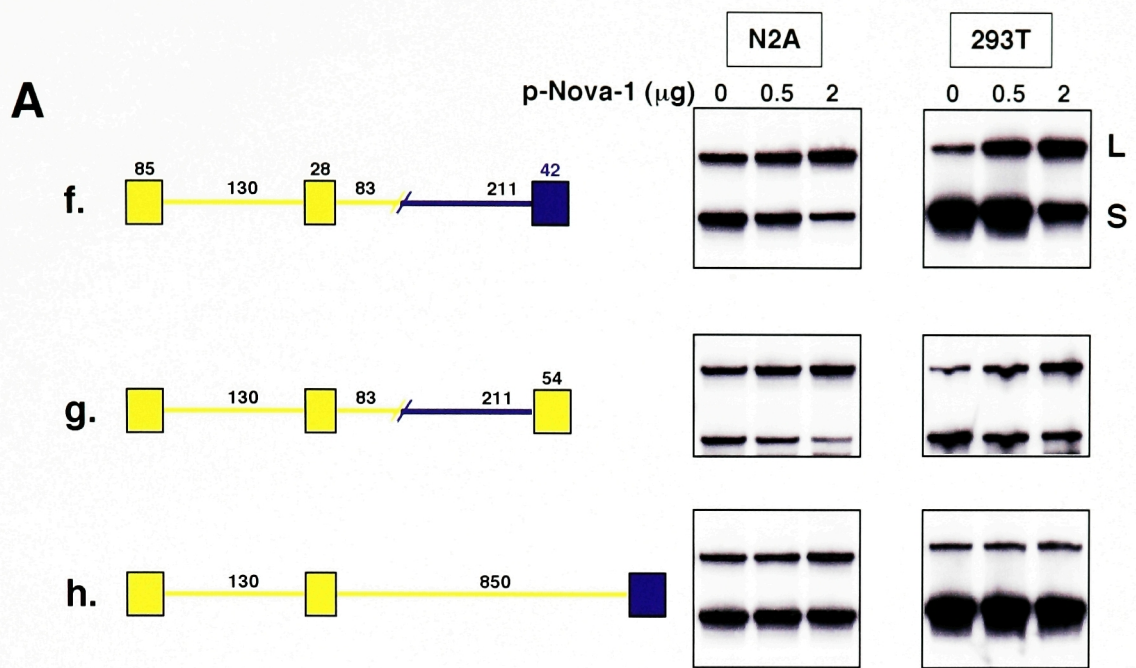


Figure 18: Sequence comparison of intronic regions upstream of GABA_AR γ 2 E10.

Genomic sequence upstream of GABA_AR γ 2 exon 10 (blue box) from mouse (mus) and human (hum) was aligned by ClustalW alignment (MacVector). Conserved YCAY motifs are underlined in red, non-conserved motifs in purple. The green box denotes the putative branch-point adenosine based on sequence analysis. Blue arrows indicate the region corresponding to *in vitro* transcribed RNA 9C (see Figure 21). Mutations (C to A) in YCAY repeats used in following experiments are shown in red.

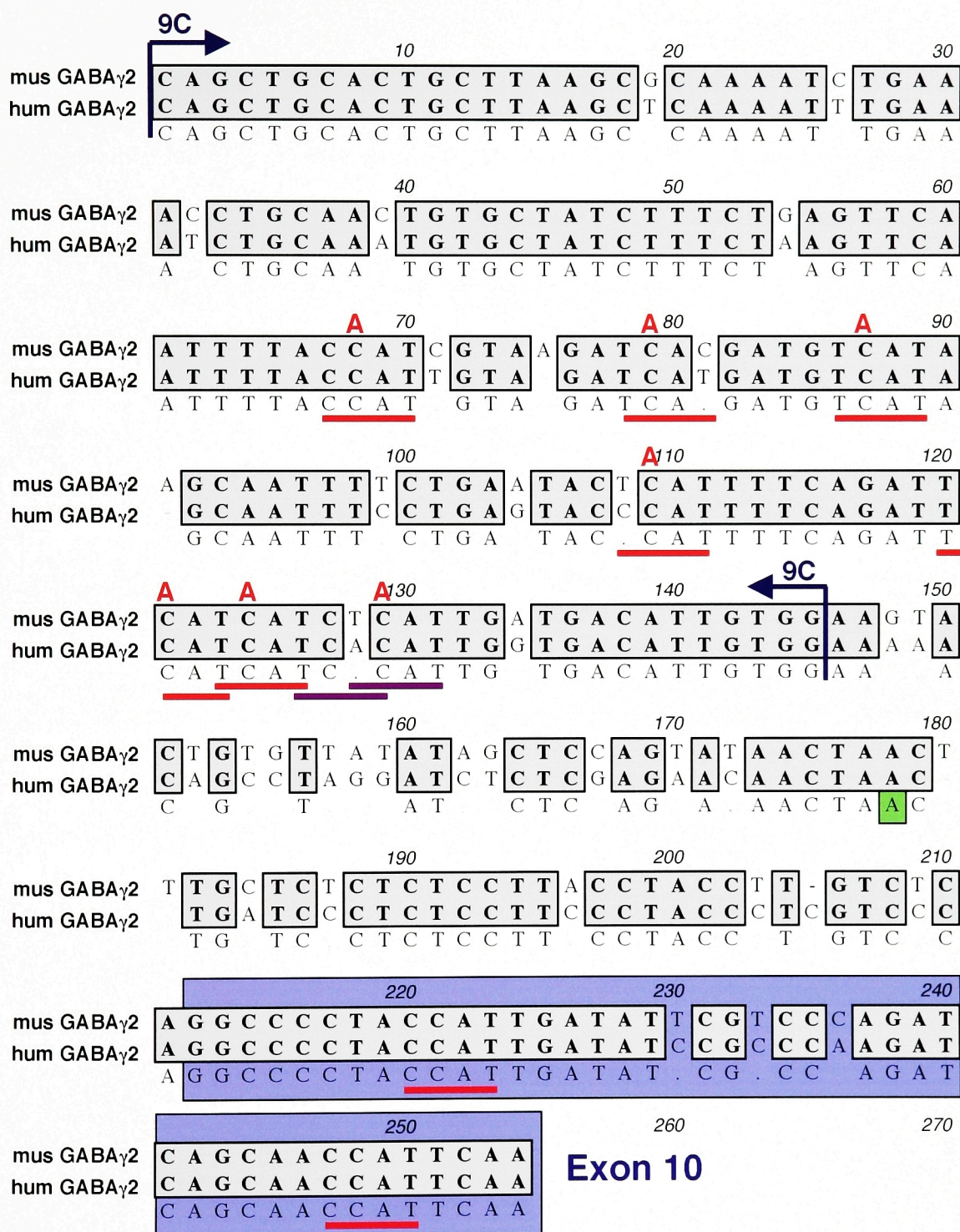
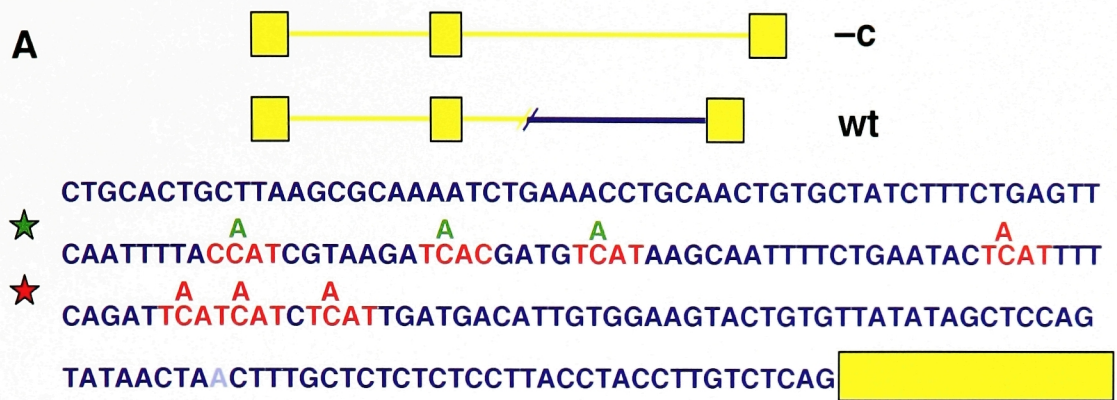


Figure 19: Mutagenesis of the YCAY repeats abrogates Nova-dependent regulation of chimeric minigene alternative splicing.

- (A) Schematic representation of minigenes used in (B). The DNA sequences derived from GABA_AR γ 2 intron 9 contained within the chimeric minigene is shown. The green star denotes one set of 3 C to A mutations (green As), the red star denotes a second set of 4 C to A mutations (red As) made in the minigene.
- (B) Minigenes depicted in (A) were cotransfected into I. N2A or II. 293T cells with 2.0 μ g of pNova-1 or empty vector and spliced products measured by RT-PCR and phosphorimage analysis as in Figure 10 (shown on the left). The transfected Nova-1 expression was monitored by western blot (not shown). Quantitation of the data is presented on the left as the ratio of L/S spliced products.
- (C) Presentation of the data shown in (B) as the change in the ratio of L/S spliced products upon addition of Nova-1 (i.e. the ratio of L/S with no added Nova is normalized to 1).



B

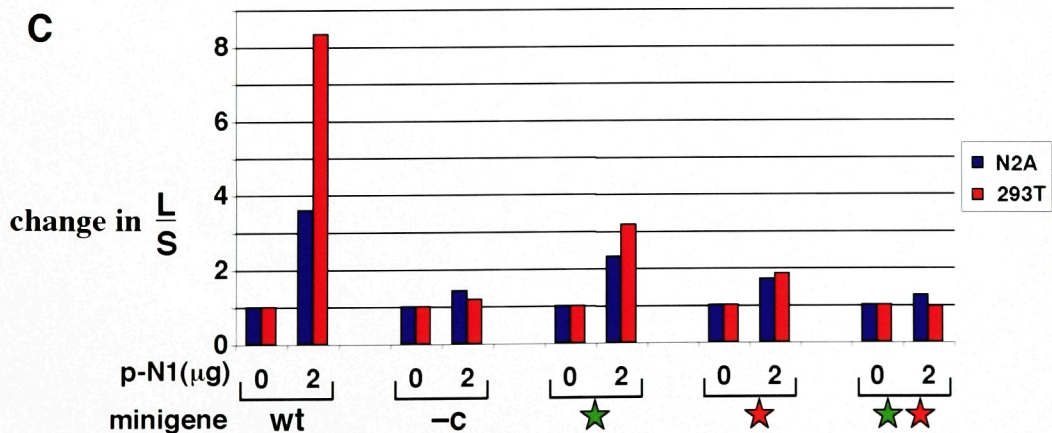
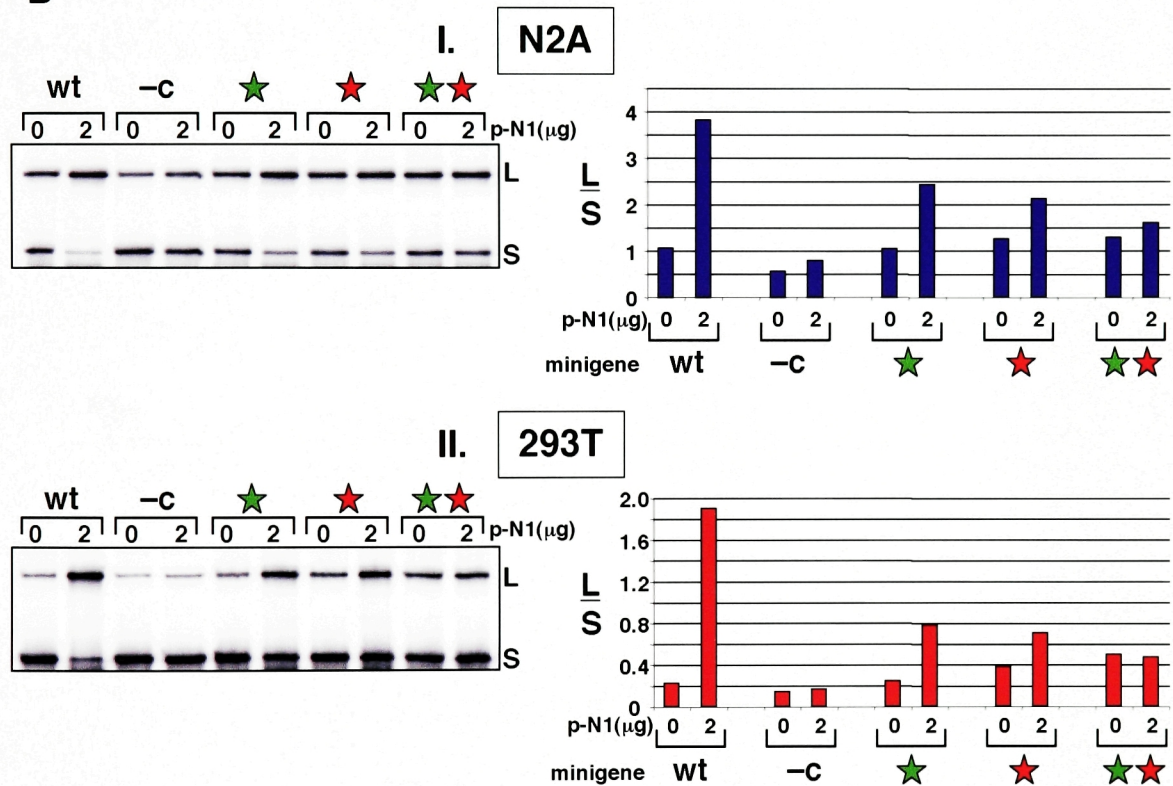


Figure 20: Mutagenesis of the YCAY repeats close to E10 abrogates Nova-dependent regulation of GABA_AR γ 2 minigene alternative splicing.

- (A) Mutations were made (depicted by stars) within the intronic region of the GABA_AR γ 2 minigene upstream of E10. The DNA sequence in the region of the mutations is shown. The green star denotes one set of 3 C to A mutations (green As), the red star denotes a second set of 4 C to A mutations (red As) made in the minigene.
- (B) Minigenes depicted in (A) were cotransfected into 293T cells with increasing amounts of pNova-1 and spliced products measured by RT-PCR and phosphorimage analysis as in Figure 10. The titration of transfected Nova-1 expression was monitored by western blot (not shown).
- (C) Quantitation of the data presented in (A) presented graphically as the ratio of γ 2L/ γ 2S spliced products.

A



★ CTGCACTGCTTAAGCGCAAATCTGAAACCTGCAACTGTGCTATCTTTCTGAGTT
 CAATTTTACCATCGTAAGATCACGATGTCATAAGCAATTTTCTGAATACTCATT
 ★ CAGATTATCATCTCATTGATGACATTGTGGAAGTACTGTGTTATATAGCTCCAG
 TATAACTAACTTTGCTCTCTCTCCTTACCTACCTTGTCTCAG

B



C

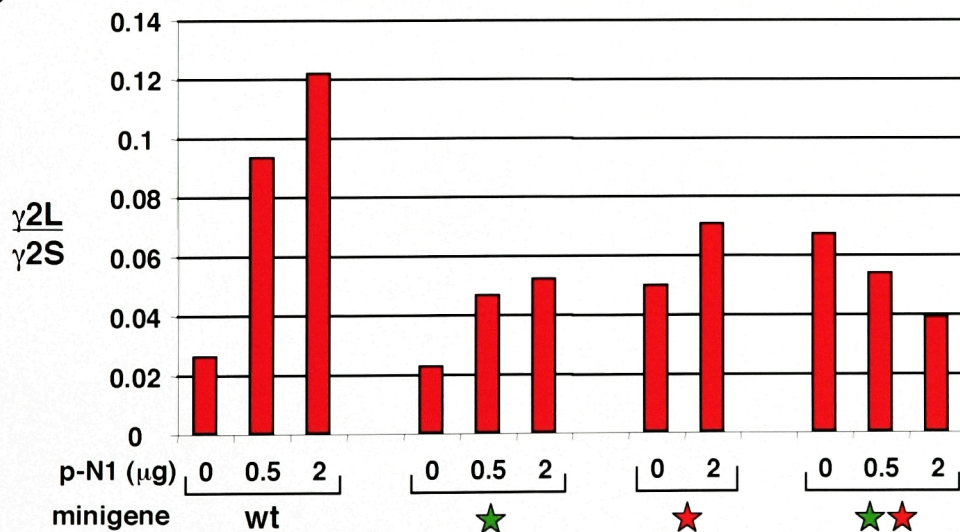


Figure 21: Nova-1 binds with high affinity to GABA_AR γ 2 RNA *in vitro*

- (A) His-tagged Nova-1 fusion protein was over expressed in *E.coli* and purified over a nickel column. The protein was eluted from the column and collected in 2 fractions. Aliquots of increasing concentrations were analyzed by SDS-PAGE followed by staining with Coomassie stain. Fraction 1 contained higher molecular weight contaminants in addition to Nova fusion protein. Fraction 2 appeared to be much more pure, so was used for subsequent experiments. M=full-range rainbow markers, CL= column load, NFP=Nova-1 fusion protein.
- (B) Nitrocellulose filter binding assays were performed using Nova-1 fusion protein shown in (A) and four *in vitro* transcribed RNAs. 9C = RNA corresponding to the region of GABA_AR γ 2 intron 8 highlighted in Figure 18 with the addition of the sequences GGGAG at the 5' end and CUAGCAAA at the 3' end derived from the PCR primers used to amplify the template prior to *in vitro* transcription. 9Cmut = RNA identical to 9C with the exception of 7 C to A mutations as shown in Figure 18. SB2 = RNA purified by SELEX with Nova-1 (Buckanovich and Darnell, 1997). glo = RNA derived from human B-globin which spans regions of exon 1 and intron 1, and contains no YCAY motifs.

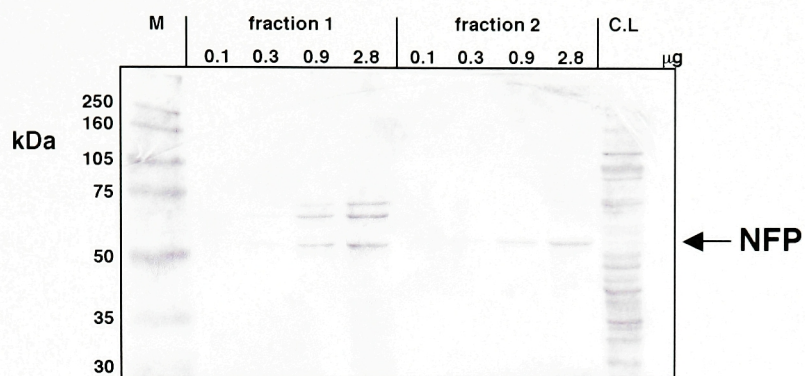
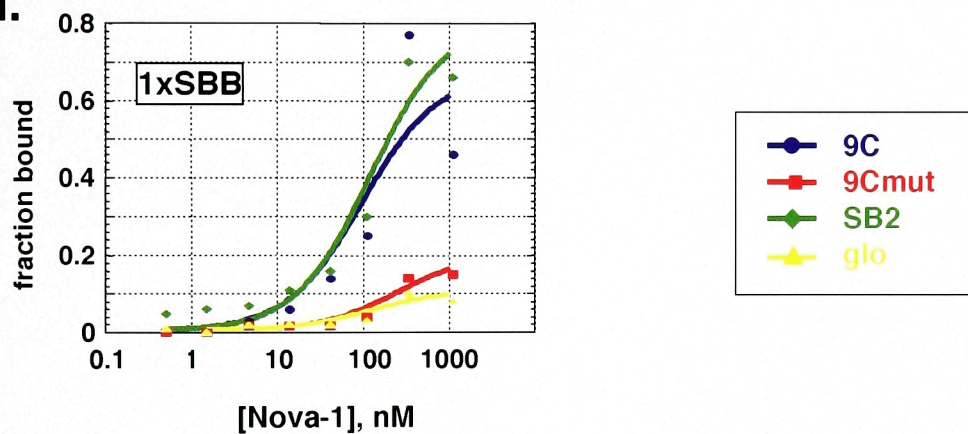
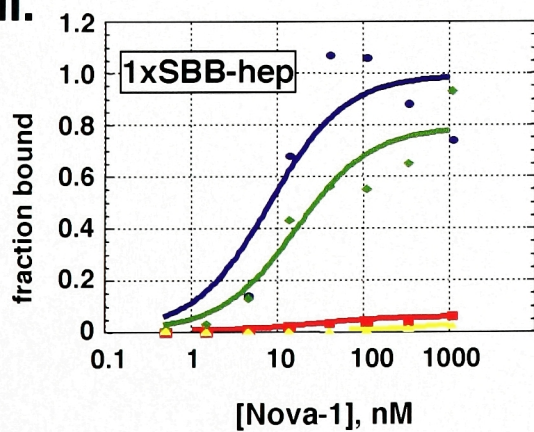
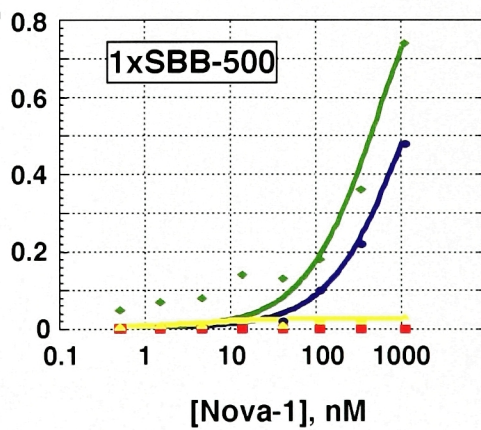
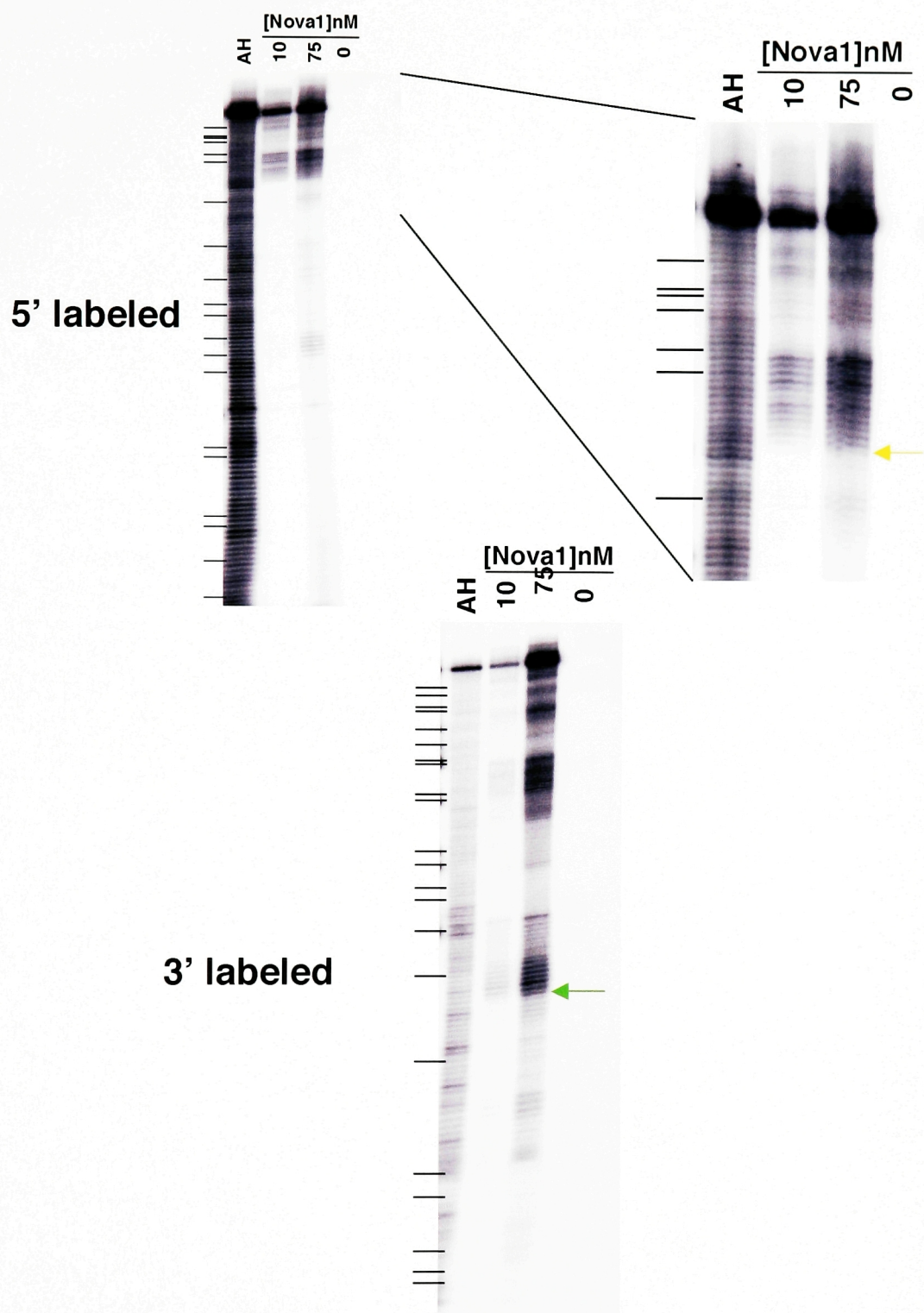
A**B****I.****II.****III.**

Figure 22: Mapping the boundaries of Nova-1 binding to GABA_AR γ 2 9C RNA

9C RNA was labeled with ^{32}P at either the 5' or 3' end and subjected to mild alkaline hydrolysis (AH). The RNA was then incubated with Nova-1 fusion protein at a final concentration of 10nM or 75nM and protein:RNA complexes captured by filtration through nitrocellulose filters. Bound RNAs were eluted and analyzed by denaturing PAGE. The ladders represent the position of G residues (determined by RNase T1 digestion). Boundaries are highlighted by arrows and indicated on the 9C RNA sequence shown at the bottom. Small letters represent sequence derived from primers.



gggagCAGCUGCACUGCUUAAGCGCAAAAUCUGAAACCUGCAACUGUGCUAUC

UUUCUGAGUUCAAUUUUUACCAUCGUAAGAUCACGAUGUCAUAAGCAAUUUU

5' boundary ↓ 3' boundary ↓

CUGAAUACUCAUUUUCAGAUUCAUCAUCUCAUUGAUGACAUUGUGGcuagcaaaa

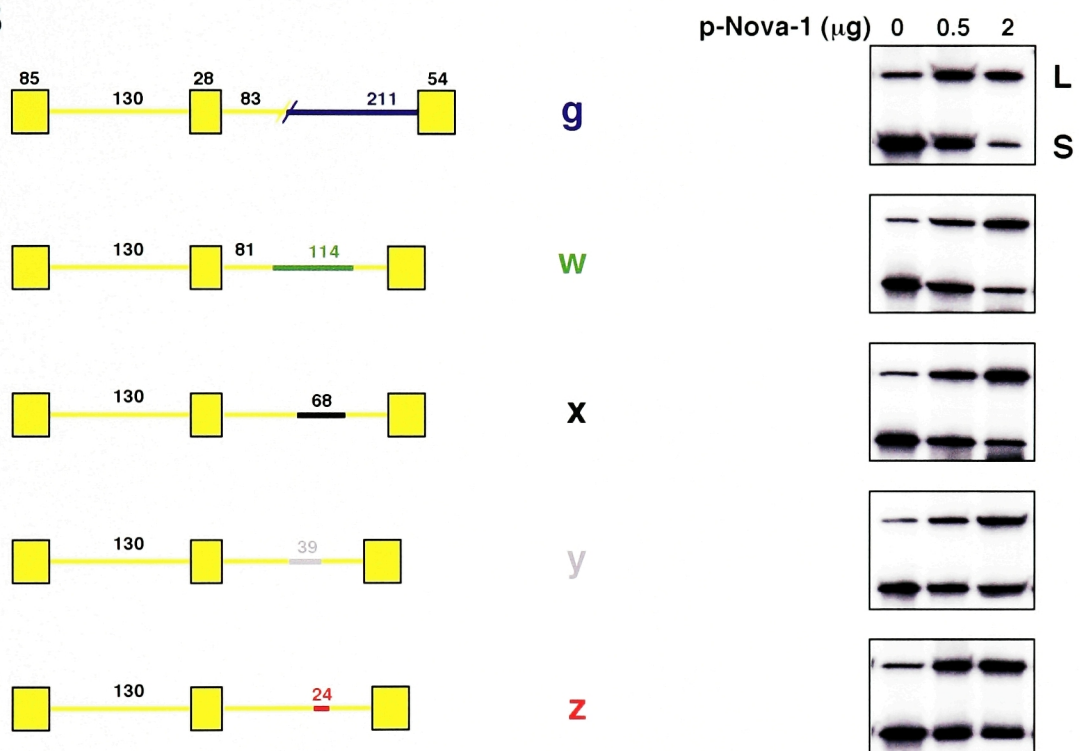
Figure 23: 24nt intronic sequence is sufficient for regulation of alternative splicing by Nova-1.

- (A) DNA sequences derived from GABA_AR γ 2 intron 9 contained within the chimeric minigenes shown in B. The colored lines highlight the region cloned into the B-globin minigene labeled in the corresponding color in B.
- (B) The chimeric minigenes depicted on the left were cotransfected into 293T cells with increasing amounts of pNova-1 and spliced products measured by RT-PCR and phosphorimage analysis as in Figure 10 (right panels). The titration of transfected Nova-1 expression was monitored by western blot (not shown).
- (C) Quantitation of the data presented in (A). The data is presented as the ratio of L/S spliced products.

A

CTGCACTGCTTAAGCGCAAAATCTGAAACCTGCAACTGTGCTATCTTTCTGAGTT
 CAATTTTACCATCGTAAGATCACGATGTCATAAGCAATTTTCTGAATACTCATTTT
 CAGATTTCATCATCTCATTGATGACATTGTGGAAGTACTGTGTTATATAGCTCCAG
 TATAACTA~~ACTTTGCTCTCTCTCCTTACCTACCTTGTCTCAG~~

B



C

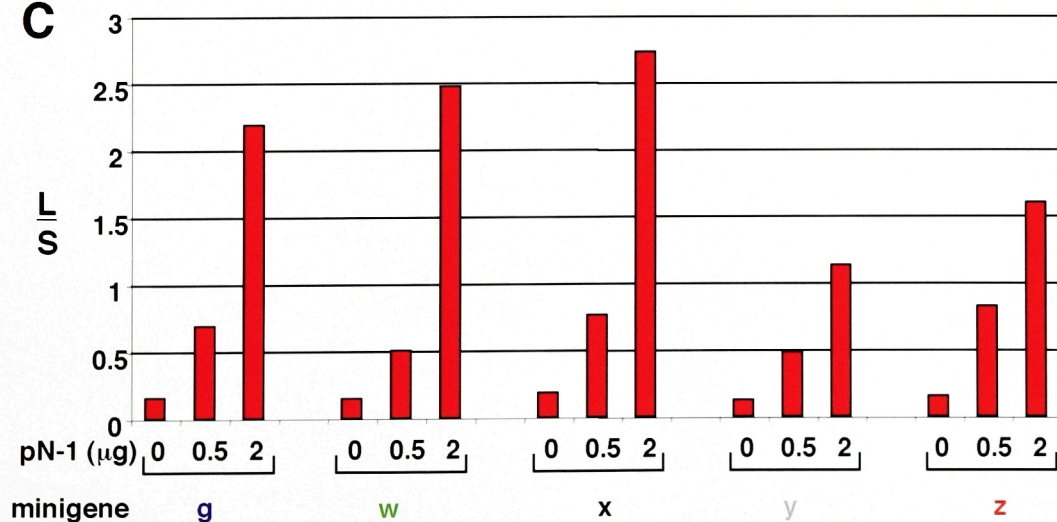
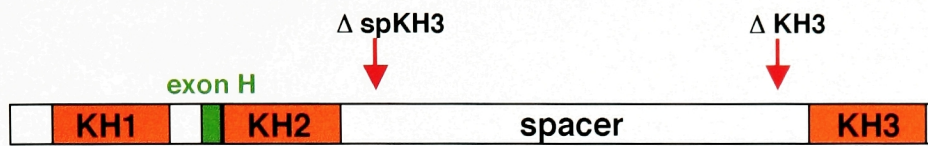


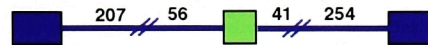
Figure 24: KH3 and the spacer region contribute to the function of Nova-1 in regulating alternative splicing.

- (A) Schematic representation of Nova-1 protein, and the GABA_AR γ 2 minigene used in this experiment. Mammalian expression constructs were generated which express truncated forms of Nova-1 which lack KH3 (Δ KH3) or KH3 and the spacer region (Δ spKH3).
- (B) N2A cells were cotransfected with the truncated GABA_AR γ 2 minigene D12/2A2 and increasing amounts of plasmids expressing full-length or truncated Nova-1 cDNAs. Spliced products were analyzed by RT-PCR and phosphorimage analysis as in Figure 10. The titration of transfected Nova-1 expression was monitored by western blot (not shown). The data is presented as the ratio of γ 2L/ γ 2S spliced products. Error bars for addition of 0 and 1.0 μ g pNova represent deviation from the mean in 2 separate trials.
- (C) 293T cells were transfected and the spliced products analyzed as in (B). Error bars represent deviation from the mean in 2 separate trials.

A

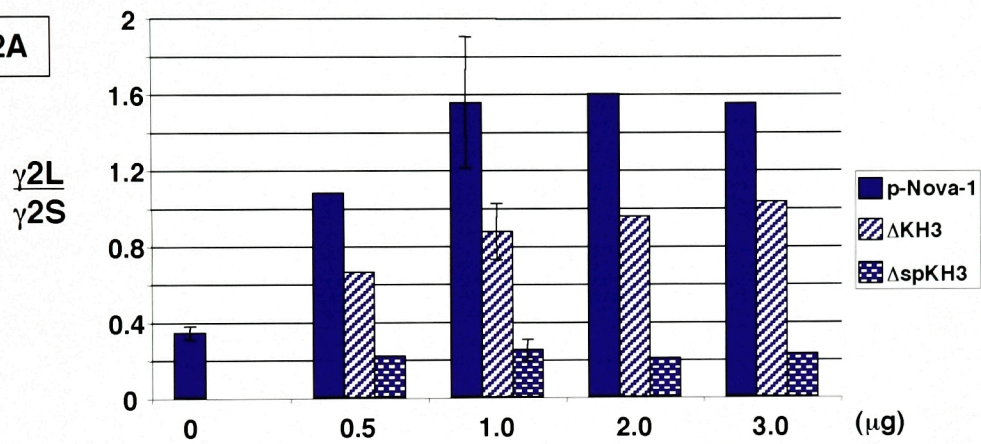


D12/2A2



B

N2A



C

293T

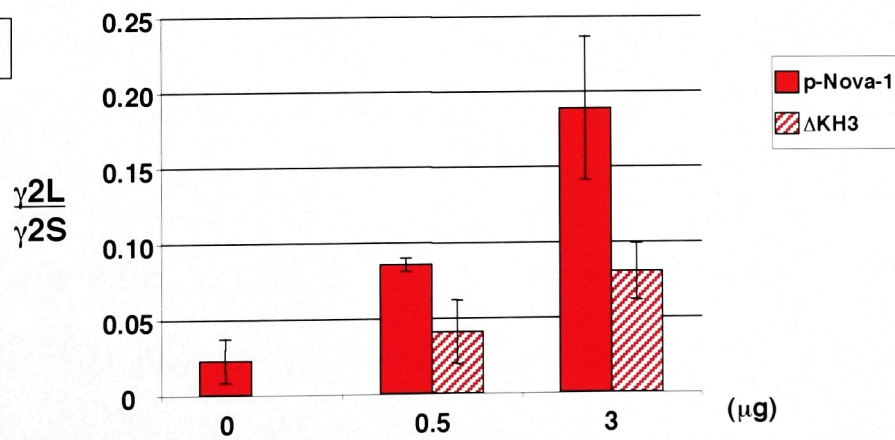
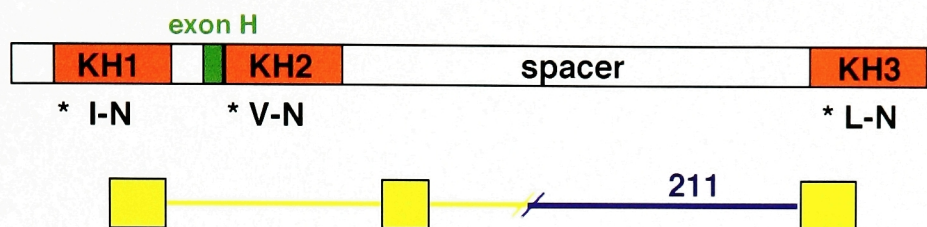


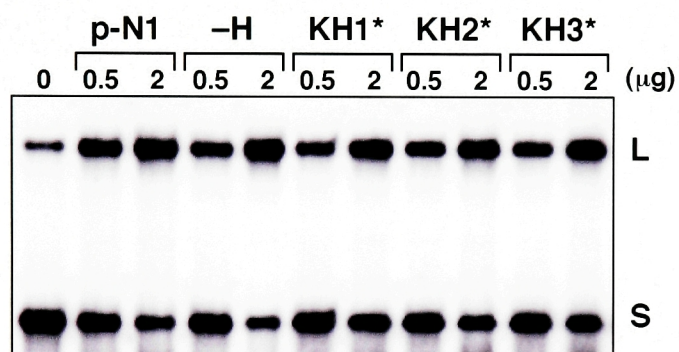
Figure 25: All 3 KH domains contribute to the function of Nova-1 in regulating alternative splicing.

- (A) Mammalian expression constructs were generated which either lack exon H or harbor point mutations in one of the KH domains as noted. These were used in cotransfection experiments with the minigene depicted.
- (B) 293T cells were cotransfected with the chimeric minigene and increasing amounts of Nova-1 expression plasmid. Spliced products were analyzed by RT-PCR and phosphorimage analysis as in Figure 10. The transfected Nova-1 expression was monitored by western blot (not shown).
- (C) Quantitation of the data presented in (B), plus 2 additional experiments. The data is presented as the average ratio of L/S spliced products over the three trials, error bars display the standard deviation.

A



B



C

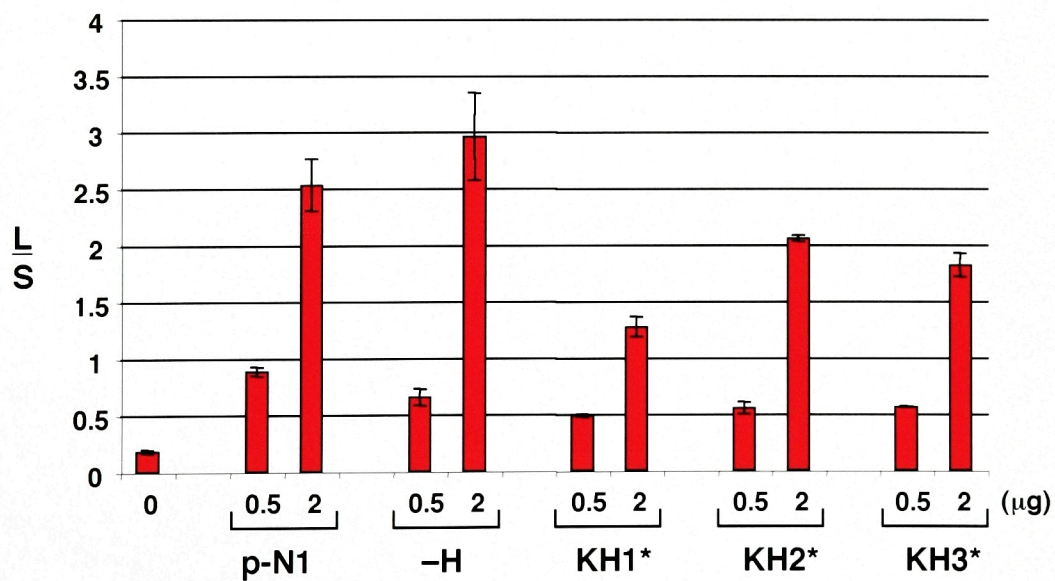
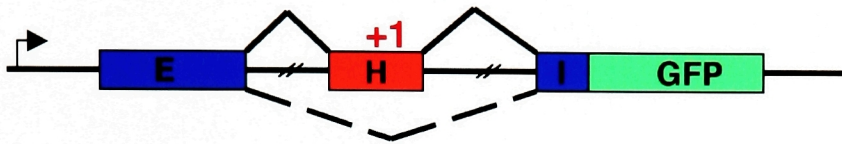


Figure 26: Nova-1 regulates alternative splicing of its own message in tissue culture cells.

- (A) Schematic representation of a Nova-1 minigene generated for this study. Details of the construction of the minigene are discussed in the text.
- (B) A version of the minigene which results in the expression of in-frame EGFP if exon H is excluded (pG-) was transfected into N2A cells with increasing amounts of p-Nova-1 expression plasmid. Cells were fixed and analyzed for EGFP expression by fluorescence microscopy.

A



+ H = GFP out of frame
- H = GFP in frame

B

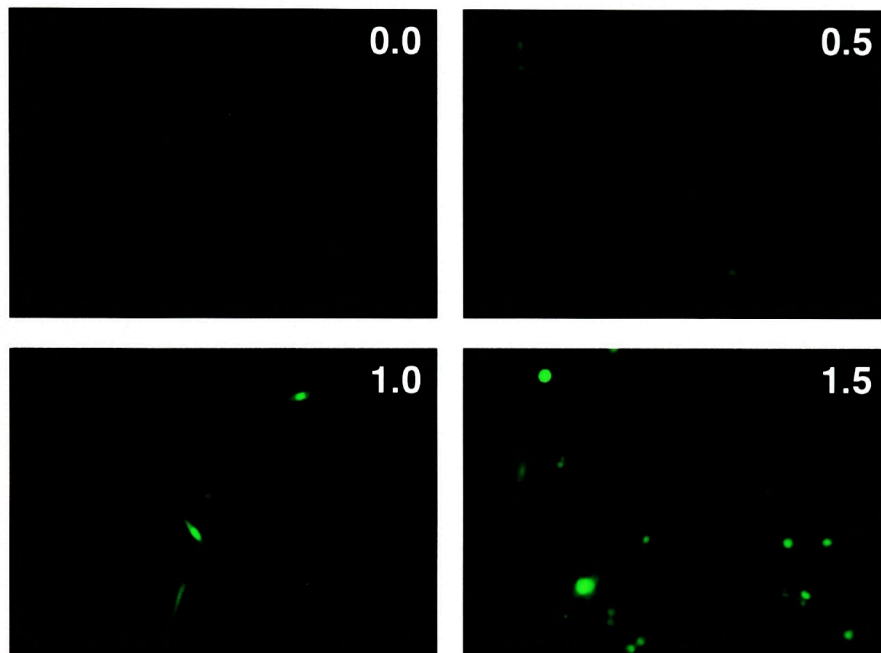
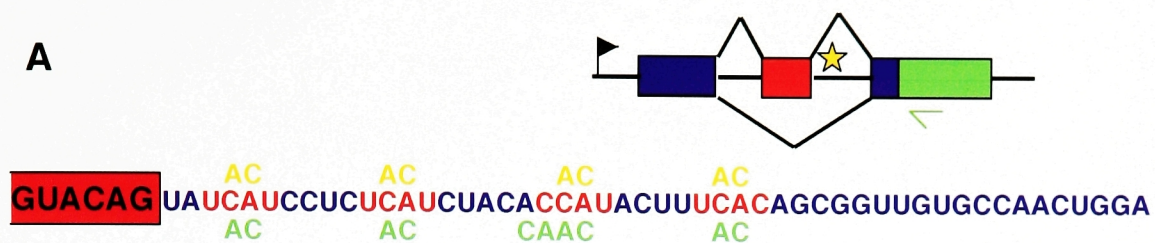


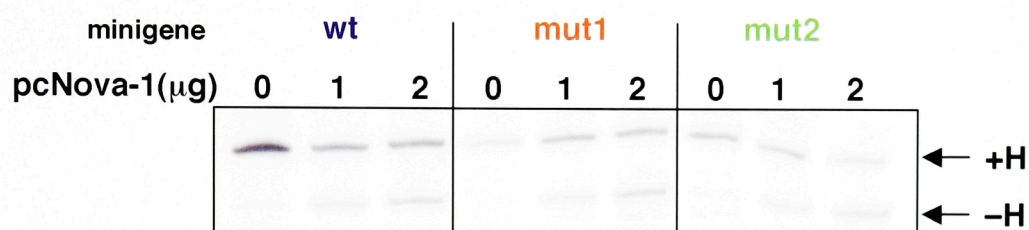
Figure 27: Mutations within the downstream intron do not interfere with Nova's ability to regulate exon H alternative splicing.

- (A) The mouse genomic Nova-1 sequence immediately downstream of exon H contains four YCAY repeats (red). Mutations made in this region of the minigene are shown in orange and green.
- (B) N2A cells were cotransfected with the wildtype or mutated minigenes and increasing amounts of Nova-1 expression plasmid. Spliced products were analyzed by primer extension using a labeled primer to EGFP and phosphorimage analysis. The transfected Nova-1 expression was monitored by western blot (not shown).
- (C) Quantitation of the data in (B). Bars represent the ratio of exon H included (+H) to excluded (-H) spliced forms.
- (D) Presentation of the data shown in (B) as the change in the ratio of +H/-H spliced products upon addition of Nova-1 (i.e. the ratio of +H/-H with no added pNova-1 is normalized to 1).

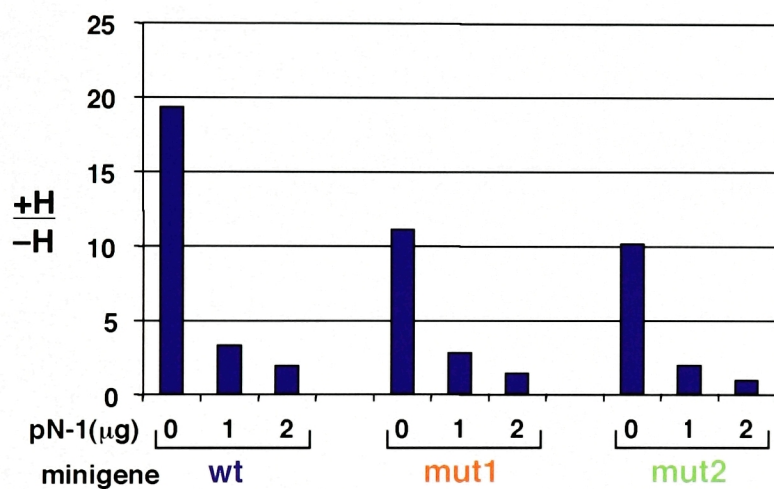
A



B



C



D

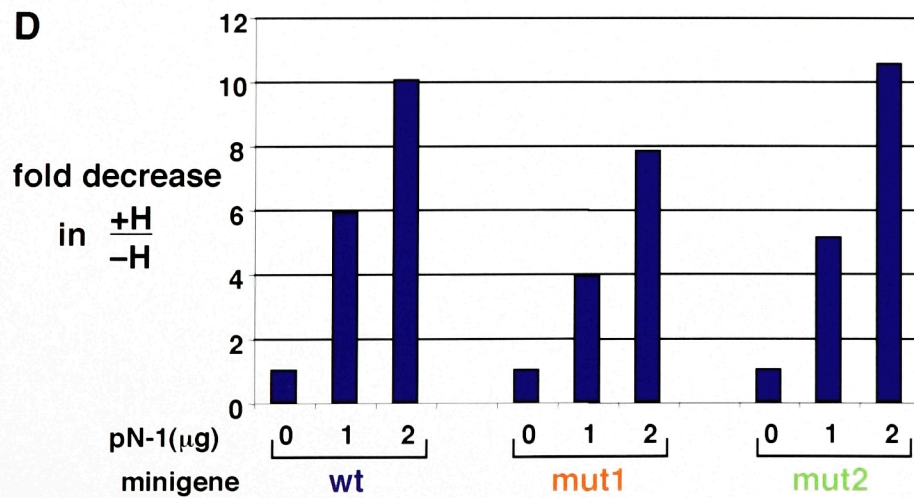


Figure 28: Alternative splicing of a truncated Nova-1 minigene is still regulated by Nova-1 protein: narrowing down the sequence elements required.

- (A) Mouse genomic Nova-1 sequence surrounding exon H (red box) which is included in the minigene shown in Figure 26. YCAY repeats are shown in red. Chimeric constructs were generated containing this entire region or the sequences contained between the arrows.
- (B) Schematic representation of the minigenes used in this experiment. Sizes of exonic or intronic regions are shown in bp.
- (C) Minigenes depicted in (B) were cotransfected into N2A or 293T cells with increasing amounts of pNova-1. RNA was harvested 40 hours post-transfection and analyzed by RT-PCR using primers to globin exons 1 and 3, followed by phosphorimage analysis.
- (D) Quantitation of the data presented in (C). Bars represent the fraction of messages containing exon H as a percentage of the total spliced products.

A

GATCCCTAAAT**TCAT**GTGGAATCAGCTTGTTGGTGATGTTCAACATATTAAGTAGGTGGA
TACTTTTATGCCAAAGTAAACT**TCATTTCACCAC**TTCTTCAAATCTCCGACTGTAT**TCATT**
GATAGGAAAGTGGCTCTTCCAATTTTAT**TCAT**CTAACTTCTTGAATTTTCTTTGACATCTC
TTTTCCCCGAAGCAAATTAGAGGGAAAGAAATGGGACTGCTCT**TCAT**GCATAAGGGCAG
TCTGCCTTTAGCAC**TCAT**GTGAGTTCCTATCAG**TCACCACCAT**GATTTTCGAG**TCAT**GAA
TTTATTTTACATGCTTATGCATGCATATTTACATATATTAGTTACTCAGTTGACCTTAAGT
T**CCAT**TAAATCTGAACGACTAGGTGGAACTTTGT**CCAT**AGGATTTTGTTCGTTATAT
TACATTTTATTTGTTTTGTTTTGTTTAATGATCAGCTTAAGGC**TCAT**AAACGTTGATTTG
CTCCTGAACTGCTTTGTTCCCA**ACATTGCCATCTTCCCCAACTACCACCAAGTCCTCT**
CCATCTGGACC**CCATGACCACCTCCAGAGCCAATCAG**GTACAGTAT**TCAT**CCTC**TCATC**
TACA**CCAT**ACTT**TCAC**AGCGGTTGTGCCAACTGGAGAAACAGATACTGGTGGGTTAAG
GATTTATTCGT**TCATTCTCAT**ATATGC**CCAT**GATCAGATTCCAAATAACCTAATAAATAA
ATATCAATCATTTTTTTTAAATTTACATGCATTTTAACT**TCAT**TAACAAAAAATATTCAGGT
GGTAGACTTTATCAGAATTTTGGAGTTCTTAAACAGTGAT**TCAC**TTTACAAAGCCCTGAC
TGTCATTATTGAGTTGGTCTTT**TCAT**CTCTTTCAATATATCCTCTTAGTAGTTTTTAGAA
ATAAATTA**TCAT**ATTACATGGGTTATGAATTGTATTAAATTGAGCATCGACCTG

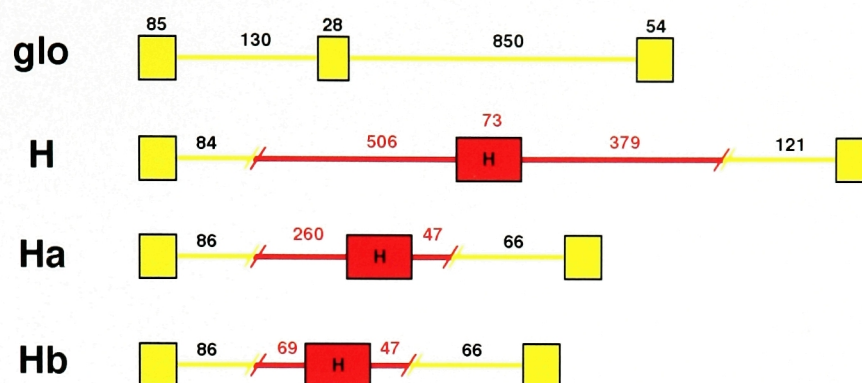
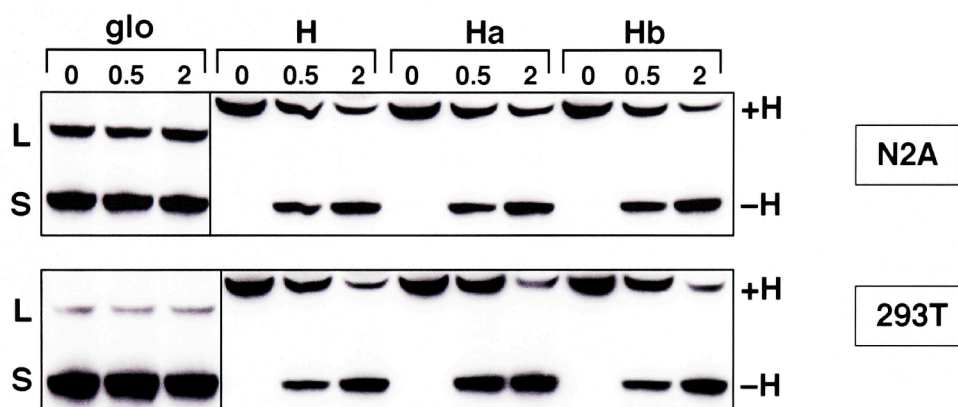
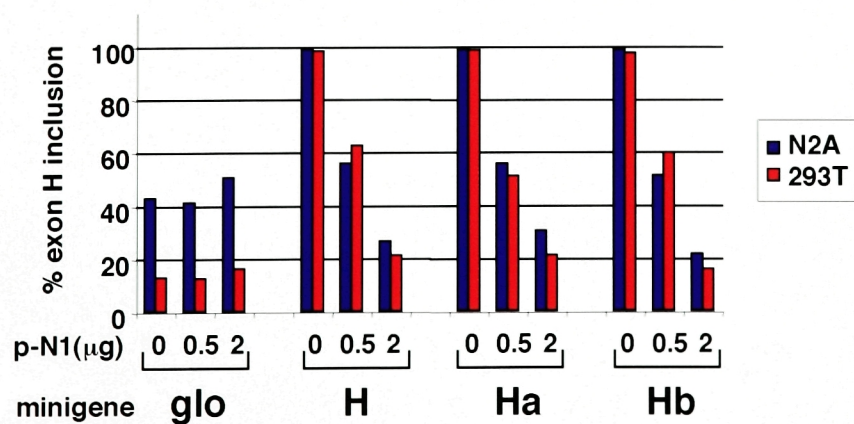
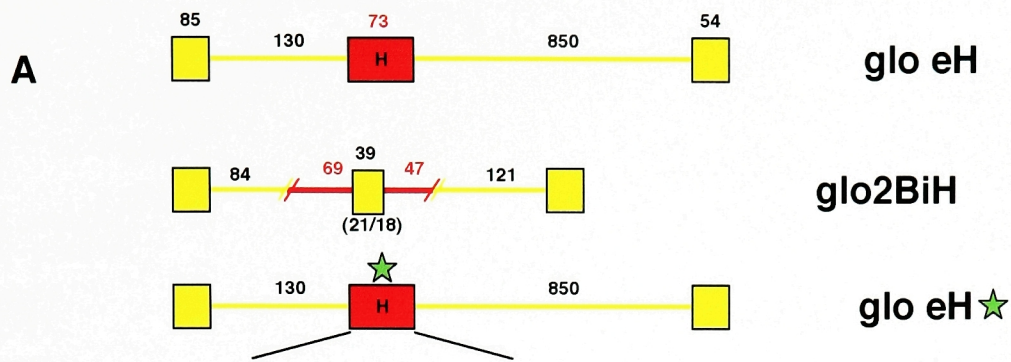
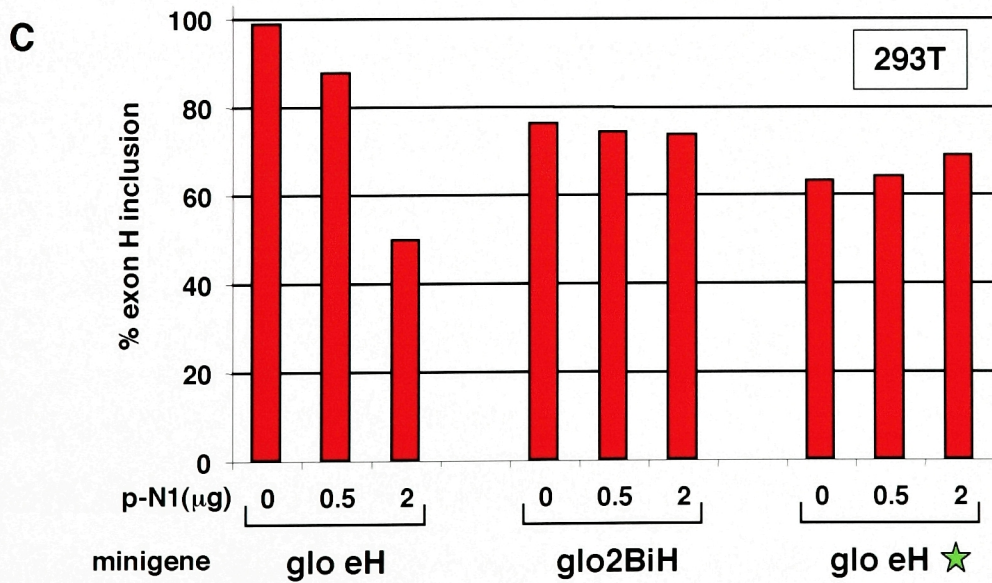
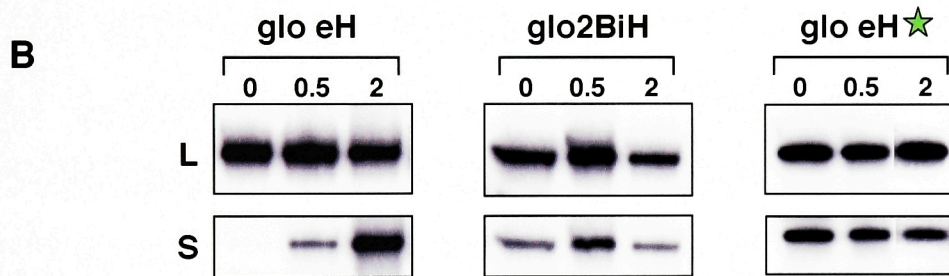
B**C****D**

Figure 29: Sequence within Nova-1 exon H is necessary and sufficient for autoregulation of alternative splicing.

- (A) Schematic representation of the minigenes used in this experiment. The sequence of exon H is shown in the red box. YCAY repeats are shown in red, mutations in green. The blue G indicates the 1bp insertion made in the original minigene construct (Figure 26) in order to create a frame shift.
- (B) Minigenes depicted in (A) were cotransfected into 293T cells with increasing amounts of pNova-1. Spliced products were analyzed as in Figure 28.
- (C) Quantitation of the data presented in (B). Bars represent the fraction of messages containing exon H as a percentage of the total spliced products.



A C A C
 ACATTGCCATCTTCCCCAACTACCAAGTCCTCTC
 TA A C A C
 CATCTGGACCCCATGACCACCTCCAGAGCCAATCAG



Chapter 5 – Nova-1 subcellular localization

Introduction

A number of distinct compartments have been visualized in the nuclei of mammalian cells, using both electron microscopy and indirect immunofluorescence against the proteins contained within them. Photobleaching and other live cell imaging experiments using GFP (and its variants) fusion proteins have shown that many of these structures are highly dynamic and feature rapid protein exchange between the compartment and the nucleoplasm (reviewed in (Misteli, 2001)). It has been suggested that by analogy to the compartmentalized organization of the cytoplasm, the existence of numerous subnuclear compartments suggests that particular processes occur in specific locations within the nucleus (Misteli, 2001). In addition, most of these compartments disassemble during M phase of the cell cycle and reassemble rapidly in daughter cells, indicating high structural plasticity. Whilst the biological functions of many of these domains are unknown, much information can be surmised from the types of proteins and nucleic acids that comprise them. Not surprisingly given the roles they play in nuclear processes, RNA binding proteins are often key components of these structures.

Nucleolus

The nucleolus is formed by the clustering of ribosomal genes on different chromosomes into chromosomal loci called nucleolar organizers. They are the site of rRNA transcription, processing and assembly into ribosomal subunits, and their formation is both transcription and cell-cycle dependent. Nucleoli are generally not associated with

poly-A mRNA, however some transcripts including MyoD and N-myc have been detected within them (Bond and Wold, 1993). In addition, mutations that interfere with mRNA transport, or heat shock result in poly-A mRNA accumulation in the nucleoli of yeast (Schneiter et al., 1995), (Tani et al., 1995). Taken together with the observation that inactivation of nucleoli prevents export of nonribosomal RNAs in mammalian cells (Harris et al., 1969), these results have led to the suggestion that the nucleolus may also be involved in mRNA export and/or degradation (Lamond and Earnshaw, 1998).

Cajal (coiled) bodies and gems (gemini of coiled bodies)

Cajal bodies (also known as coiled bodies) were first described by Ramon y Cajal in vertebrate neural tissues. Although the function of these small nuclear organelles is still largely unknown, they may play a role in snRNP transport, maturation, or both (Lamond and Earnshaw, 1998) or in the assembly of RNA polymerase complexes (Gall, 2001). In addition to snRNPs, they are highly enriched in basal transcription and cell cycle factors, the nucleolar proteins fibrillarin (Raska et al., 1990) and Nopp140 ascent pre-mRNA or non-snRNP splicing proteins, thus they are unlikely to be sites of pre-mRNA transcription or splicing. Coilin is often used as a marker of Cajal bodies which generally number between 1 and 10 per cell. Like nucleoli, Cajal bodies are associated with specific genomic loci, in this case the snRNA and histone gene clusters (Gall, 2000). Combined with the fact that Cajal bodies contain the U7 snRNP, which cleaves the 3' end of histone pre-mRNA, a role in histone 3' end processing has also been proposed (Frey and Matera, 1995).

SMN, the target antigen of spinal muscular atrophy (SMA) localizes to nuclear structures often paired with Cajal (or coiled) bodies. SMN interacts with both the Sm

class of snRNP proteins and SIP-1 (Liu et al., 1997), and the SMN-SIP-1 complex plays an essential role in spliceosomal snRNP biogenesis (Fischer et al., 1997), as discussed in Chapter 1.

Nuclear speckles (interchromatin granule clusters) and perichromatin fibrils.

Pre-mRNA splicing factors accumulate in approximately 25-50 nuclear speckles (or intrachromatin granule clusters), as well as being diffusely distributed throughout the nucleoplasm (Spector, 1993). The diffuse localization is attributed to snRNPs interacting with nascent RNAs. Nuclear speckles are highly dynamic and their components appear to cycle between sites of active transcription and interchromatin granule speckles (Misteli et al., 1997). This cycling may be dependent on phosphorylation since inhibition of kinase or phosphatase activities alters the appearance of the speckles (Misteli and Spector, 1996), (Gui et al., 1994). Upon inhibition of transcription, the diffuse staining disappears and speckles become enlarged (Carmo-Fonseca et al., 1992; Spector et al., 1991).

Several functions have been proposed for nuclear speckles. For example, they may play a role in mRNA transcription or regulation based on evidence that the mRNA from some highly transcribed genes is enriched near speckles, or in mRNA export since they do not incorporate Br-UTP and lack detectable DNA, but do contain poly-A RNA (reviewed in (Lamond and Earnshaw, 1998)). Alternatively, speckles have been suggested to function as supply depots from which factors are recruited to sites of transcription (perichromatin fibrils), or regions of accumulation of snRNPs bound either to excised introns after their release from the spliceosome, or partially spliced pre-mRNA (Singer and Green, 1997; Spector, 2001). Not all splicing factors localize in an identical

manner. For example, SC35 is highly concentrated in discrete regions within the nucleus, in a pattern that is a distinct subset of that seen with anti-snRNP antibodies (Fu and Maniatis, 1990). Thus it was hypothesized that SC35 plays an active role in splicing and as a result is localized in perichromatin fibrils along with Pol II, whereas snRNPs and other SR proteins are mostly localized to nuclear speckles.

PML bodies

PML bodies are nuclear structures specifically disrupted in individuals suffering from acute promyelocytic leukemia (APL). PML bodies ordinarily number 10-30 per cell nucleus, however APL cells show a larger number of smaller PML-containing foci resembling broken up PML bodies. This is the result of a translocation event which fuses the PML gene to the retinoic acid receptor alpha (RAR α) gene (Goddard et al., 1991). Treatment with retinoic acid triggers degradation of PML- RAR α , restores PML bodies and results in remission of the cancer. In addition to the RING-finger motif containing protein, PML, these bodies contain a number of other proteins including the autoantigen Sp100 and SUMO1. The function of PML bodies is not known, though they have been suggested to play a role in transcription regulation, and appear to be targets of viral infection (Spector, 2001). The PML protein itself exists in two forms, one of which is dispersed throughout the nucleoplasm. The other is a form conjugated to SUMO1 (small ubiquitin-like modifier), which is exclusively localized to PML bodies (Muller et al., 1998).

In order to gain additional insight into the function of Nova, analysis of the subcellular localization of Nova by immunofluorescent microscopy was undertaken to

assess whether Nova, like many other splicing factors, localizes to discrete compartments within cell nuclei, and if so, is localization to a known compartment?

Results

Nova localizes to a novel subnuclear compartment.

Nova is a predominantly nuclear antigen that plays a role in alternative splicing regulation. To determine the subnuclear distribution pattern of Nova, N2A cells, which express endogenous Nova proteins by western blot analysis (data not shown), were analyzed by immunofluorescent microscopy. Nova resides in a number (usually 2-10) of distinct, heavily staining foci as can be seen in the first column of Figure 30 (green). These foci are not seen by staining with secondary antibody alone, or in cell lines that do not express Nova (data not shown). These cells were co-stained with antibodies to proteins known to reside in particular subnuclear compartments. In the first row cells are co-stained with an antibody which recognizes the core Sm proteins of spliceosomal snRNPs (sm), and thus stains nuclear speckles, in addition to diffuse staining throughout the nucleoplasm. The merged panel shows that whilst both antibodies show punctate staining within the nucleus, they clearly recognize distinct foci, thus Nova does not localize to classical nuclear speckles.

Nova also does not reside in gems, despite the similarity in the size and numbers of foci recognized by the two antibodies in the second row (Sip-1). Nova does not co-localize with the splicing factor SC35, or in PML bodies and is clearly excluded from nucleoli as can be seen by co-staining for the nucleolar protein, Nopp140, and by the presence of unstained regions within the nucleus of some cells (particularly evident in

first (Sm) row). In addition, Nova does not reside in Cajal bodies (p80 coilin) or in perichromatin fibrils (Pol II). Thus Nova in N2A cells localizes to a subnuclear compartment distinct from nucleoli, Cajal bodies, gems, nuclear speckles, PML bodies or perichromatin fibrils.

Actinomycin-D disrupts Nova foci.

Inhibition of transcription by agents such as actinomycin-D, α -amanitin and D, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) cause significant redistribution of many nuclear factors and alter the morphologies of nucleoli and nuclear speckles (Carmo-Fonseca et al., 1992; Spector et al., 1991). These agents cause enlargement of nuclear speckles and SC35 containing bodies (Fox et al., 2002). To determine whether transcription inhibition has a similar effect on Nova foci, cells were treated with actinomycin-D (5 μ g/ml) for 3 hours prior to fixation (Figure 31B). Untreated cells (A) received fresh medium. Cells were also treated with cyclohexamide (20 μ g/ml, 3 hours) to determine whether blocking protein synthesis had any effect on Nova localization (C), or actinomycin-D together with cyclohexamide (D). In contrast to other splicing factors, Nova bodies disperse upon actinomycin-D treatment, in the presence or absence of cyclohexamide, consistent with the hypothesis that the formation and maintenance of Nova foci is transcription dependent. Actinomycin D has also been shown to inhibit nuclear import of some shuttling proteins, including hnRNP K (Michael et al., 1997). Thus another possibility is that Nova foci are dependent on the presence of a protein that normally shuttles in a transcription-dependent manner, which gets trapped in the cytoplasm upon actinomycin D treatment. Alternatively, Nova itself could shuttle in a transcription-dependent manner resulting in dispersion of foci on actinomycin D

treatment. Cyclohexamide treatment had little or no effect on Nova distribution indicating that rapid new synthesis of proteins is not required for maintenance of Nova containing bodies.

Transfection of a splicing target does not disrupt Nova foci.

If Nova bodies act as reservoirs for the storage of inactive splicing factors as has been proposed for the role of nuclear speckles, high levels of transcription of a Nova target could be expected to disrupt Nova localization. To test this hypothesis, N2A cells were transfected with a region of GABA_AR γ 2 RNA known to contain a Nova-binding site and to be regulated by Nova-1 at the level of alternative splicing. The GABA_AR γ 2 region of the minigene D12/2A2 (Figure 15A) was cloned into a different parental vector, but still under the control of a CMV promoter. On the same construct, EGFP was expressed from an SV40 promoter in order to easily identify cells that had been transfected with the plasmid. Similar to the original minigene construct, GABA_AR γ 2 E9 inclusion was enhanced by the addition of exogenous Nova-1 (data not shown). As a control, cells were also transfected with the parental vector to ensure that any changes seen in Nova distribution could be attributed to GABA_AR γ 2 minigene expression. A schematic representation of these constructs is shown in Figure 32A. Figure 32B shows that the morphology of Nova foci is not changed upon high levels of transcription of a Nova target, or due to EGFP expression under the conditions tested. No difference was seen in cells analyzed 12 hours after transfection when EGFP expression is barely detectable (not shown), or 36 hours post-transfection (Figure 32B).

Nova forms subnuclear foci in mouse spinal cord

To determine whether Nova localizes to discrete foci in tissue, mouse spinal cord sections were stained with human anti-Nova serum and analyzed by confocal microscopy. Figure 33 shows that distinct, brightly staining foci (arrows) were detected in the nuclei of Nova positive cells in the ventral spinal cord, often in the vicinity of the nucleolus. Similar to the staining pattern in N2A cells, Nova appears to be completely excluded from nucleoli. These foci were much more difficult to detect in these cells compared to N2A cells, in part due to a higher level of diffuse Nova staining in tissue. In addition, fewer foci (at most 2 per cell) were seen in tissue compared to N2A cells. However, the visualization of these foci in tissue implies that the localization observed in cell cultures corresponds to a biologically significant process observable in the context of the whole brain.

Discussion

The compartmentalization of the nucleus into discrete domains contributes to the complexity of processes involved in gene expression and its regulation. Immunofluorescent microscopy was used to determine that Nova in N2A cells localizes to a subnuclear compartment distinct from known structures, including nucleoli, Cajal bodies, gems, nuclear speckles, PML bodies or perichromatin fibrils. Nova domains become dispersed upon inhibition of transcription with actinomycin-D, unlike nuclear speckles which harbor constitutive splicing factors and become enlarged upon transcription inhibition. This finding is consistent with the hypothesis that the formation and maintenance of Nova foci is an active process and is transcription dependent.

At least one other protein, YT521-B, which localizes to subnuclear bodies (called YT bodies) disperses upon treatment with actinomycin-D, but not other transcriptional inhibitors such as α -amanitin or DRB. Since actinomycin-D inhibits transcription by binding to GC rich regions of double stranded DNA, preventing RNA elongation, it was suggested that YT bodies form independently of transcriptional activity, possibly through a direct or indirect DNA-protein interaction (Nayler et al., 2000). Thus it would be interesting to further elucidate the role of transcription in the appearance of Nova foci using more direct inhibitors such as α -amanitin which inhibits transcription through direct inhibition of RNA polymerase II or DRB which blocks transcription through inhibition of an upstream kinase of RNA polymerase II (Zandomeni and Weinmann, 1984). Experiments involving visualization of regions of transcription by analysis of Br-UTP incorporation could also be used to determine whether Nova- containing bodies are recruited to sites of active transcription.

Transcription of a Nova target minigene from a highly active promoter (CMV) did not result in alterations in the appearance of Nova foci in N2A cells 12 or 36 hours post-transfection. This does not rule out the possibility that transient disruption occurs or that these domains become highly dynamic but do not disperse when presented with an abundance of a pre-mRNA which is spliced in a Nova-dependent fashion. Live cell imaging of cells transfected with GFP-tagged Nova proteins which also localize to discrete foci in N2A cells (A. D. Polydorides and R.B Darnell, unpublished data), could be used to address these concerns.

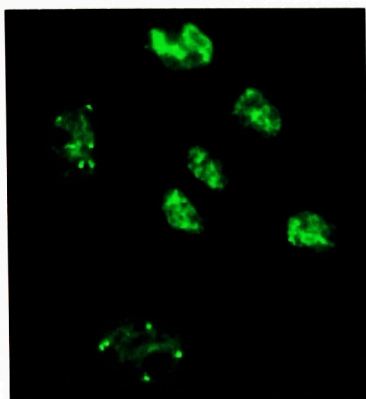
The results shown in this chapter, and the further studies suggested here are of some importance since Nova is also seen in foci in tissue (Figure 33), implying that the

localization observed in cell cultures corresponds to a process of biological significance in the context of the whole brain. Thus information regarding the localization and dynamics of Nova foci and their role in nuclear processes could be extended (cautiously) to the role of Nova in the brain.

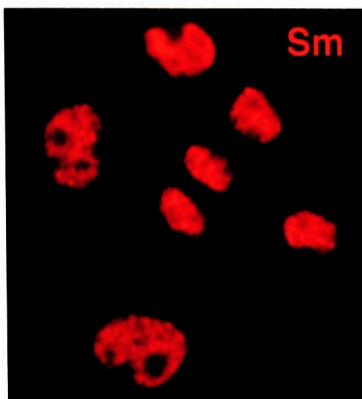
Figure 30: Nova localizes in to a novel subnuclear compartment.

N2A cells were fixed and stained with anti-Nova human serum and visualized with Cy2-conjugated anti-human secondary antibody (green, first column). Notice the distinct, bright-staining intranuclear foci. Cells were also co-stained with a second primary antibody to the proteins indicated which was visualized using the appropriate Cy3-conjugated secondary antibody (red, second column), and counterstained with DAPI (blue) to visualize cell nuclei. The third column is the merged composite of all three channels. The antibodies and concentrations used and the specific proteins recognized by each are listed in Chapter 2 (Materials and Methods).

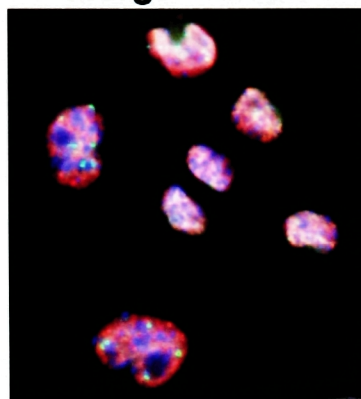
Nova



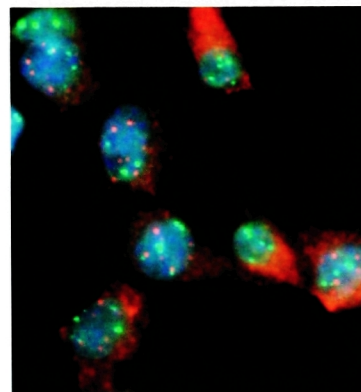
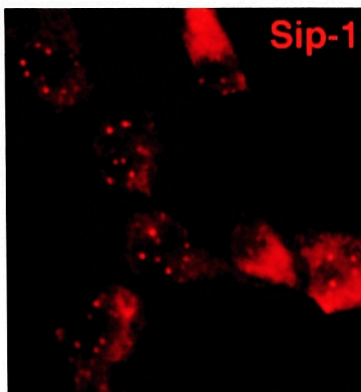
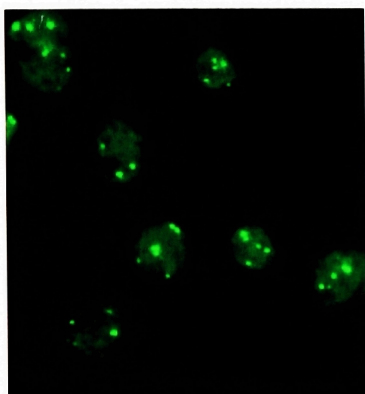
Sm



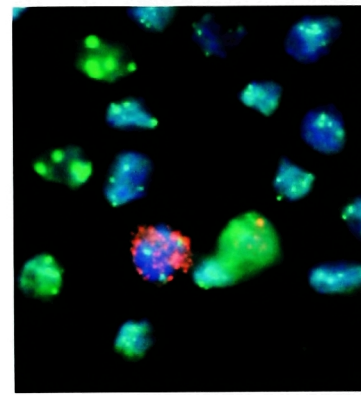
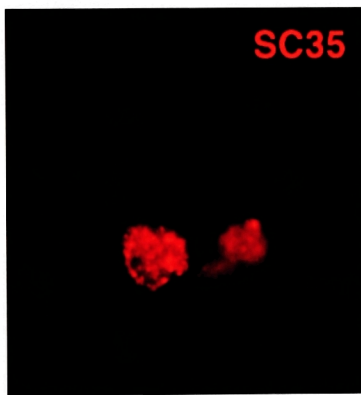
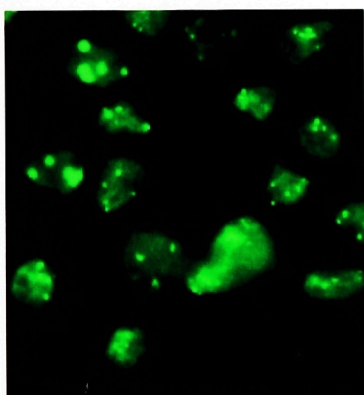
Merge + DAPI



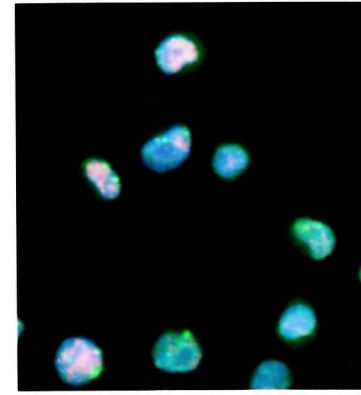
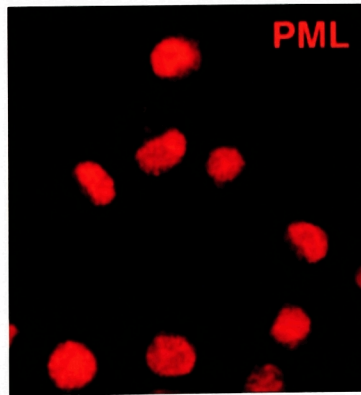
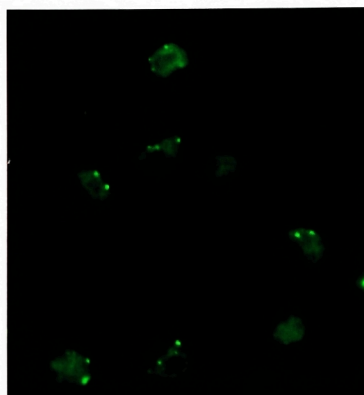
Sip-1



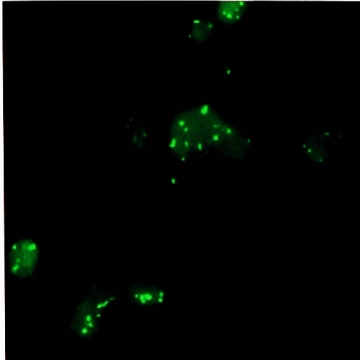
SC35



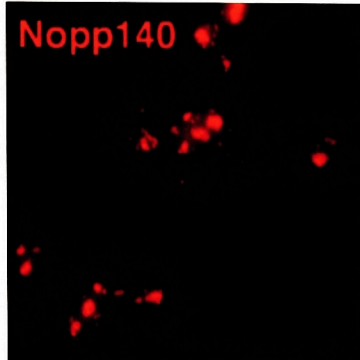
PML



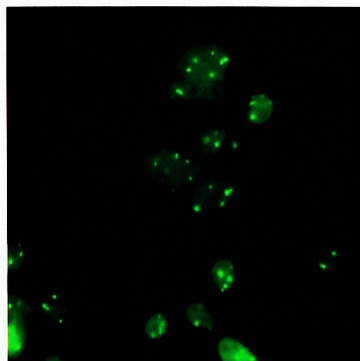
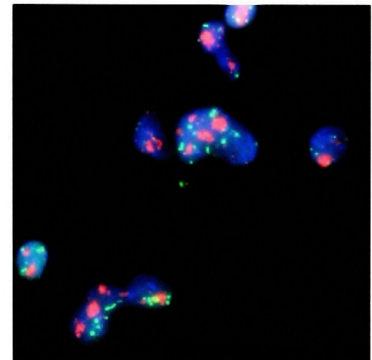
Nova



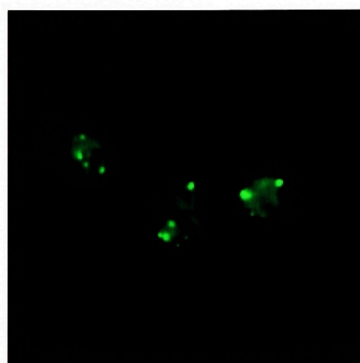
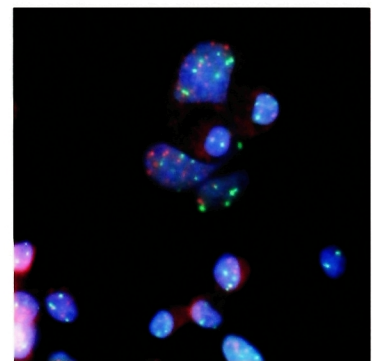
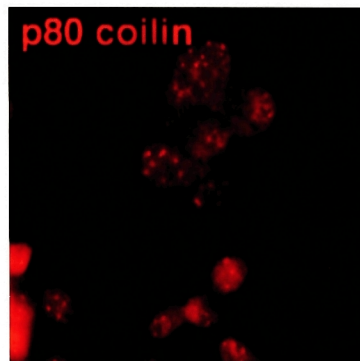
Nopp140



Merge + DAPI



p80 coilin



Pol II(CTD)

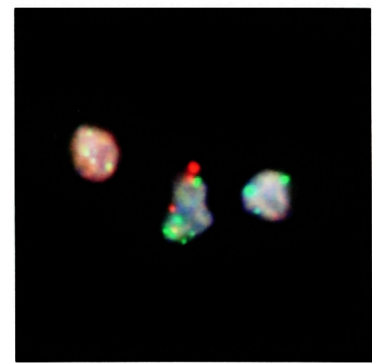
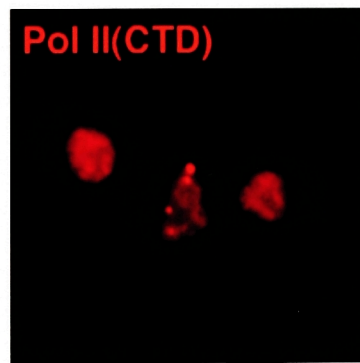


Figure 31: Actinomycin-D disrupts Nova-containing foci, cyclohexamide does not.

N2A cells were fixed and stained with anti-Nova human serum and visualized with Cy2-conjugated anti-human secondary antibody (green, first column) after the following treatments:

- (A) Untreated.
- (B) Actinomycin-D: 5 μ g/ml, 3hours.
- (C) Cyclohexamide: 20 μ g/ml, 3hours
- (D) Actinomycin-D + cyclohexamide: as in (B) and (C).

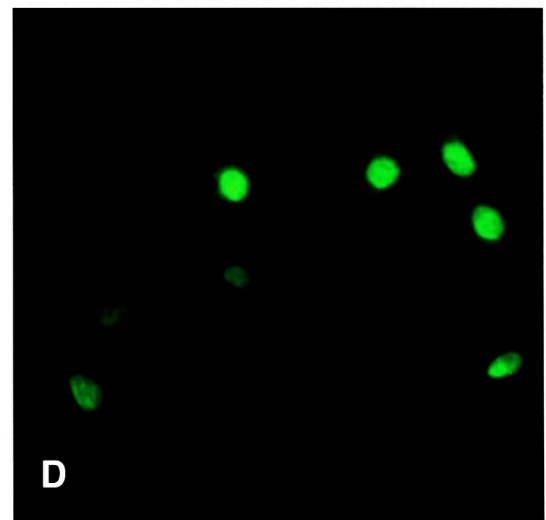
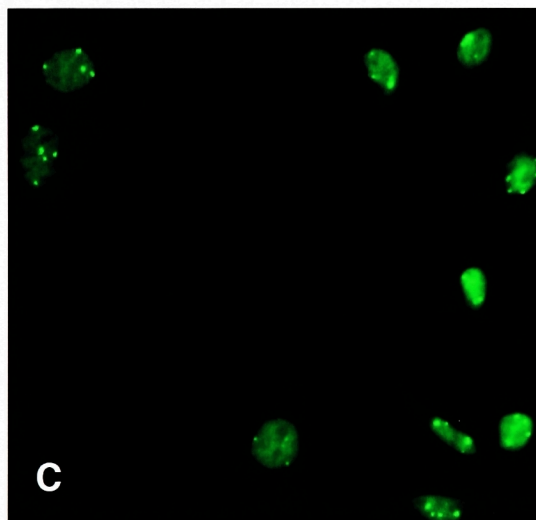
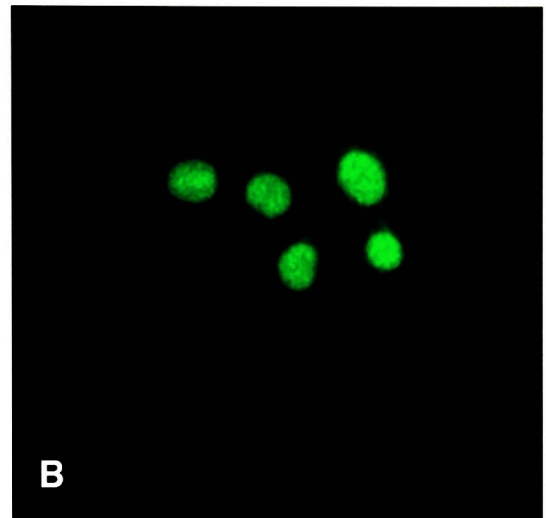
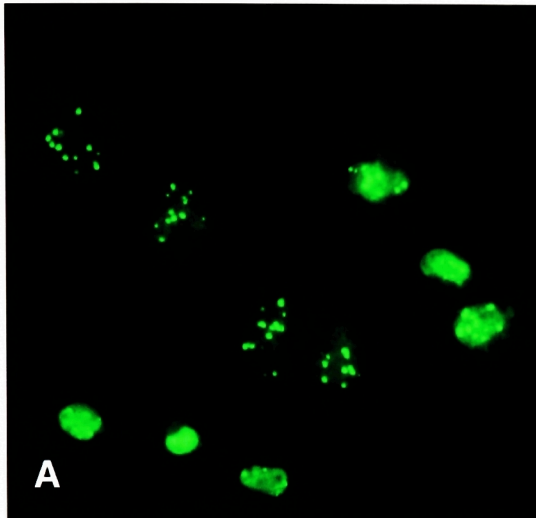
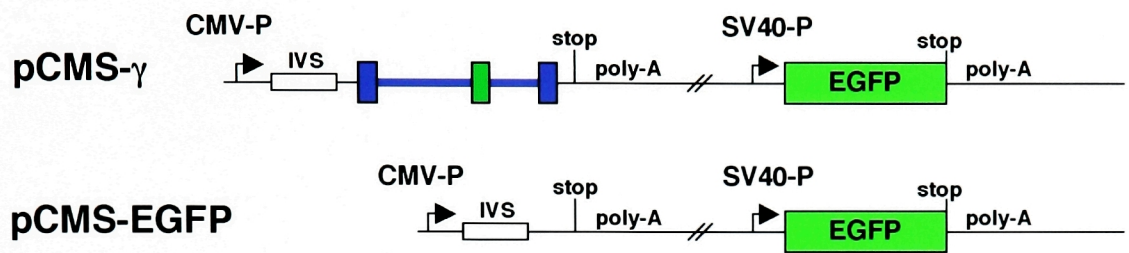


Figure 32: Transfection of a splicing target does not disrupt Nova foci.

- (A) Schematic representation of plasmids used in this study. pCMS- γ contains the truncated GABA_AR γ 2 minigene region from plasmid D12/2A2 (see Figure 15A) inserted into the multiple cloning site of pCMS-EGFP (Clontech). The Nova target (minigene) RNA is transcribed from a CMV promoter. Transfected cells express EGFP from an SV40 promoter contained within the same construct.
- (B) N2A cells were grown on chamber slides and transfected with pCMS- γ or the parental vector pCMS-EGFP. 12 hours (not shown) or 36 hours post-transfection, cells were fixed and stained with anti-Nova human serum and visualized with Cy3-conjugated anti-human secondary antibody (red, second column). EGFP expression is shown in the first column (green). Cells were also counter-stained with DAPI to visualize cell nuclei. The third column is the merged composite of all three channels.

A



B

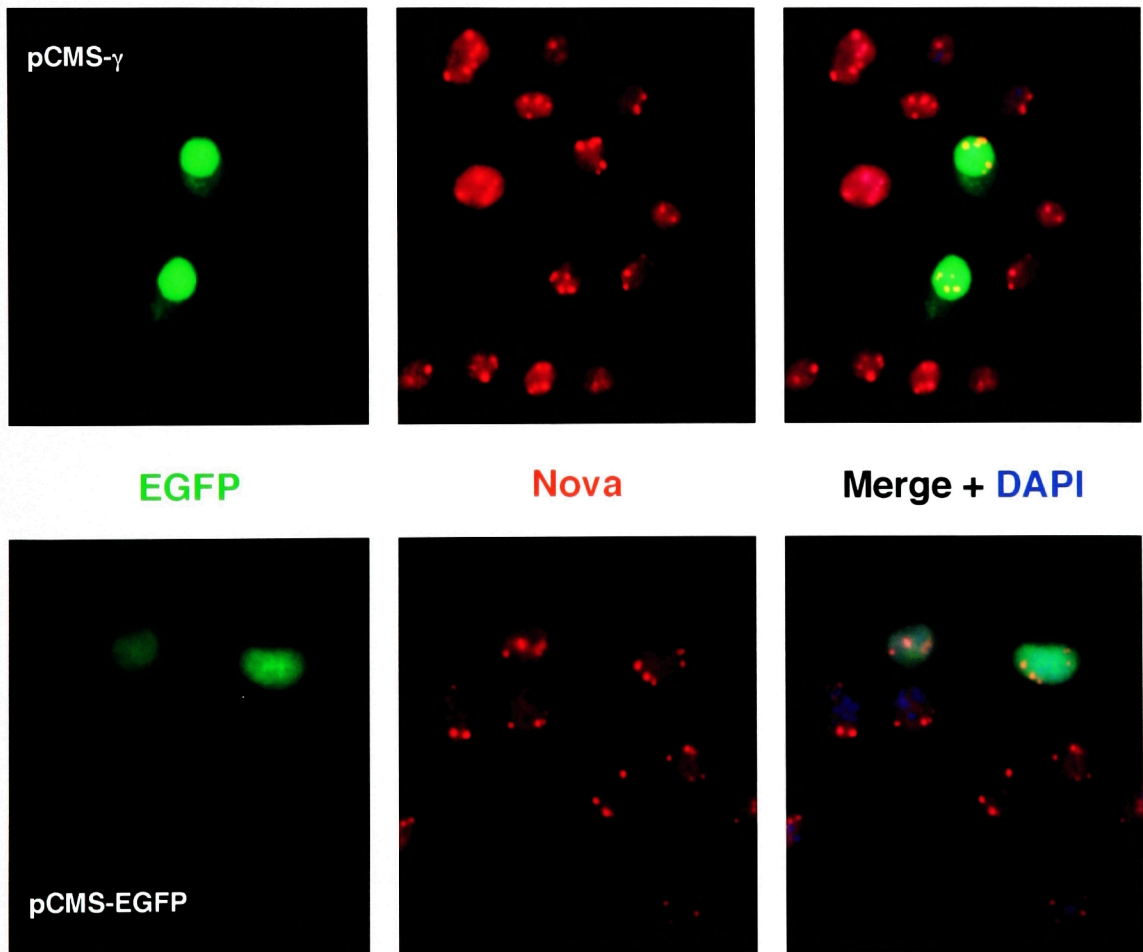
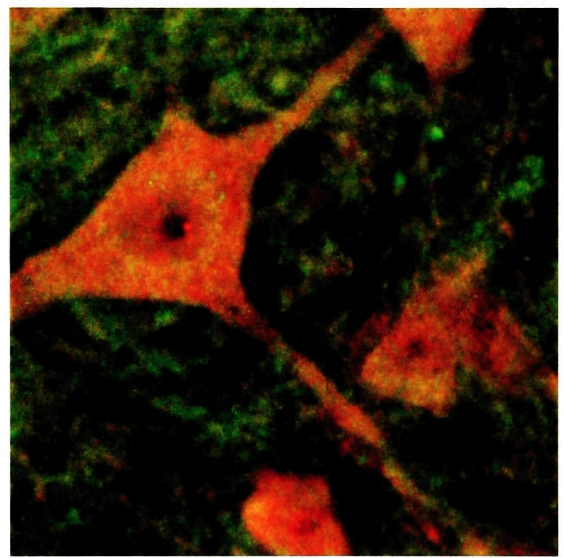
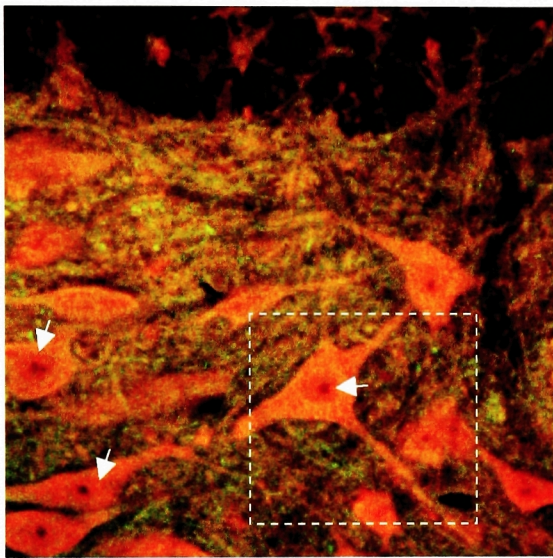


Figure 33: Nova forms subnuclear foci in mouse spinal cord.

Confocal laser image of Nova expression pattern in P5 {check} mouse ventral spinal cord. Horizontal spinal cord sections were stained with anti-Nova human serum and visualized with Cy5-conjugated anti-human secondary antibody (red). Co-staining is with anti-MAP2 antibody and Cy2-conjugated secondary antibody (green). Heavily staining Nova foci are highlighted by arrows. The boxed area (dashed lines) is shown at higher magnification on the right.



Nova

MAP2

Chapter 6 – Differential gene expression screens

Introduction

Differences in gene expression underlie a vast number of cellular processes from fate determination and specification of cell type to formation and maintenance of synapses in the brain. In addition stimuli such as drug exposure, environmental changes, deprivation of required factors can all influence gene expression which may in turn exert dramatic changes on cellular phenotype, fate and function, so it is not surprising that comparisons of gene expression between populations of cells is of great interest in many biological systems. Numerous different methods have arisen which aim to identify these genes in order to better understand the processes involved at a molecular level. The various methods differ in their applicability to different systems, their ease of execution, sensitivity and reliability.

Non-selective methods:

1) Differential screening involves screening a library (or libraries) in duplicate with cDNA probes generated from two different RNA populations of interest. Colonies or plaques harboring clones representing differentially expressed RNAs should hybridize differentially to the two probes and thus can be isolated and analyzed further. This approach is readily available, rapid and relatively inexpensive and has been used successfully even at the single cell level (Dulac and Axel, 1995). However, differential screening is relatively insensitive, so it can only be used in situations when the RNAs involved are abundant and the differences in expression level are high.

2) Serial analysis of gene expression (SAGE), developed by Velculescu et al. (Velculescu et al., 1995) allows quantitative and simultaneous analysis of a large number of transcripts. Briefly, double stranded cDNA is generated using biotinylated oligo(dT) primer and cleaved with a frequently cutting restriction endonuclease (i.e. one with a 4bp recognition site). The 3'-most fragment of each transcript is then isolated by binding to streptavidin coated beads. This pool is then divided in half and ligated to one of two linkers containing the recognition site for a restriction enzyme which cleaves at a site distant from its recognition site. Cleavage with this enzyme results in the release from the beads of the linker with a short piece of the cDNA, generating sequence tags. These tags are then amplified, concatenated, cloned and sequenced. Comparison of the frequency of each tag in each of two populations then forms the basis of the analysis of differential gene expression. The disadvantages of this technique, however, are that it requires a dedicated sequence facility and also biases towards the 3' ends of cDNAs.

3) cDNA arrays are becoming more widely available for these types of studies and offer a rapid method of screening a vast number of discrete sequences. These may take the form of membranes or chips spotted with an array of different cDNAs or oligonucleotides (either commercially available or custom made). The use of chips allows orders of magnitude more sequences to be analyzed at one time and detection is usually more sensitive than that achieved with membrane arrays, however the cost involved is also orders of magnitude higher! In addition, arrays are not currently very useful for distinguishing between alternative spliced forms of mRNAs.

4) For differential display (DDRT-PCR) (Liang and Pardee, 1992) random PCR primers are used to generate a series of bands which are then separated in polyacrylamide

gels. Bands differing in intensity between two populations are isolated from the gel, cloned and sequenced. In order to cover a statistically significant portion of the population, a large number of primer pairs and sequencing gels are involved making this technique quite labor intensive. Abundant non-differentially expressed species can also obscure the detection of other species of interest, and high rates of false positives have been reported (Wan and Erlander, 1997).

Selective Methods

1) Subtractive hybridization is the basis behind the first selective methods reported for isolating differentially expressed genes (for review see (Milner et al., 1995)). Two single stranded cDNA or RNA libraries are generated; one from the population of interest (the “tester”) and a second, complementary (antisense) library from the control (“driver”) population, and mixed such that the driver is in excess. Hybridization of the two libraries is followed by subtraction of double stranded hybrids which represent species shared by the two populations. This is achieved either using hydroxyapatite columns (Wan and Erlander, 1997) or by tagging the driver cDNA with biotin and removing the driver-containing species through streptavidin binding and organic extraction (Akopian and Wood, 1995; Sive and St John, 1988). The remaining single stranded species of the sense strand are then either used as probes for library screening or converted to double stranded cDNA and cloned. Although highly successful for some applications for example cloning of the T-cell receptor (Hedrick et al., 1984) and identification of genes that are activated at the gastrulation stage of *Xenopus laevis* (Sargent and Dawid, 1983) there are two primary drawbacks to subtractive hybridization. Firstly, small differences in abundance will not be detected because if the fold increase of

a particular species in the tester population is less than the fold excess of driver used in the hybridization reaction, these species will be lost during the selection procedure. Secondly, the selection procedure is a negative one, that is unwanted species are removed from the reaction rather than desired species being positively selected. If the selection does not proceed with 100% efficiency, which is likely since hybridization kinetics approach but never reach completion, unwanted species will be retained in the selected population resulting in a higher rate of false positives and possible masking of desired species of low abundance.

2) Suppression hybridization kits are available commercially (eg. PCR-Select, Clontech) in which abundant species, deemed to be of less interest, are suppressed by selective PCR of single-stranded species after hybridization of tester and driver populations. This then allows rare differentially expressed species to be identified by traditional non-selective methods somewhat more easily and with less false positives (Diatchenko et al., 1996; von Stein et al., 1997), however there is the risk of losing modest differences which are suppressed.

3) Competitive hybridization. In this instance double stranded tester and driver populations are hybridized together rather than single stranded libraries, so there is competition between the formation of heterohybrids deriving one strand from each of the tester and driver populations, and homohybrids containing either two tester strands or two driver strands. Assuming random assortment, molecules more abundant in the tester population will form a higher proportion of tester homohybrids relative to tester/driver heterohybrids than molecules which are expressed equally in both species. Selection of tester homohybrids thus results in an enrichment of species more abundant in the tester

population. Enrichment of such species will occur even in instances when the difference in abundance is less than the excess of driver to tester used, a clear advantage over subtractive hybridization approaches. Enrichment to detectable levels, especially of rare species, is achievable but may require several rounds of selection and PCR amplification which in itself can also introduce problems. Several methods of selection of tester homohybrids after competitive hybridization have been published. These include:

- (a) Biotinylation of the driver species followed by streptavidin binding and organic extraction (Wang and Brown, 1991).
- (b) Enzymatic degradation selection (EDS) in which tester molecules are tagged with thiolated nucleotides, thus rendering tester homohybrids resistant to digestion with a combination of exonucleases III and VII (Zeng et al., 1994).
- (c) Cloning of only tester homohybrids by the presence of compatible restriction site overhangs only on tester molecules (Klickstein, 1995).
- (d) Representational difference analysis (RDA) (Lisitsyn and Wigler, 1993). PCR primers are ligated only to tester molecules which results in exponential amplification of tester homohybrids only.
- (e) Biotin and restriction enrichment (SABRE) (Lavery et al., 1997). Tester homohybrids are selected by biotin/streptavidin binding and restriction enzyme reconstitution.

The first two selection methods suffer from the issues of negative selection discussed above for non-selective procedures, (c) and (d) are positive selection procedures, but both require substantial manipulation between rounds of selection. For competitive hybridization to be successful hybridization must be efficient to avoid the loss of rare species, in addition, like all methods utilizing PCR amplification of the

populations, bias can be introduced through preferential amplification of some species. The SABRE system developed by Lavery et al (Lavery et al., 1997), addresses these problems whilst still benefiting from the advantages of competitive hybridization for isolation of rare species displaying moderate to low (less than ten-fold) differences in abundance.

Two of these methods, screening of nylon cDNA arrays and SABRE, were undertaken to identify mRNA species differentially expressed in Nova-1 null mice compared to their wild-type littermates. The SABRE technology is of particular interest as it has the potential to identify changes in only a region of a message which is differentially expressed, for example an alternative exon whose level of inclusion differs between the two populations being tested. Several species cloned after SABRE selection appear to fall into this category and warrant further investigation.

Results

cDNA Arrays

ATLASTM cDNA expression arrays (Clontech) containing 588 mouse cDNAs spotted in duplicate were screened using RNA isolated from the spinal cords of P12 Nova-1 null and wild-type littermate mice, or the hindbrains of P0 mice. Both regions express Nova-1 at high levels and are thus likely to show greatest changes in gene expression in Nova-1 null mice. Uniformly labeled single stranded cDNA was generated using primers supplied in the kit specific for the range of genes to be screened using polyA⁺ RNA as a template. The resulting libraries of fragments were size fractionated via column elution then used to probe identical blots. Figure 34 shows the results after

hybridization of the P12 spinal cord probes. Some differences in gene expression can be detected readily by eye, for example the cDNA spots corresponding to insulin-like growth factor binding protein 2 (IGF-BP2) appear darker on the blot probed with the library generated from wild-type mice, indicating that this message occurs with lower abundance in Nova-1 null mice.

Quantification was performed by phosphorimager analysis including normalization with respect to a selection of housekeeping genes (bottom row of spots, Figure 34), the levels of which are assumed to be the same in the two RNA populations. A summary of the differentially expressed genes detected by this method is listed in Table 1. Only positions which showed moderate to high levels of hybridization, and where differences in hybridization signals between Nova-1 null and wild-type probes were apparent are shown. Care was taken to ensure that any differences were indeed the result of higher levels of hybridization to both of the duplicate spots on one filter compared to the other for a particular gene, not the result of high levels of background labeling in a particular region of one filter. No dramatic differences (>2 fold change in both experiments) were seen in the expression of any genes represented on the filters. Of the five messages (shown in bold in Table 1) which showed differences in abundance in both tissues tested, P0 hindbrain and P12 spinal cord, four showed modestly higher abundance in wild-type tissues compared to those from Nova-1 null mice. The fifth, **BAG-1**, was decreased in wild-type P0 hindbrain, but increased in P12 spinal cord compared to Nova-1 null tissues. This could be the reflection of differences in cellular responses to a lack of Nova-1 in the two different tissues or at the two different ages or,

more likely, the result of natural variation between individual mice. Further analysis is required to confirm or deny these results.

SABRE (Selective amplification via biotin- and restriction-mediated enrichment)

SABRE was performed using libraries prepared from the hindbrains, and separately the spinal cords, of P0 Nova-1 null and wild-type mice in order to assess differential gene expression of the whole complement of transcripts expressed in Nova-1 rich regions of the CNS. This methodology was also chosen as it has the potential to aid in identification of additional candidate messages regulated by Nova-1 at the level of alternative splicing. P0 mice were chosen because at this age no neuronal degeneration is evident in Nova-1 null mice and thus changes in gene expression detected are less likely to be due downstream effects of neuronal dysfunction, and thus more likely to be a direct result of the absence of Nova-1. Figure 35 outlines the protocol used. Double stranded cDNA libraries were generated from RNA extracted from spinal cords or hindbrains of 20 Nova-1 null or wild-type littermate mice. The cDNA was then digested with *Sau3A*, ligated to linkers of fixed sequence, and size selected by agarose gel electrophoresis. These libraries were then amplified by PCR using primers specific to the linker regions; each library was amplified using either the tester-specific (*Bam*HI containing, biotinylated) primer T1, and the driver-specific primer D1, in separate reactions.

Following amplification, the two populations were mixed with a 30-fold excess of driver, ethanol precipitated and resuspended in hybridization buffer. Hybridization was performed using a modification of the phenol emulsion reassociation technique (PERT) developed by Miller and Riblet (Miller and Riblet, 1995) in a thermal cycler. Under

these conditions DNA reassociation equivalent to that achieved after one week of hybridization under standard conditions in drawn out capillary tubes is achieved overnight. At the end of the hybridization procedure, single stranded (unhybridized) species were digested with S1 nuclease and the mixture was incubated with streptavidin coated magnetic beads. The beads were washed to remove unbiotinylated species, thus retaining only hybrids harboring at least one strand derived from the tester population. Further enrichment was achieved by eluting only tester-tester homohybrids by restriction digest with *Bam*H1. This population was then further amplified using primer T1 for use in successive rounds of selection.

To minimize false positives introduced as a result of preferential amplification of certain sequences by PCR, and other manipulations, a control hybridization and selection reaction is performed using the control population (the driver in the experimental hybridization) as both driver and tester. This population is used as the driver in subsequent rounds of selection. In theory, this population should be identical to the starting population. By comparing experimental and control populations after selection, species with an increased concentration in both populations can be excluded from further analysis.

Selection in this manner was performed such that RNA species whose steady state levels are increased or decreased will be detected by using the Nova-1 null libraries as either the tester population, or alternatively, as the driver population. Figure 36A shows the results of one such experiment using spinal cord cDNA libraries and selecting for species of higher abundance in the Nova-1 null mice. A fraction of both the experimental (or tester, T) and control (driver, D) reactions was body labeled with $\alpha^{32}\text{P}$ -dATP during

PCR amplification after each round of selection and separated by denaturing PAGE. Species enriched in the tester but not the driver population after five rounds of selection were excised from the gel, PCR amplified, cloned and sequenced. Figure 36B shows a region of the gel from which 3 enriched species were identified. The identification of one of these bands as LacZ was highly encouraging given that this gene is driven from the Nova-1 promoter in the null mice. Table 2 lists the species identified in this manner as enriched in Nova-1 null (A,B, spinal cord and hindbrain, respectively) or wild-type (C,D, spinal cord and hindbrain, respectively) tissues. Clones which corresponded to ribosomal RNA or appeared in both populations (i.e. upregulated in Nova-1 null and wild-type libraries) were not included. These comprised approximately 20% of the independent clones sequenced.

Confirmation of these results by Northern blot, RT-PCR or RPA is required. Figure 37 shows RT-PCR confirmation of clone 1H36 which corresponds to contactin associated protein 1 (Cntnap1), a neurexin-like axonal transmembrane molecule mainly localized at the paranodal junction. Cntnap1 (also called neurexin IV or Paranodin) mRNA level is increased in the hindbrains of Nova-1 null mice compared with heterozygous or wild-type littermates by a factor of 1.64 fold (± 0.22 , $n=6$). Taken together with the identification of LacZ, these results indicate that the SABRE procedure was successful in identifying all-or-none differences in gene expression between two samples (i.e. LacZ which is only expressed in null mice, not wild-types) and subtle alterations as was seen for Cntnap1.

Database searches using sequences obtained from SABRE clones showed that a number of these clones correspond to one of two or more alternative isoforms of the

mRNA identified. For example, MAP1A mRNA is transcribed from 2 alternative promoters, generating a long and a short transcript (Nakayama et al., 2001). Clone 2S31 matches a region of the gene which is specific to the long transcript and extends into the region common to both (depicted in Figure 38A). Attempts to assess Nova's effect on the levels of these two transcripts by transfection of N2A cells (which express both transcripts, (Nakayama et al., 2001)) with exogenous Nova-1 were complicated by the fact that the ratio of the two mRNA forms was altered by transfection of the empty expression vector (data not shown).

Clone 1H60 matches a region of a large intron present in rat Shank3a. When this rat mRNA sequence is compared to mouse sequences in the database (by BLAST analysis), the closest match is a transcript, Shank3b, which is almost identical to the rat sequence, but lacks this large exon (exon 8A, Figure 38B). Since this clone was selected from a mouse library, it is hypothesized that this may represent an alternatively spliced exon in mice. RT-PCR using primers to exons 8 and 9 or exons 8A and 9 confirmed the existence of both species in mouse hindbrain (not shown). Further analysis by Northern blot or RPA is required to determine whether the enrichment of this clone during the SABRE procedure is the result of a difference in overall message level, or a change in alternative splicing of this exon. Analysis of splicing by RT-PCR similar to the approach used to assess GABA_AR γ 2 alternative splicing would not be appropriate given the large size (2.3kb) of the exon to be spanned.

EST data suggests that clone 1H53 also corresponds to one of two alternative isoforms of an mRNA encoding a protein of unknown function. In this instance, it appears that the clone extends into one of two alternative 3'-terminal exons as depicted in

Figure 38C. No ESTs or other cDNA sequences were found that contained both exon 6A and exon 7 in the same transcript, however these two exons lie within close proximity in genomic DNA (Celera). Again, further analysis by is required to determine whether the enrichment of this clone is the result of a difference in alternative splicing of this exon, or overall message level of both transcripts.

Discussion

Differential gene expression screens are powerful tools for comparing the mRNA profiles of two or more populations. As discussed in the introduction of this chapter, there are many methodologies for performing such screens, each with their own set of advantages and disadvantages and the use of several of these techniques to address a specific question can be particularly powerful. cDNA libraries prepared from Nova-1 null and wild-type littermate mice were screened using two of these methods; cDNA arrays and SABRE. The steady state levels of a number of transcripts were found to be different in the two populations.

Differential gene expression screens are usually employed to detect differences in the level of transcription of messages in different biological samples; however, what they really detect is differences in the steady state levels of messages. RNA processing events in the nucleus and cytoplasm are linked in ways that are just beginning to be explored. Alterations at any step of the process can have profound effects on the downstream processing of a transcript. For example, splicing of non-coding regions of a transcript can result in incorporation of different signals in the form of sequence or associated proteins which result in altered stability, polyadenylation, nuclear export and/or translation of a message, and ultimately changes in steady state mRNA level. Thus analysis of Nova-1

null mice by these approaches not only assesses the transcriptional alterations, some of which are most likely due to indirect effects of the lack of Nova-1 expression such as neuronal dysfunction and degeneration, but also the downstream effects of misregulation of Nova-1 target RNAs. The accumulation of GlyR α 2 mRNA in Nova-1 null mice detected during experiments presented in chapter 3 is hypothesized to be the result of incorrect processing of this message, perhaps inefficient nuclear export, as a result of the lack of Nova regulation of alternative splicing. Similarly, other messages may be incorrectly spliced and as a result incorrectly processed, and detected by the methods described in this chapter.

Interestingly, at least 3 of the clones isolated during the SABRE screen appear to correspond to alternative isoforms of the mRNA identified. In one instance (Shank), the difference between the two transcripts appears to be at the level of alternative splicing, consistent with the known role of Nova-1 in neurons. A second difference is detected in a transcript (clone 1H53, unknown function) with an alternative 3'-terminal exon and thus at the level of both alternative splice choice and regulation of polyadenylation. PTB has been shown to regulate both exon inclusion and polyadenylation of the alternative 3' terminal exon, exon 4 of CT/CGRP (Lou et al., 1999), so by analogy Nova-1 may also play a dual role in splicing and polyadenylation of the message corresponding to clone 1H53.

Finally, one clone corresponded to a message transcribed from an alternative promoter (2S31, MAP1A). FMRP is predicted to regulate translation and/or stability by binding to a region of the 5'UTR of the long transcript of MAP1B (Darnell et al., 2001), and J.C. Darnell (personal communication), which has an almost identical gene structure

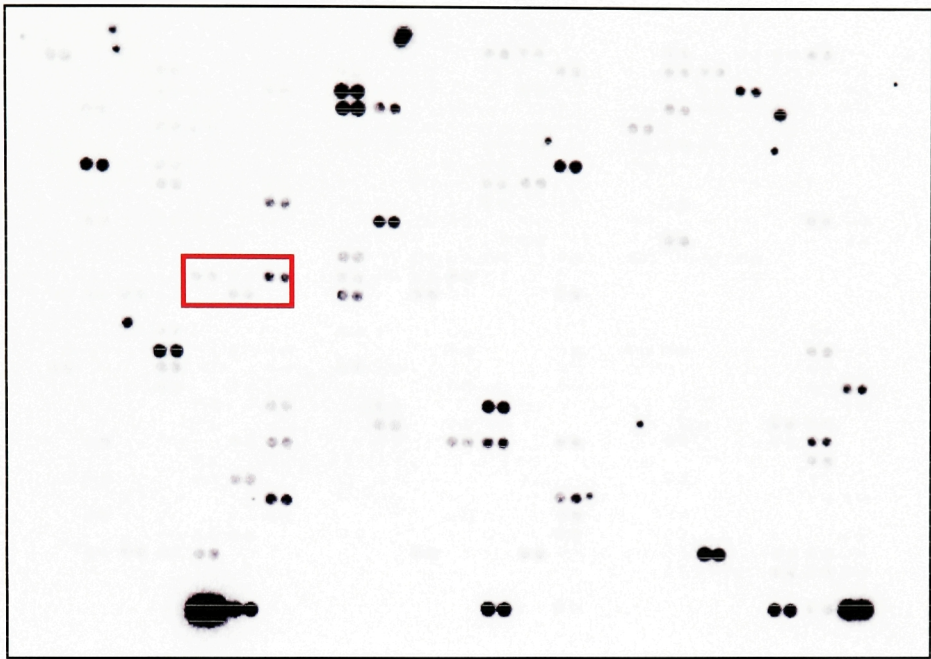
to MAP1A. By analogy, the difference in steady-state level of this transcript in Nova-1 null mice may reflect specific association of Nova-1 with one of the alternative transcript and subsequent regulation of its post-transcriptional processing. Another formal possibility is that Nova plays a role in the transcriptional regulation of one of the MAP1A transcripts. Although no role in transcription regulation has been suggested for Nova proteins, other RNA binding proteins such as hnRNP K have been hypothesized to function as transcriptional regulators (Takimoto et al., 1993). In addition, spliceosomal U snRNPs have been shown to promote transcriptional elongation, probably through interactions with the elongation factor TAT-SF1, leading to the hypothesis that recruitment of U snRNPs near the elongating polymerase is important for transcription (Fong and Zhou, 2001).

In addition to identifying a number of interesting candidate Nova-1 targets, this work has paved the way for additional, more complicated experiments to be performed. These experiments, which are currently ongoing in the laboratory, incorporate additional manipulations in order to address the aim of identifying Nova-1 RNA targets more directly, but are in essence very similar to the screens discussed here. One such experiment involves the co-immunoprecipitation of Nova, followed by assessment of the bound RNAs by microarray analysis. A similar technique has been used successfully in the identification of RNA targets of FMRP (Brown et al., 2001). A second approach involves the cross-linking of Nova to target RNAs *in vivo* by UV irradiation and the subsequent co-immunoprecipitation, isolation and identification of cross-linked species. These approaches, combined with those presented in this chapter should yield valuable information regarding additional Nova targets and thus Nova function *in vivo*.

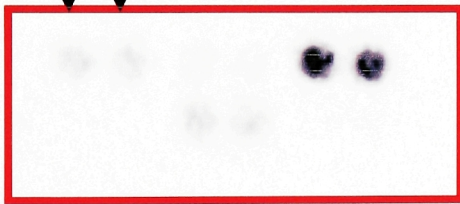
Figure 34: cDNA expression arrays

Duplicate cDNA arrays (Clontech) were probed using uniformly labeled single stranded cDNA libraries generated from RNA isolated from Nova-1 null (top) or wildtype littermate (bottom) mice at ages P0 (hindbrain, not shown) and P12 (spinal cord, shown). Each cDNA is spotted in duplicate. Quantification was performed by phosphorimager analysis with normalization to a selection of housekeeping genes, the levels of which are assumed to be the same in the two populations. Regions displaying different levels of hybridization to the two different probes allow identification of messages which are differentially regulated in the two populations, for example insulin-like growth factor binding protein (IGF-BP2) (inset, highlighted by arrows).

-/-



IGF-BP2



IGF-BP2



+/+

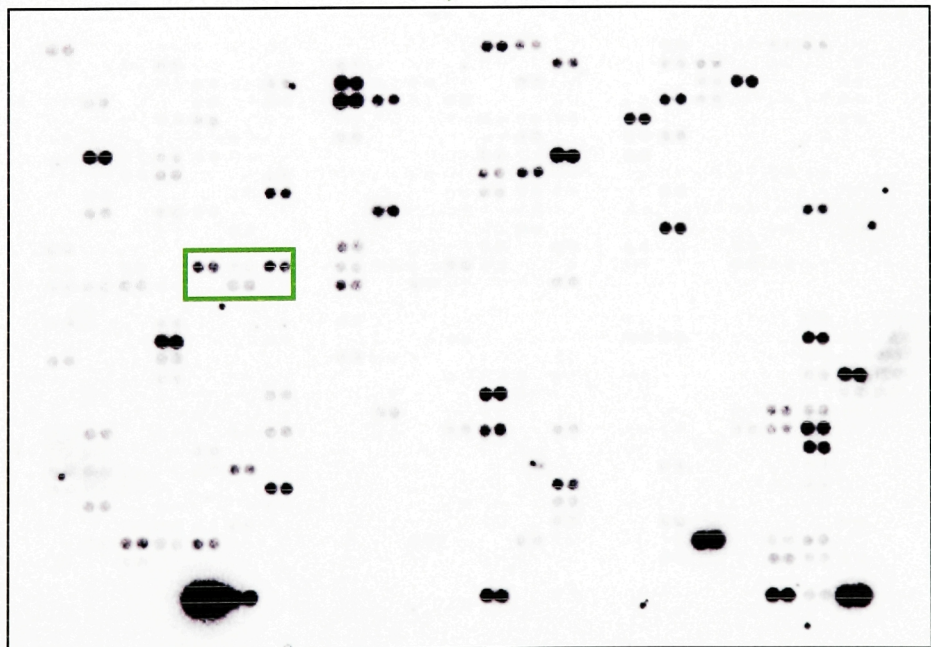


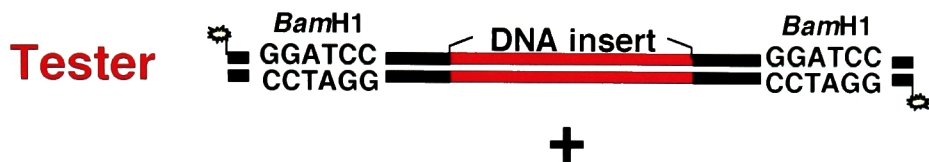
Table 1: Differentially expressed genes identified using Clontech cDNA arrays.

Quantitation of the data presented in Figure 34, and from arrays probed with libraries generated from the spinal cords of P0 mice. Only positions which showed moderate to high levels of hybridization, and where differences in hybridization signals between Nova-1 null and wildtype probes were apparent are shown.

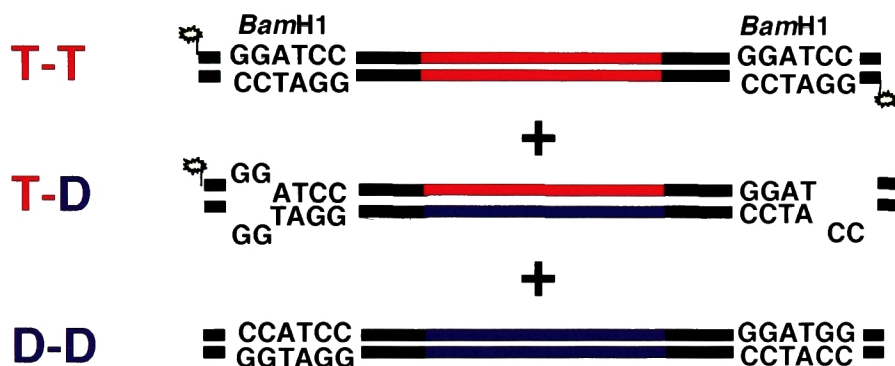
	P0 Hindbrain		P12 Spinal cord	
	<u>Gene</u>	<u>wt/ko</u>	<u>Gene</u>	<u>wt/ko</u>
Oncogenes and tumor suppressors	p53	0.31	TTF-1	1.56
	IGFBP-2	2.33	IGFBP-2	1.95
Cell cycle regulators	p21	5.38		
	p19	2.50		
Stress response proteins	HSP84	0.54		
	HSP86	0.67		
Intracellular signal transduction modulators and effectors	TF A10	5.01	Fyn	2.05
			MAPKK 1	1.52
			Gem	1.70
			Erk 1	1.51
			PKC-theta	1.52
Apoptosis-related proteins	BAG-1	0.61	BAG-1	1.69
	Bax	2.13		
	c-Akt	2.18	c-Akt	1.78
DNA synthesis, repair and recombination proteins	HR23spA	5.53	MHR23B	2.30
Transcription factors and general DNA binding proteins			Ada-1	1.42
			DP-1	1.92
			EPS8	2.46
			HMG-14	2.37
			LKLF	1.77
Neurotransmitter receptors			5-HT Rc 1c	0.38
Cell surface antigens and cell adhesion proteins	Lamimin Rc1	2.99		
	N-cadherin	1.84		
Cytoskeleton and motility proteins	Vimentin	2.21	Vimentin	1.74
			Nm myosin LC3	2.17
			CDC42	3.09
	Cam KII	2.14	CamK II	1.89
Proteases and inhibitors			ACE	1.84
			Cathepsin B	2.08
			CTLA-1	2.40
			PN-1	2.11
Growth factors, cytokines and chemokines			Neuroleukin	1.87

Figure 35: Selection of tester homohybrid species by SABRE

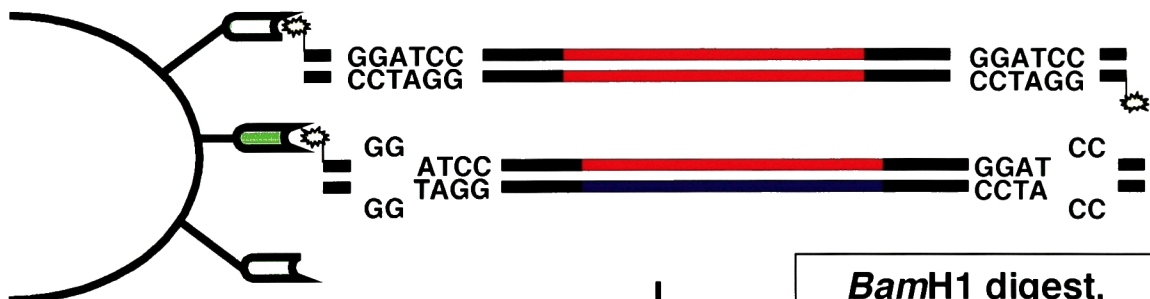
Tester DNA, biotinylated at each 5' end (yellow star) and with a *Bam*HI restriction site in each primer sequence, is hybridized with an excess of driver DNA, which is unbiotinylated and contains a mutated *Bam*HI site in the primer sequence. Three types of double-stranded molecules are formed: tester homohybrids, driver homohybrids, and driver-tester heterohybrids. Tester homohybrids and driver-tester heterohybrids are purified by capture of biotin-containing hybrids on streptavidin (green)-coated magnetic beads. Next, digestion with *Bam*HI specifically releases the tester homohybrids from the beads. The released molecules are then reamplified by PCR for further analysis and selection. Figure adapted from (Lavery et al., 1997).



Denature, rehybridize



Bind to streptavidin-coated magnetic beads



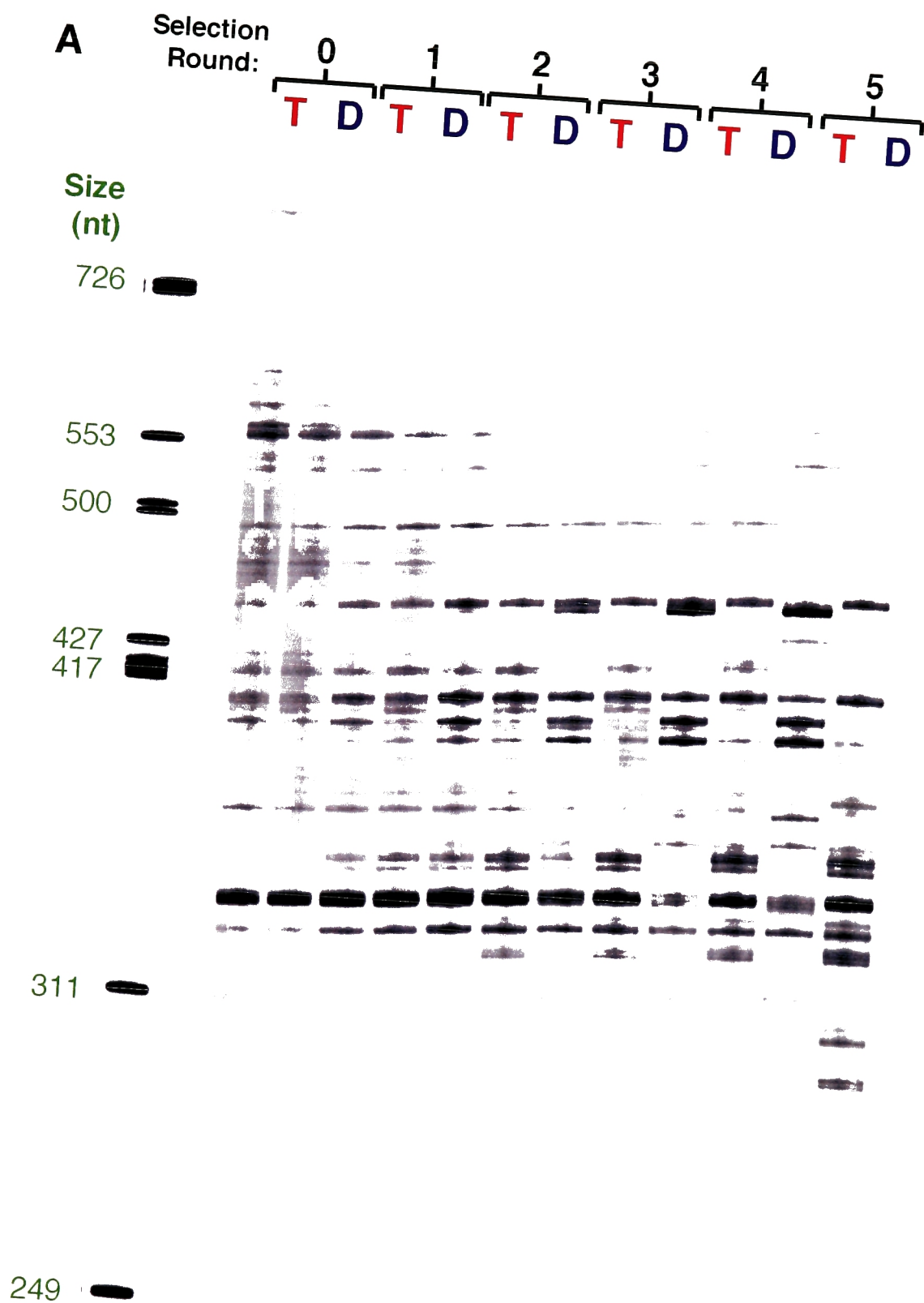
*Bam*H1 digest, take supernatant



PCR amplify with

Figure 36: Enrichment of cDNA fragments overexpressed in spinal cords of Nova-1 null mice.

- (A) Representative autoradiogram showing enrichment of specific bands following successive rounds of SABRE selection. SABRE was performed on *Sau3A*-digested cDNA libraries from spinal cord mRNA samples isolated from either homozygous Nova-1 null (-/-) or wild-type (+/+) mice, with -/- cDNA as tester and +/+ cDNA as driver (lanes marked T). Control hybridizations were performed with +/+ cDNA as tester and +/+ cDNA as driver (Lanes marked D). After five selection rounds, several prominent species enriched in the experimental -/- vs. +/+ selection (round 5, lane T) but not in the control +/+ vs. +/+ selection (round 5, lane D) were cloned and sequenced.
- (B) Region of the gel shown in (A), enlarged (Lanes T and D, round 5). Indicated bands were identified by sequence analysis.



B

T = -/- **D** = +/+

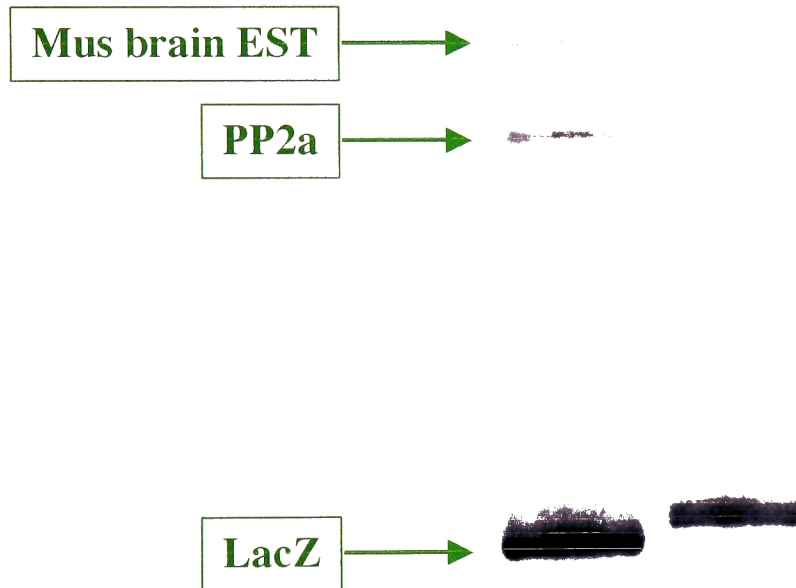


Table 2. Differentially expressed genes identified by SABRE selection.

Summary of differentially regulated genes identified by this method. Identity of fragments cloned after SABRE was performed on:

- (A) Spinal cord cDNAs. Tester = $-/-$, Driver = $+/+$.
- (B) Spinal cord cDNAs. Tester = $+/+$, Driver = $-/-$.
- (C) Hindbrain cDNAs. Tester = $-/-$, Driver = $+/+$.
- (D) Hindbrain cDNAs. Tester = $+/+$, Driver = $-/-$.

A. mRNAs increased in P0 Nova-1 null spinal cord

Clone ref	Identity
1S1	Lac Z
1S2	No match
1S6	Rattus norvegicus mRNA for beta-tubulin T beta15.Mus musculus, Similar to tubulin, beta polypeptide.
1S42	C11ORF2. Unknown
1S46	protein phosphatase 2, regulatory subunit B (B56), beta isoform (PPP2R5B).
1S47	mPer.
1S49	Mus musculus hypothalamus cDNA clone. Unknown.
1S121	Mus musculus cDNA clones Homo sapiens partner of RAC1 (arfaptin 2)
1S142	Mus musculus adult Cb cDNA. Unknown.

B. mRNAs increased in P0 Nova-1 null hindbrain

Clone ref	Identity
1H15	N-myc downstream regulated 2 (Ndr2)
1H16	Unknown. Proline rich region.
1H18	Unknown. Match to region of human genome with no known genes (UCSC).
1H30	ribosomal protein L11
1H32	Mus musculus dishevelled segment polarity protein homolog (Dvl-1)
1H34	Mus musculus tumor differentially expressed 1 (Tde1) / membrane protein TMS-1 mRNA / transfer RNA-Ser synthetase (SERS)
1H35	Mus musculus apolipoprotein E
1H36	Mus musculus contactin associated protein 1 (Cntnap1)
1H37	Homo sapiens guanosine-diphosphatase like protein mRNA (mus nucleoside triphosphate -related)
1H53	Unknown. Chromosome segregation protein SMC-related
1H60	Rattus norvegicus Shank postsynaptic density protein 3a
1H64	double cortin and calcium/calmodulin-dependent protein kinase-like 1 (Dcamk11) Mus musculus CPG16
1H68	igfbp-6
1H70	Homo sapiens a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 4 (ADAMTS4)
1H73	Unknown
1H87	Unknown

C. mRNAs increased in P0 wildtype spinal cord

Clone ref	Identity
2S1	Mus musculus syndecan 3 (Sdc3)- transmembrane heparan sulfate proteoglycan
2S5	Mus musculus cDNA clone similar to inositol polyphosphate 4-phosphatase. VAV proto-oncogene related
2S6	Mus musculus alpha-mannosidase
2S8	Mus musculus carnitine acetyltransferase (Crat)
2S10	Mus musculus cyclin ania-6b (partial match)
2S12	Mus musculus potassium channel, subfamily K, member 2 (Kcnk2)/ TREK-1 K+ channel subunit
2S22	Homo sapiens OSBP-related protein 7 mRNA similar to oxysterol-binding protein; ORP7
2S24	Mus musculus MORF-related gene X (Mirgx)
2S25	Mus musculus kinesin family member 21B (Kif21b)
2S26	Unknown
2S31	Mus musculus microtubule-associated protein MAP1A (Mtap1) mRNA, alternative promoter product
2S33	Unknown
2S35	Homo sapiens, myeloid leukemia factor 2

D. mRNAs increased in P0 wildtype hindbrain

Clone ref	Identity
2H1	Mus musculus similar to nischarin / Mus musculus nischarin (Nisch)
2H2	Mus musculus adult male hippocampus cDNA. Unknown.
2H3	Unknown
2H5	zinc finger protein of the cerebellum 1 (Zic1)
2H10	Mus musculus cDNA clone similar to human melanocyte protein PMEL
2H14	Unknown
2H19	Mus musculus MRPL4 mRNA for mitochondrial ribosomal protein L4
2H20	Unknown
2H23	Homo sapiens brother of CDO (BOC) mRNA - member of Ig superfamily
2H25	Unknown.
2H30	Homo sapiens myotubularin related protein 7 mRNA
2H36	Rattus norvegicus plectin (Plec1), mRNA

Figure 37: RT-PCR confirmation of contactin associated protein 1 (Cntnap1, clone 1H36) upregulation in Nova-1 null mice.

RNA isolated from hindbrains of Nova-1 null and control (heterozygous or wildtype littermates) at the indicated ages was analyzed by RT-PCR. Primers were designed to recognize a region of the mRNA contained in the SABRE clone, and to span 2 exon/exon boundaries. PCRs were multiplexed with primers to β -actin mRNA (QuantumRNA™ β -actin Internal Standards, Ambion). Data was quantitated by phosphorimager analysis and presented as the ratio of message in Nova-1 null samples vs. control (control level set to 1, indicated by thick line) after correction for actin.

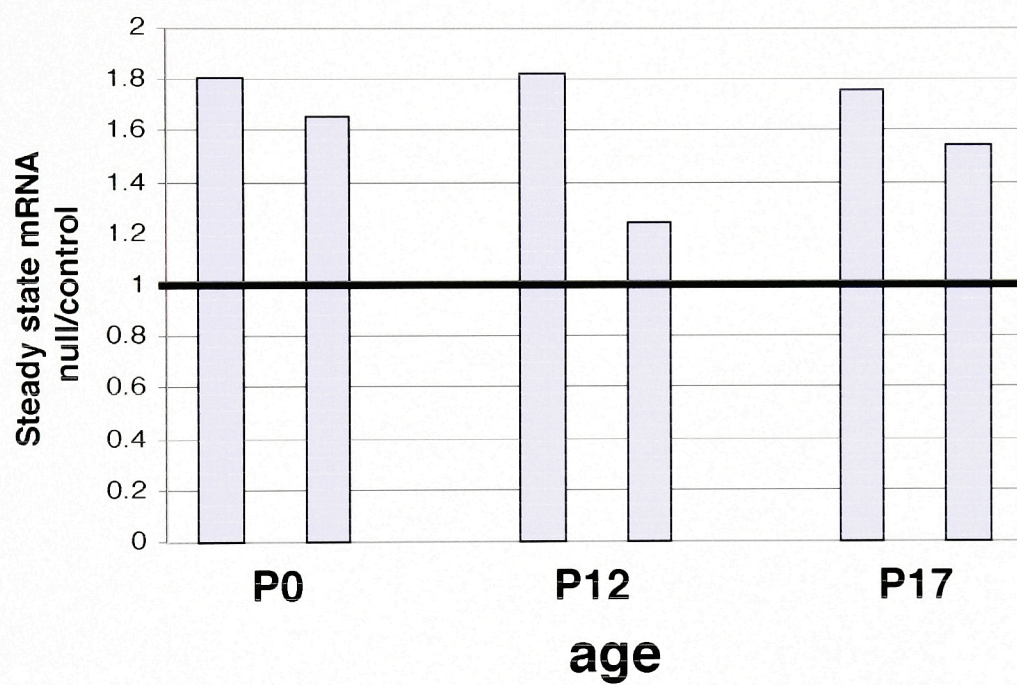


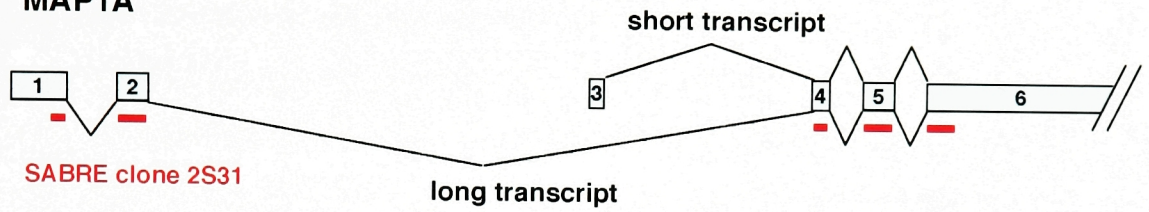
Figure 38: Several SABRE clones correspond to alternative mRNA isoforms.

Schematic representations of the genomic regions of 3 genes identified by SABRE as differentially expressed in Nova-1 null mice compared to wildtype littermates. Red bars show regions contained within the SABRE clones. Exon numbers were assigned based on comparisons of cDNA and genomic sequences in the databases.

- (A) SABRE clone 2S31 (increased in wildtype spinal cord) spans 5 exons of the long transcript of MAP1A. The two transcripts are generated from alternative promoters (Nakayama et al., 2001).
- (B) SABRE clone 1H60 (increased in Nova-1 null hindbrain) is contained within a large (2.3kb) exon (designated 8A) in rat Shank3a mRNA. BLAST searches of rat Shank3a mRNA against mouse sequences pulls out mouse Shank 3b which lacks exon 8A.
- (C) SABRE clone 1H53 (increased in Nova-1 null hindbrain) spans 3 exons of a gene of unknown function. Analysis of EST data reveals the likelihood of 2 alternative 3'-terminal exons (and 2 polyA sites). Clone 1H53 extends into the first of these exons.

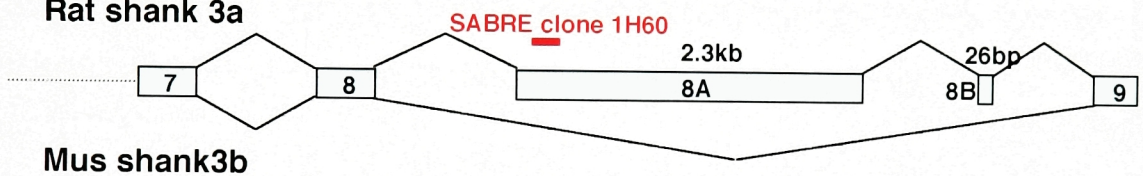
A

MAP1A



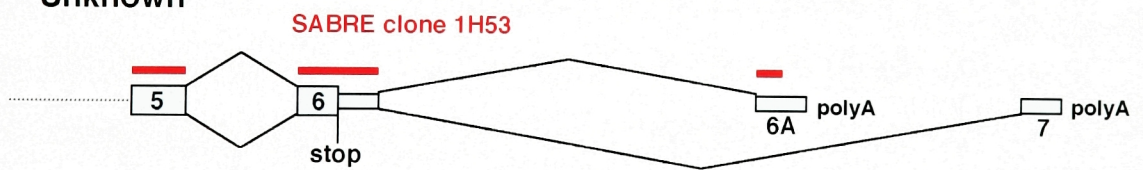
B

Rat shank 3a



C

Unknown



Chapter 7 - Discussion

Physiological consequences of Nova-dependent splicing dysregulation.

Several reports have implicated splicing defects as a cause of motor neuron death in the spinal cord. Dreyfuss and colleagues have determined that a protein mutated in the human disorder spinal muscular atrophy plays a critical role in the generation of snRNPs, and have suggested that the proper regulation of splicing is necessary for post-natal survival of motor neurons (Fischer et al., 1997). In addition, Lin et al. have reported that splicing defects in the glutamate transporter, EAAT2, are associated with the motor neuron disorder amyotrophic lateral sclerosis (Lin et al., 1998). The phenotype of Nova-1 null mice, which phenocopies many aspects of the human POMA syndrome, together with the biochemical evidence presented here, provides a particularly compelling case illustrating splicing defects that are associated with neuronal death in the ventral spinal cord.

The symptoms of POMA patients and the phenotypes of Nova null mice share many of the characteristic hallmarks of failure of inhibitory responses in the central nervous system. The finding that two of the known targets of Nova action on alternative splicing are subunits of inhibitory neurotransmitter receptors, and the third is the Nova-1 message itself (which may add further complexity to the regulation of the first two targets), is very intriguing. Mice lacking the long splice variant of GABA_AR γ 2 (γ 2L) display a higher level of anxiety than control mice (Homanics et al., 1999), and increased

sensitivity to benzodiazapines (Quinlan et al., 2000). Targeted disruption of the entire GABA_AR γ 2 gene results in benzodiazapine insensitive mice which, like Nova null mice display retarded growth postnatally and drastically reduced lifespan in addition to sensorimotor dysfunction (Gunther et al., 1995). Furthermore, epilepsy has been correlated with mutations in the GABA_AR γ 2 gene (Baulac et al., 2001; Wallace et al., 2001), and altered ratios of γ 2L and γ 2S mRNAs in schizophrenics have also been reported (Huntsman et al., 1998). Several human and animal disorders of glycine neurotransmission, such as hyperekplexia, the murine mutations *spastic*, *spasmodic* and *oscillator*, and the bovine, equine and possibly canine forms of myoclonus reflect symptoms similar to POMA patients and to Nova null mice (glycine disorders reviewed in (Rajendra et al., 1997)). These observations attest to the importance of correct functioning of these receptors *in vivo*, and are consistent with the hypothesis that failure of correct Nova-dependent processing of these messages may contribute, at least in part, to the neuronal dysfunction and phenotypes seen in Nova null mice and POMA patients.

A delicate balance between different alternatively spliced forms of individual messages is clearly important for correct functioning of the nervous system, as evidenced by the finding of subtle alterations in splice site selection in FTDP-17, SMA, DM and the mouse model of POMA. The alterations in the ratio of alternatively spliced forms of GABA_AR γ 2 and GlyR α 2 in Nova-1 null mice are relatively subtle, however these ratios were measured at the tissue level. Nova-1 is expressed in only a subset of neurons, which is overlapping with the subset of those neurons that express GABA_AR γ 2 and GlyR α 2 subunits. Thus it is likely that the differences at the single cell level are far more profound. In agreement with this, the change in alternative exon usage in tissue culture

cells transfected with minigenes with or without Nova is greater than that seen in Nova null mice.

Splicing has also been linked to several downstream processes such as nuclear export, translation and cytoplasmic localization. The failure of these RNAs to go through a Nova-dependent splicing pathway could have even more profound effects on relative protein levels or distributions. Nova protein and GlyR α 2 mRNAs co-localize in the dendrites of ventral horn spinal cord neurons of the adult rat (C. Racca, A. Triller and R. B. Darnell unpublished data). In addition, GlyR α 2 and α mRNAs differ in their subcellular localization depending on the neuronal population suggesting that different mechanisms for the localization of these messages are used by distinct populations of neurons (Racca et al., 1998). Furthermore, GlyR α 2 mRNAs are not transported to dendrites in dorsal horn neurons of the spinal cord, cells in which Nova-2 but not Nova-1 is expressed, which points at a possible functional difference between these two proteins. Taken together, these observations suggest that Nova-1 may play a role in the dendritic localization of GlyR α 2, and by analogy, GABA $_A$ R γ 2 mRNAs, and that this localization may be splicing-dependent. Thus in Nova-1 null mice, these messages are spliced independently of Nova-1, so may not acquire the appropriate hnRNP composition necessary for dendritic localization, possibly including the deposition of Nova-1 itself onto the spliced message. Conceivably, Nova could be associated with one alternatively spliced form of the message only. Thus a relatively subtle dysfunction in alternative splicing in Nova-1 null mice could in fact reflect a much more dramatic effect on the protein expression at distal sites.

The observation that steady-state levels of GlyR α 2 mRNA are increased the spinal cords of Nova-1 null mice compared to wild-type littermates may also be the result of incorrect processing of these RNAs subsequent to splicing. Nova proteins may shuttle between the nucleus and cytoplasm, and in doing so may facilitate the export of bound mRNAs. A lack of Nova-1 may then result in accumulation of GlyR α 2 message in the nucleus. As a result of either a decrease in cytoplasmic turnover of these messages or a positive feedback on transcription, or both, an overall increase in message level can be envisaged. The fact that a similar observation was not made in the case of GABA $_A$ R γ 2 may reflect a difference in the processing of these messages, or in the physiological response to aberrations in their processing.

Proposed mechanisms of Nova-dependent splicing regulation.

Several unanswered questions regarding tissue-specific splicing are addressed by the finding that Nova-1 regulates alternative splicing in neurons. RNA binding proteins such as Sxl and Tra/Tra-2 in *Drosophila*, identified by genetic approaches, act in a sequence-specific manner to regulate alternative splicing of specific target transcripts. However, genetics has not previously been applied to the study of RNA binding proteins in mammals, and biochemical studies applied to complex splicing problems have led not to the identification of tissue-specific splicing factors, but to the identification of ubiquitously expressed RNA binding proteins. These studies have yielded one clear model in which differential regulation of splicing in mammals is generated through relatively subtle changes in the levels of such ubiquitous splicing factors. Changing the

ratio of the constitutively expressed hnRNP protein hnRNP A1 to the SR protein ASF/SF2 mediates quantitative changes, ranging from 1.25-fold to several-fold in cell transfection studies, in the utilization of proximal versus distal splice site choices at alternatively spliced exons (Caceres et al., 1994). The data presented here demonstrates that in addition to the role general factors play in regulating alternative splicing, tissue-specific factors that recognize specific RNA ligands exist in mammals, and may confer more robust changes than those seen with constitutive factors alone.

In previous studies of the mechanism of neuron-specific splicing, intronic enhancer elements similar in nature to the GlyR α 2 (UCAUY)₃ sequences have been identified in other transcripts. These include multiple elements that promote neuron-specific exon inclusion in the c-src, calcitonin/CGRP, GABA_A γ 2 and agrin genes (Black, 1992; Lou et al., 1999; Wei et al., 1997; Zhang et al., 1996). Regulation of splicing appears complex given that multiple elements, both positive and negative, influence alternative splicing events. For example, in c-src, in addition to positive acting signals identified as targets of an RNA binding protein complex involving KSRP, hnRNP F and hnRNP H (Chou et al., 1999; Min et al., 1997), negative elements are found in the polypyrimidine tract upstream of the neuronal n-src exon, which interact with PTB (Chan and Black, 1997), and downstream of the n-src exon, which interact with brain-enriched PTB (Markovtsov et al., 2000).

The mechanism of neuron-specific splicing in the GABA_A γ 2 gene has similar complexities (Ashiya et al., 1995; Zhang et al., 1996; Zhang et al., 1999). A number of negative elements surrounding the γ 2 exon were found to bind PTB, and, evidence was also found for positive acting splicing elements. These observations, together with our

data, suggest that Nova-1 may act in concert with general factors such as PTB or brain-enriched PTB to regulate GABA_A γ 2L exon utilization, which would be consistent with the finding that Nova interacts with brain PTB (Polydorides et al., 2000). The work presented here has led to the identification of a sequence element necessary and sufficient for Nova-dependent enhancement of GABA_AR γ 2L exon utilization. This enhancer element lies approximately 1,250 nt downstream of the alternatively spliced exon, E9, and 80 nt upstream of the next constitutively included (last) exon.

Several examples of downstream enhancer elements have been documented in the literature. For example, as noted above, n-src splicing is regulated in part by the binding of KSRP to the downstream control element (DCS), possibly by recruiting U1 snRNP to the 5' splice site and enhancing removal of the downstream intron (Min et al., 1997). In processing of the CT/CGRP RNA, an intron enhancer is located downstream of the regulated exon, exon 4, which is included in thyroid cells, but excluded in neuronal cells. Increasing PTB levels results in an increase in exon 4 inclusion (Lou et al., 1999). This enhancer contains a core element which resembles a pseudoexon, featuring all the necessary splicing signals; 3' and 5' splice sites, polypyrimidine tract and branch point, with 3' and 5' splice sites abutted such that they contain no internal sequences. PTB binds to this pseudoexon element and excludes binding of U2AF, thereby preventing sequestration of U2AF at the cryptic splice site, and favoring binding of U2AF to the upstream splice site, and subsequent splicing of exon 4. If this site is mutated to contain a consensus U2AF⁶⁵ binding site, enhancement of exon inclusion is prevented (Lou et al., 1999). Thus although PTB acts as a splicing enhancer in this system, the mechanism of action is very similar to the role of PTB in negative regulation of other alternative exons,

namely GABA_AR γ 2L and src exon N1. This mechanism of action is highly reminiscent of the regulation of *tra* splicing by Sxl (Valcarcel et al., 1993); Sxl bind to an upstream 3' splice site in *tra*, which precludes binding of U2AF⁶⁵ and thereby promotes U2AF⁶⁵ binding to the downstream, weaker 3' splice site.

The splicing of human nonmuscle myosin heavy chain (NMHC)-B neural-specific alternative exon, N30, is also regulated by a downstream enhancer element (Kawamoto, 1996). This element, termed the intronic distal downstream enhancer (IDDE) resides 1.5kb downstream of the exon it regulates. *In vitro* splicing assays using substrates containing only two exons, N30 and either the upstream or downstream exon, demonstrated that this element facilitates splicing of the upstream intron, not the one in which it resides (Guo and Kawamoto, 2000). This upstream intron contains a weak polypyrimidine tract, optimization of which obviates the need for the downstream enhancer for exon inclusion, and destroys neural specificity. This observation, combined with transfection experiments involving overexpression of a truncated U2AF⁶⁵ lacking the RNA binding domain which acts in a dominant negative fashion to inhibit N30 inclusion, led the authors to conclude that this enhancer element facilitates recognition of the 3' splice site in the upstream intron via a series of protein-protein interactions which recruit U2AF to the suboptimal polypyrimidine tract. However, the nature of the factors which directly bind to this element and the way in which neural specificity is achieved is unknown.

An overriding theme in the regulation of alternative splicing is a balance between positive and negative (or suboptimal) signals. It appears that in many instances there are limiting amounts of splicing factors that assemble on a pre-mRNA, and that weak splice

sites may be skipped by default in situations where factors are limited. To tip the balance between positive and negative signals several scenarios can be envisaged. One, enhancement of splicing could be mediated through inhibition of a negative signal, thus allowing a particular splicing event to occur – for example, PTB's action on CT/CGRP which involves the inhibition of a competing pseudoexon. Alternatively, enhancement could be achieved by bona fide positive action on the mechanics of splicing such as recruitment of essential splicing factors to an otherwise poorly utilized site, as has been proposed for KSRP enhancement of src N1 inclusion, or the action of the downstream enhancer of (NMHC)-B N30 inclusion. These possibilities are not necessarily mutually exclusive.

The first scenario would predict that in the absence of the inhibitory signal, the enhancer element would not be required, and in fact would fail to act as an enhancer. The finding that a small (as short as 24nt) sequence element corresponding to the region of Nova-1 binding is sufficient to mediate Nova-dependent enhancement of an upstream exon in a heterologous system (as was shown in Figure 21) would argue against this hypothesis of Nova action on GABA_AR γ 2L exon utilization, unless this 24nt element also harbored the inhibitory element, or Nova acts in a manner reminiscent of PTB as a splicing inhibitor. This seems highly unlikely given the small size of the element, the lack of any obvious pseudo-splice site within the element (like that in CT/CGRP).

Nova-dependent enhancement of GABA_AR γ 2L exon utilization is more likely to involve facilitation of splicing of an intron adjacent to E9. It is unclear as yet whether this facilitation involves the intron upstream (similar to (NMHC)-B N30) or downstream (similar to KSRP/src N1) of E9. *In vitro* splicing assays utilizing simple two exon RNA

substrates similar to those performed by Guo and Sawamoto (Guo and Kawamoto, 2000) would be helpful in determining this. Comparison with Nova's action on GlyR α 2 splicing, however, would predict that Nova enhances splicing of the intron in which it resides. By binding to these intronic enhancer elements, Nova may facilitate spliceosome assembly. Yeast-2-hybrid results have indicated that Nova can bind to spliceosomal elements present at both the 3' and 5' splice sites, namely U1-70K and U2AF⁶⁵ as well as some SR proteins, including SRp20 (G. Stefani and R.B. Darnell, unpublished results). Through interactions such as these, Nova may serve a bridging function between the nearby 3' splice site and the next upstream 5' splice site. Figure 39A illustrates such a model.

The position of the Nova binding site so close to the splice acceptor signals of exon 10 is intriguing. One other possibility for the mechanism of Nova action is that Nova simply slows down the assembly of the spliceosome at exon 10 by interfering with binding or assembly of essential factors at this site. This could then enhance splicing to E9 in a kinetic manner, essentially by allowing more time for assembly to take place at an otherwise weakly recognized site, and thus increasing the chances of recognition of this exon. This hypothesis does not require (though it also does not exclude) the need for Nova to recruit or bind to other splicing factors. However, it is difficult to reconcile this hypothesis with what is known about Nova regulation of GlyR α 2 splicing. The Nova-binding element shown to be necessary for enhancement of E3A inclusion is in a very similar location with respect to the downstream exon, but in this instance the downstream exon is the regulated exon (E3A). If Nova was to act on GlyR α 2 in a manner similar to

the hypothesis stated above, Nova binding would be expected to result in a decrease in E3A inclusion.

Splicing regulation of Nova-1 exon H is also likely to represent a balance between positive and negative signals. In contrast to the two other known targets of Nova-1 splicing regulation, in this instance it appears that the default pathway involves exon H inclusion and Nova acts as an inhibitor via binding to an exonic silencer sequence. Thus Nova is a multifunctional splicing factor, able to act as both a repressor and enhancer of alternative exons. The most obvious difference between the targets of Nova splicing regulation is that in both instances where Nova acts to enhance alternative exon utilization, the Nova-binding site lies within an intron, albeit in a different position relative to the regulated exon, whereas the Nova-binding site in its own message is contained within exon H.

Position can have a profound effect on splicing enhancers. Changing their location can alter their dependence on particular trans-acting factors. For example, Tra is no longer required to activate female-specific splicing of exon 4 of *dsx* if the splicing enhancer is moved closer to the 3' splice site (Tian and Maniatis, 1994). Sequences identical to this *dsx* enhancer are also present in the gene *fruitless (fru)* where they direct utilization of a downstream 5' splice site in females, a process also dependent on Tra (Heinrichs and Baker, 1995). This difference implies that a particular type of splicing enhancer complex can promote different outcomes on different genes, triggered by the same developmental regulator, Tra, but involving a different range of molecular interactions depending on the position within the pre-mRNA.

Position can even transform a positive element into a negative one, as the data presented in this work would suggest occurs for Nova regulation of its own message compared to that of GlyR α 2 or GABA $_A$ R γ 2. For example, an SR-protein-binding site positioned just upstream of a branch point causes splicing inhibition in an adenovirus pre-mRNA by blocking access of U2snRNP. The same element can act as an exonic splicing enhancer if it is placed downstream of the 3' splice site (Kanopka et al., 1996). Interestingly, this inhibition is dependent on phosphorylation of the SR proteins; inhibition is relieved upon dephosphorylation of the bound SR proteins (Kanopka et al., 1998). Similarly, Nova-binding to the site in exon H may inhibit access of necessary splicing factors such as SR proteins to this exon. Since Nova can be phosphorylated *in vivo* (G. Stefani and R. B. Darnell, unpublished data) the action of Nova on splicing could also conceivably be influenced by phosphorylation state, though there is no evidence to support this hypothesis yet.

Nova binding to exon H could inhibit splicing in a number of ways. Binding could simply sterically inhibit the binding or association of other necessary components of the spliceosome at this exon, resulting in exon skipping, as illustrated in Figure 39B.I. Alternatively Nova binding could lead to recruitment of constitutive factors, as was suggested for regulation of GlyR α 2 and GABA $_A$ R γ 2 alternative splicing, but to the wrong position relative to the RNA and thus in a manner inhibitory for exon inclusion. For example, since KH binding to RNA is directional as evidenced by the co-crystallization of a Nova KH domain bound to a target RNA (Lewis et al., 2000), Nova binding to RNA elements is restricted in a unidirectional fashion. If Nova interactions with other protein factors are also directional, Nova could dictate the position of these factors relative to the

RNA. By extension of the GlyR α 2 / GABA $_A$ R γ 2 model shown in Figure 39A, if Nova is only able to bind U170K on the upstream side and U2AF⁶⁵ on the downstream side, this would result in the recruitment of these factors to the wrong splice site if Nova was bound to an exon, and hence exon exclusion (illustrated in Figure 39B.II).

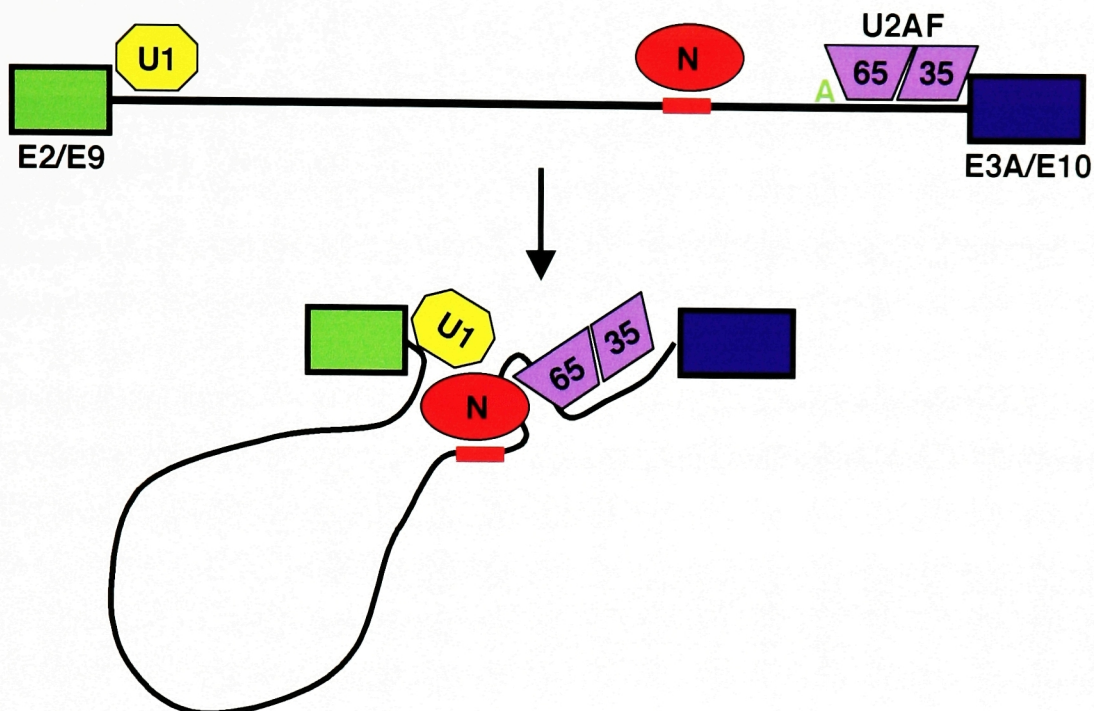
Summary

A delicate balance between different alternatively spliced forms of individual pre-mRNAs is clearly important for correct functioning of the nervous system, as evidenced by the finding of subtle alterations in splicing of particular messages in a number of neurological diseases. The mechanisms by which splicing is regulated so tightly and specifically are not generally known, but likely involves both general factors and neuron-specific (or enriched) factors. The finding that Nova-1 acts as a neuron-specific alternative splicing factor is an important one, since it is the first example of such a factor found exclusively in the mammalian brain. Elucidation of the mechanism of Nova-dependent splicing regulation through identification of splicing targets and the elements necessary and sufficient for Nova regulation of them, will help to understand the exquisite control and complexity of gene expression in the brain. Ultimately, studies such as these will also aid in the understanding of a number of neurological diseases.

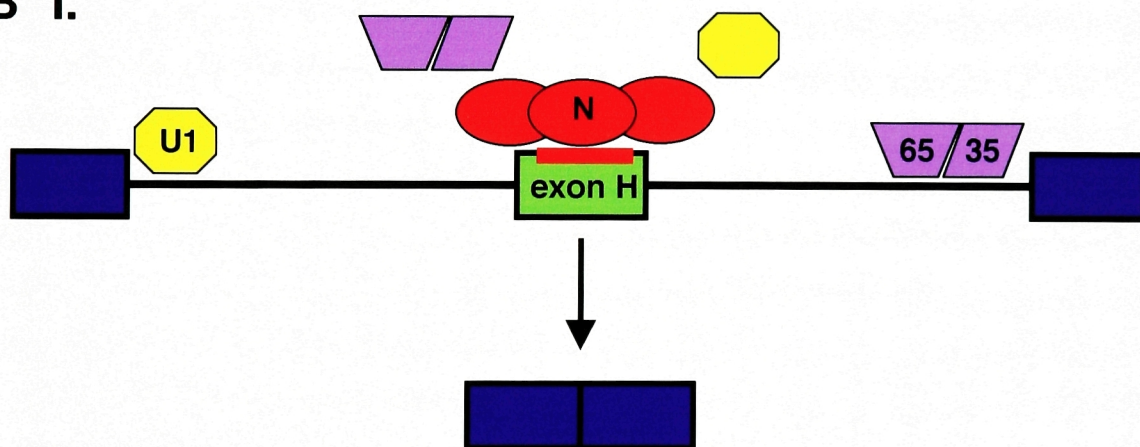
Figure 39: Model for Nova regulation of alternative splicing.

- (A) Nova (red ovals) binds to an intronic region in GlyR α 2 and GABA $_A$ R γ 2 pre-RNAs, near the 3' splice site. Binding may occur in either the intron upstream (as is the case for GlyR α 2 E3A) or downstream (as for GABA $_A$ R γ 2 E9) of the alternatively spliced exon. Nova then bridges the interaction between the adjacent 5' and 3' splice sites via interactions with proteins at both sites, such as U170K, a component of U1snRNP (yellow octagons) at the 5' splice site and U2AF⁶⁵ (purple trapezoid) at the 3' splice site, thereby enhancing splicing of the intron. Nova binding may recruit factors already bound to the adjacent splice sites (as shown) and stabilize the interaction. Alternatively, Nova recruitment of protein factors may aid in their association with otherwise weakly recognized splice sites. Exons are represented by green and blue rectangles.
- (B) Nova recognizes an exonic element in Nova-1 exon H (red bar). I. Binding of Nova to this element may sterically inhibit binding of other factors necessary for splicing to this exon. II. Alternatively, Nova may recruit spliceosomal factors as in (A), but binding may be directional, resulting in the recruitment of these factors to the wrong position relative to the RNA and subsequent inhibition of exon inclusion.

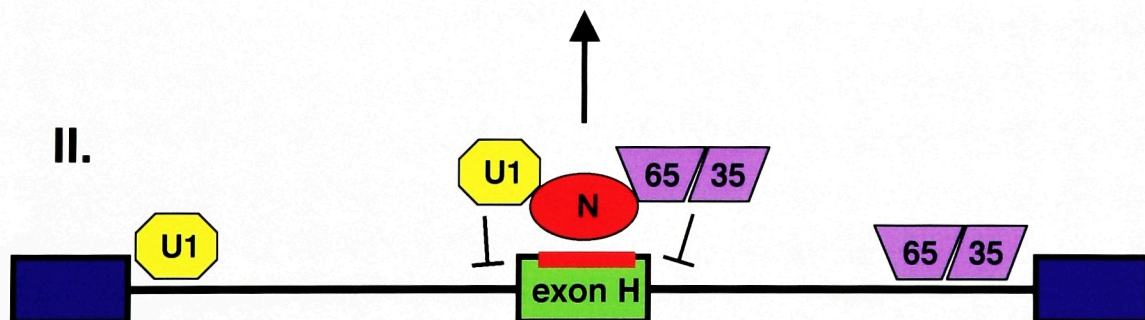
A



B I.



II.



References

- Adams, M. D., Rudner, D. Z., and Rio, D. C. (1996). Biochemistry and regulation of pre-mRNA splicing, *Curr Opin Cell Biol* 8, 331-9.
- Akopian, A. N., and Wood, J. N. (1995). Peripheral nervous system-specific genes identified by subtractive cDNA cloning, *J Biol Chem* 270, 21264-70.
- Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S., and Evans, R. M. (1982). Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products, *Nature* 298, 240-4.
- Arning, S., Gruter, P., Bilbe, G., and Kramer, A. (1996). Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA, *RNA* 2, 794-810.
- Ashiya, M., and Grabowski, P. J. (1997). A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart, *RNA* 3, 996-1015.
- Ashiya, M., Zhang, L., and Grabowski, P. J. (1995). Regulated splicing of gamma2 pre-messenger RNA in neuronal cells, *Nucleic Acids Symp Ser* 33, 215-6.
- Bashaw, G. J., and Baker, B. S. (1997). The regulation of the *Drosophila* msl-2 gene reveals a function for Sex-lethal in translational control, *Cell* 89, 789-98.
- Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J. F., Baulac, M., Brice, A., Bruzzone, R., and LeGuern, E. (2001). First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene, *Nat Genet* 28, 46-8.
- Benne, R., Van den Burg, J., Brakenhoff, J. P., Sloof, P., Van Boom, J. H., and Tromp, M. C. (1986). Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA, *Cell* 46, 819-26.

- Berget, S. M. (1995). Exon recognition in vertebrate splicing, *J Biol Chem* 270, 2411-4.
- Black, D. L. (1992). Activation of c-src neuron-specific splicing by an unusual RNA element in vivo and in vitro, *Cell* 69, 795-807.
- Blanc, V., Navaratnam, N., Henderson, J. O., Anant, S., Kennedy, S., Jarmuz, A., Scott, J., and Davidson, N. O. (2001). Identification of GRY-RBP as an apolipoprotein B RNA-binding protein that interacts with both apobec-1 and apobec-1 complementation factor to modulate C to U editing, *J Biol Chem* 276, 10272-83.
- Bond, V. C., and Wold, B. (1993). Nucleolar localization of myc transcripts, *Mol Cell Biol* 13, 3221-30.
- Braddock, D. T., Louis, J. M., Baber, J. L., Levens, D., and Clore, G. M. (2002). Structure and dynamics of KH domains from FBP bound to single-stranded DNA, *Nature* 415, 1051-6.
- Brennan, C. M., Gallouzi, I. E., and Steitz, J. A. (2000). Protein ligands to HuR modulate its interaction with target mRNAs in vivo, *J Cell Biol* 151, 1-14.
- Brennan, C. M., and Steitz, J. A. (2001). HuR and mRNA stability, *Cell Mol Life Sci* 58, 266-77.
- Brown, R. H., Jr. (1996). Superoxide dismutase and familial amyotrophic lateral sclerosis: new insights into mechanisms and treatments, *Ann Neurol* 39, 145-6.
- Brown, V., Jin, P., Ceman, S., Darnell, J. C., O'Donnell, W. T., Tenenbaum, S. A., Jin, X., Feng, Y., Wilkinson, K. D., Keene, J. D., *et al.* (2001). Microarray identification of FMRP-associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome, *Cell* 107, 477-87.
- Brzustowicz, L. M., Lehner, T., Castilla, L. H., Penchaszadeh, G. K., Wilhelmsen, K. C., Daniels, R., Davies, K. E., Leppert, M., Ziter, F., Wood, D., and *et al.* (1990). Genetic mapping of chronic childhood-onset spinal muscular atrophy to chromosome 5q11.2-13.3, *Nature* 344, 540-1.

- Buckanovich, R. J., and Darnell, R. B. (1997). The neuronal RNA binding protein Nova-1 recognizes specific RNA targets in vitro and in vivo, *Mol Cell Biol* *17*, 3194-201.
- Buckanovich, R. J., Posner, J. B., and Darnell, R. B. (1993). Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system, *Neuron* *11*, 657-72.
- Buckanovich, R. J., Yang, Y. Y., and Darnell, R. B. (1996). The onconeural antigen Nova-1 is a neuron-specific RNA-binding protein, the activity of which is inhibited by paraneoplastic antibodies, *J Neurosci* *16*, 1114-22.
- Caceres, J. F., Stamm, S., Helfman, D. M., and Krainer, A. R. (1994). Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors, *Science* *265*, 1706-9.
- Cao, W., Jamison, S. F., and Garcia-Blanco, M. A. (1997). Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro, *RNA* *3*, 1456-67.
- Carmo-Fonseca, M., Pepperkok, R., Carvalho, M. T., and Lamond, A. I. (1992). Transcription-dependent colocalization of the U1, U2, U4/U6, and U5 snRNPs in coiled bodies, *J Cell Biol* *117*, 1-14.
- Carstens, R. P., McKeehan, W. L., and Garcia-Blanco, M. A. (1998). An intronic sequence element mediates both activation and repression of rat fibroblast growth factor receptor 2 pre-mRNA splicing, *Mol Cell Biol* *18*, 2205-17.
- Cartegni, L., and Krainer, A. R. (2002). Disruption of an SF2/ASF-dependent exonic splicing enhancer in *SMN2* causes spinal muscular atrophy in the absence of *SMN1*, *Nat Genet* *30*, 377-384.
- Chan, R. C., and Black, D. L. (1995). Conserved intron elements repress splicing of a neuron-specific c-src exon in vitro [published erratum appears in *Mol Cell Biol* 1997 May;17(5):2970], *Mol Cell Biol* *15*, 6377-85.

Chan, R. C., and Black, D. L. (1997). The polypyrimidine tract binding protein binds upstream of neural cell- specific c-src exon N1 to repress the splicing of the intron downstream, *Mol Cell Biol* 17, 4667-76.

Chevallier, P. (1993). Pest sequences in nuclear proteins, *Int J Biochem* 25, 479-82.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction, *Anal Biochem* 162, 156-9.

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

Conn, P. J., and Pin, J. P. (1997). Pharmacology and functions of metabotropic glutamate receptors, *Annu Rev Pharmacol Toxicol* 37, 205-37.

D'Souza, I., Poorkaj, P., Hong, M., Nochlin, D., Lee, V. M., Bird, T. D., and Schellenberg, G. D. (1999). Missense and silent tau gene mutations cause frontotemporal dementia with parkinsonism-chromosome 17 type, by affecting multiple alternative RNA splicing regulatory elements, *Proc Natl Acad Sci U S A* 96, 5598-603.

Dalmau, J., Furneaux, H. M., Cordon-Cardo, C., and Posner, J. B. (1992a). The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues, *Am J Pathol* 141, 881-6.

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

Daoud, R., Da Penha Berzaghi, M., Siedler, F., Hubener, M., and Stamm, S. (1999). Activity-dependent regulation of alternative splicing patterns in the rat brain, *Eur J Neurosci* 11, 788-802.

- Darnell, J. C., Jensen, K. B., Jin, P., Brown, V., Warren, S. T., and Darnell, R. B. (2001). Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function, *Cell* 107, 489-99.
- Darnell, R. B. (1996). Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity, and the brain, *Proc Natl Acad Sci U S A* 93, 4529-36.
- David, C., McPherson, P. S., Mundigl, O., and de Camilli, P. (1996). A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals, *Proc Natl Acad Sci U S A* 93, 331-5.
- Davis, B. M., McCurrach, M. E., Taneja, K. L., Singer, R. H., and Housman, D. E. (1997). Expansion of a CUG trinucleotide repeat in the 3' untranslated region of myotonic dystrophy protein kinase transcripts results in nuclear retention of transcripts, *Proc Natl Acad Sci U S A* 94, 7388-93.
- De Boulle, K., Verkerk, A. J., Reyniers, E., Vits, L., Hendrickx, J., Van Roy, B., Van den Bos, F., de Graaff, E., Oostra, B. A., and Willems, P. J. (1993). A point mutation in the FMR-1 gene associated with fragile X mental retardation, *Nat Genet* 3, 31-5.
- De Camilli, P., Thomas, A., Cofield, R., Folli, F., Lichte, B., Piccolo, G., Meinck, H. M., Austoni, M., Fassetta, G., Bottazzo, G., and et al. (1993). The synaptic vesicle-associated protein amphiphysin is the 128-kD autoantigen of Stiff-Man syndrome with breast cancer, *J Exp Med* 178, 2219-23.
- Deshler, J. O., Highett, M. I., Abramson, T., and Schnapp, B. J. (1998). A highly conserved RNA-binding protein for cytoplasmic mRNA localization in vertebrates, *Curr Biol* 8, 489-96.
- Diatchenko, L., Lau, Y. F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E. D., and Siebert, P. D. (1996). Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries, *Proc Natl Acad Sci U S A* 93, 6025-30.

- Dominski, Z., and Kole, R. (1991). Selection of splice sites in pre-mRNAs with short internal exons, *Mol Cell Biol* 11, 6075-83.
- Dropcho, E. J., Chen, Y. T., Posner, J. B., and Old, L. J. (1987). Cloning of a brain protein identified by autoantibodies from a patient with paraneoplastic cerebellar degeneration, *Proc Natl Acad Sci U S A* 84, 4552-6.
- Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals, *Cell* 83, 195-206.
- Ehlers, M. D., Tingley, W. G., and Huganir, R. L. (1995). Regulated subcellular distribution of the NR1 subunit of the NMDA receptor, *Science* 269, 1734-7.
- Engbrecht, J. A., Voelkel-Meiman, K., and Roeder, G. S. (1991). Meiosis-specific RNA splicing in yeast, *Cell* 66, 1257-68.
- Fan, X. C., and Steitz, J. A. (1998a). HNS, a nuclear-cytoplasmic shuttling sequence in HuR, *Proc Natl Acad Sci U S A* 95, 15293-8.
- Fan, X. C., and Steitz, J. A. (1998b). Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs, *EMBO J* 17, 3448-60.
- Fathallah-Shaykh, H., Wolf, S., Wong, E., Posner, J. B., and Furneaux, H. M. (1991). Cloning of a leucine-zipper protein recognized by the sera of patients with antibody-associated paraneoplastic cerebellar degeneration, *Proc Natl Acad Sci U S A* 88, 3451-4.
- Ferns, M., Hoch, W., Campanelli, J. T., Rupp, F., Hall, Z. W., and Scheller, R. H. (1992). RNA splicing regulates agrin-mediated acetylcholine receptor clustering activity on cultured myotubes, *Neuron* 8, 1079-86.
- Ferrandon, D., Elphick, L., Nusslein-Volhard, C., and St Johnston, D. (1994). Staufen protein associates with the 3'UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner, *Cell* 79, 1221-32.

- Fischer, U., Liu, Q., and Dreyfuss, G. (1997). The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis, *Cell* 90, 1023-9.
- Fitzwater, T., and Polisky, B. (1996). A SELEX primer, *Methods Enzymol* 267, 275-301.
- Fletcher, C. F., Lutz, C. M., O'Sullivan, T. N., Shaughnessy, J. D., Jr., Hawkes, R., Frankel, W. N., Copeland, N. G., and Jenkins, N. A. (1996). Absence epilepsy in tottering mutant mice is associated with calcium channel defects, *Cell* 87, 607-17.
- Foley, K. P., Leonard, M. W., and Engel, J. D. (1993). Quantitation of RNA using the polymerase chain reaction, *Trends Genet* 9, 380-5.
- Fong, Y. W., and Zhou, Q. (2001). Stimulatory effect of splicing factors on transcriptional elongation, *Nature* 414, 929-33.
- Fox, A. H., Lam, Y. W., Leung, A. K., Lyon, C. E., Andersen, J., Mann, M., and Lamond, A. I. (2002). Paraspeckles. A novel nuclear domain, *Curr Biol* 12, 13-25.
- Frey, M. R., and Matera, A. G. (1995). Coiled bodies contain U7 small nuclear RNA and associate with specific DNA sequences in interphase human cells, *Proc Natl Acad Sci U S A* 92, 5915-9.
- Fu, X. D., and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus, *Nature* 343, 437-41.
- Gall, J. G. (2000). Cajal bodies: the first 100 years, *Annu Rev Cell Dev Biol* 16, 273-300.
- Gall, J. G. (2001). A role for Cajal bodies in assembly of the nuclear transcription machinery, *FEBS Lett* 498, 164-7.
- Gallouzi, I. E., and Steitz, J. A. (2001). Delineation of mRNA export pathways by the use of cell-permeable peptides, *Science* 294, 1895-901.
- Garner, C. C., Tucker, R. P., and Matus, A. (1988). Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites, *Nature* 336, 674-7.

- Gattenlohner, S., Brabletz, T., Schultz, A., Marx, A., Muller-Hermelink, H. K., and Kirchner, T. (1994). Cloning of a cDNA coding for the acetylcholine receptor alpha-subunit from a thymoma associated with myasthenia [correction of myastenia] gravis, *Thymus* 23, 103-13.
- Gebauer, F., Merendino, L., Hentze, M. W., and Valcarcel, J. (1998). The *Drosophila* splicing regulator sex-lethal directly inhibits translation of male-specific-lethal 2 mRNA, *Rna* 4, 142-50.
- Ghosh, A., Ginty, D. D., Bading, H., and Greenberg, M. E. (1994). Calcium regulation of gene expression in neuronal cells, *J Neurobiol* 25, 294-303.
- Goddard, A. D., Borrow, J., Freemont, P. S., and Solomon, E. (1991). Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia, *Science* 254, 1371-4.
- Goedert, M., and Spillantini, M. G. (2000). Tau mutations in frontotemporal dementia FTDP-17 and their relevance for Alzheimer's disease, *Biochim Biophys Acta* 1502, 110-21.
- Goedert, M., Spillantini, M. G., Potier, M. C., Ulrich, J., and Crowther, R. A. (1989). Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain, *Embo J* 8, 393-9.
- Grabowski, P. J. (1998). Splicing regulation in neurons: tinkering with cell-specific control, *Cell* 92, 709-12.
- Granadino, B., Penalva, L. O., Green, M. R., Valcarcel, J., and Sanchez, L. (1997). Distinct mechanisms of splicing regulation in vivo by the *Drosophila* protein Sex-lethal, *Proc Natl Acad Sci U S A* 94, 7343-8.
- Graus, F., Cordon-Cardo, C., and Posner, J. B. (1985). Neuronal antinuclear antibody in sensory neuronopathy from lung cancer, *Neurology* 35, 538-43.

- Grover, A., Houlden, H., Baker, M., Adamson, J., Lewis, J., Prihar, G., Pickering-Brown, S., Duff, K., and Hutton, M. (1999). 5' splice site mutations in tau associated with the inherited dementia FTDP-17 affect a stem-loop structure that regulates alternative splicing of exon 10, *J Biol Chem* 274, 15134-43.
- Gui, J. F., Lane, W. S., and Fu, X. D. (1994). A serine kinase regulates intracellular localization of splicing factors in the cell cycle, *Nature* 369, 678-82.
- Gunther, U., Benson, J., Benke, D., Fritschy, J. M., Reyes, G., Knoflach, F., Crestani, F., Aguzzi, A., Arigoni, M., Lang, Y., and et al. (1995). Benzodiazepine-insensitive mice generated by targeted disruption of the gamma 2 subunit gene of gamma-aminobutyric acid type A receptors, *Proc Natl Acad Sci U S A* 92, 7749-53.
- Guo, N., and Kawamoto, S. (2000). An intronic downstream enhancer promotes 3' splice site usage of a neural cell-specific exon, *J Biol Chem* 275, 33641-9.
- Harris, H., Sidebottom, E., Grace, D. M., and Bramwell, M. E. (1969). The expression of genetic information: a study with hybrid animal cells, *J Cell Sci* 4, 499-525.
- Hasegawa, M., Smith, M. J., and Goedert, M. (1998). Tau proteins with FTDP-17 mutations have a reduced ability to promote microtubule assembly, *FEBS Lett* 437, 207-10.
- He, X., and Rosenfeld, M. G. (1991). Mechanisms of complex transcriptional regulation: implications for brain development, *Neuron* 7, 183-96.
- Hedrick, S. M., Cohen, D. I., Nielsen, E. A., and Davis, M. M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins, *Nature* 308, 149-53.
- Heinrichs, V., and Baker, B. S. (1995). The *Drosophila* SR protein RBP1 contributes to the regulation of doublesex alternative splicing by recognizing RBP1 RNA target sequences, *EMBO J* 14, 3987-4000.
- Hertel, K. J., Lynch, K. W., and Maniatis, T. (1997). Common themes in the function of transcription and splicing enhancers, *Curr Opin Cell Biol* 9, 350-7.

Higuchi, M., Single, F. N., Kohler, M., Sommer, B., Sprengel, R., and Seeburg, P. H. (1993). RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency, *Cell* 75, 1361-70.

Hollmann, M., and Heinemann, S. (1994). Cloned glutamate receptors, *Annu Rev Neurosci* 17, 31-108.

Homanics, G. E., Harrison, N. L., Quinlan, J. J., Krasowski, M. D., Rick, C. E., de Blas, A. L., Mehta, A. K., Kist, F., Mihalek, R. M., Aul, J. J., and Firestone, L. L. (1999). Normal electrophysiological and behavioral responses to ethanol in mice lacking the long splice variant of the gamma2 subunit of the gamma-aminobutyrate type A receptor, *Neuropharmacology* 38, 253-65.

Hong, M., Zhukareva, V., Vogelsberg-Ragaglia, V., Wszolek, Z., Reed, L., Miller, B. I., Geschwind, D. H., Bird, T. D., McKeel, D., Goate, A., *et al.* (1998). Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17, *Science* 282, 1914-7.

Honig, L. S., Chambliss, D. D., Bigio, E. H., Carroll, S. L., and Elliott, J. L. (2000). Glutamate transporter EAAT2 splice variants occur not only in ALS, but also in AD and controls., *Neurology* 55, 1082-8.

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

Huh, G. S., and Hynes, R. O. (1994). Regulation of alternative pre-mRNA splicing by a novel repeated hexanucleotide element, *Genes Dev* 8, 1561-74.

Huntsman, M. M., Tran, B. V., Potkin, S. G., Bunney, W. E., Jr., and Jones, E. G. (1998). Altered ratios of alternatively spliced long and short gamma2 subunit mRNAs of the gamma-amino butyrate type A receptor in prefrontal cortex of schizophrenics, *Proc Natl Acad Sci U S A* 95, 15066-71.

- Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., *et al.* (1998). Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17, *Nature* 393, 702-5.
- Inoue, K., Hoshijima, K., Sakamoto, H., and Shimura, Y. (1990). Binding of the *Drosophila* sex-lethal gene product to the alternative splice site of transformer primary transcript, *Nature* 344, 461-3.
- Ishihara, T., Hong, M., Zhang, B., Nakagawa, Y., Lee, M. K., Trojanowski, J. Q., and Lee, V. M. (1999). Age-dependent emergence and progression of a tauopathy in transgenic mice overexpressing the shortest human tau isoform, *Neuron* 24, 751-62.
- Izaurralde, E., Stepinski, J., Darzynkiewicz, E., and Mattaj, I. W. (1992). A cap binding protein that may mediate nuclear export of RNA polymerase II-transcribed RNAs, *J Cell Biol* 118, 1287-95.
- Jensen, K. B., Dredge, B. K., Stefani, G., Zhong, R., Buckanovich, R. J., Okano, H. J., Yang, Y. Y., and Darnell, R. B. (2000a). Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability, *Neuron* 25, 359-71.
- Jensen, K. B., Musunuru, K., Lewis, H. A., Burley, S. K., and Darnell, R. B. (2000b). The tetranucleotide UCA_Y directs the specific recognition of RNA by the Nova K-homology 3 domain, *Proc Natl Acad Sci U S A* 97, 5740-5.
- Jiang, Z., Cote, J., Kwon, J. M., Goate, A. M., and Wu, J. Y. (2000). Aberrant splicing of tau pre-mRNA caused by intronic mutations associated with the inherited dementia frontotemporal dementia with parkinsonism linked to chromosome 17, *Mol Cell Biol* 20, 4036-48.
- Kang, H., and Schuman, E. M. (1996). A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity, *Science* 273, 1402-6.

- Kanopka, A., Muhlemann, O., and Akusjarvi, G. (1996). Inhibition by SR proteins of splicing of a regulated adenovirus pre- mRNA, *Nature* 381, 535-8.
- Kanopka, A., Muhlemann, O., Petersen-Mahrt, S., Estmer, C., Ohrmalm, C., and Akusjarvi, G. (1998). Regulation of adenovirus alternative RNA splicing by dephosphorylation of SR proteins, *Nature* 393, 185-7.
- Kawamoto, S. (1996). Neuron-specific alternative splicing of nonmuscle myosin II heavy chain- B pre-mRNA requires a cis-acting intron sequence, *J Biol Chem* 271, 17613-6.
- Keegan, L. P., Gallo, A., and O'Connell, M. A. (2001). The many roles of an RNA editor, *Nat Rev Genet* 2, 869-78.
- Keene, J. D. (1999). Why is Hu where? Shuttling of early-response-gene messenger RNA subsets, *Proc Natl Acad Sci U S A* 96, 5-7.
- Kelley, R. L., Wang, J., Bell, L., and Kuroda, M. I. (1997). Sex lethal controls dosage compensation in *Drosophila* by a non-splicing mechanism, *Nature* 387, 195-9.
- Kiledjian, M., Wang, X., and Liebhaber, S. A. (1995). Identification of two KH domain proteins in the alpha-globin mRNP stability complex, *Embo J* 14, 4357-64.
- King, M. L., Zhou, Y., and Bubunencko, M. (1999). Polarizing genetic information in the egg: RNA localization in the frog oocyte, *Bioessays* 21, 546-57.
- Klickstein, L., ed. (1995). (New York, John Wiley and Sons).
- Kohmura, N., Senzaki, K., Hamada, S., Kai, N., Yasuda, R., Watanabe, M., Ishii, H., Yasuda, M., Mishina, M., and Yagi, T. (1998). Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex, *Neuron* 20, 1137-51.
- Koob, M. D., Moseley, M. L., Schut, L. J., Benzow, K. A., Bird, T. D., Day, J. W., and Ranum, L. P. (1999). An untranslated CTG expansion causes a novel form of spinocerebellar ataxia (SCA8), *Nat Genet* 21, 379-84.

- Kornstein, M. J., Asher, O., and Fuchs, S. (1995). Acetylcholine receptor alpha-subunit and myogenin mRNAs in thymus and thymomas, *Am J Pathol* 146, 1320-4.
- Kosik, K. S., Orecchio, L. D., Bakalis, S., and Neve, R. L. (1989). Developmentally regulated expression of specific tau sequences, *Neuron* 2, 1389-97.
- Koushika, S. P., Lisbin, M. J., and White, K. (1996). ELAV, a *Drosophila* neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform, *Curr Biol* 6, 1634-41.
- Koushika, S. P., Soller, M., and White, K. (2000). The neuron-enriched splicing pattern of *Drosophila* erect wing is dependent on the presence of ELAV protein, *Mol Cell Biol* 20, 1836-45.
- Krishek, B. J., Xie, X., Blackstone, C., Huganir, R. L., Moss, S. J., and Smart, T. G. (1994). Regulation of GABAA receptor function by protein kinase C phosphorylation, *Neuron* 12, 1081-95.
- Kuhse, J., Kuryatov, A., Maulet, Y., Malosio, M. L., Schmieden, V., and Betz, H. (1991). Alternative splicing generates two isoforms of the alpha 2 subunit of the inhibitory glycine receptor, *FEBS Lett* 283, 73-7.
- Lamond, A. I., and Earnshaw, W. C. (1998). Structure and function in the nucleus, *Science* 280, 547-53.
- Lavery, D. J., Lopez-Molina, L., Fleury-Olela, F., and Schibler, U. (1997). Selective amplification via biotin- and restriction-mediated enrichment (SABRE), a novel selective amplification procedure for detection of differentially expressed mRNAs, *Proc Natl Acad Sci U S A* 94, 6831-6.
- Le Hir, H., Gatfield, D., Izaurralde, E., and Moore, M. J. (2001). The exon-exon junction complex provides a binding platform for factors involved in mRNA export and nonsense-mediated mRNA decay, *EMBO J* 20, 4987-97.

- Le Hir, H., Moore, M. J., and Maquat, L. E. (2000). Pre-mRNA splicing alters mRNP composition: evidence for stable association of proteins at exon-exon junctions, *Genes Dev* 14, 1098-108.
- Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M., and et al. (1995). Identification and characterization of a spinal muscular atrophy- determining gene, *Cell* 80, 155-65.
- Lellek, H., Kirsten, R., Diehl, I., Apostel, F., Buck, F., and Greeve, J. (2000). Purification and molecular cloning of a novel essential component of the apolipoprotein B mRNA editing enzyme-complex, *J Biol Chem* 275, 19848-56.
- Lemke, G. (1993). Transcriptional regulation of the development of neurons and glia, *Curr Opin Neurobiol* 3, 703-8.
- Lewis, H. A., Chen, H., Edo, C., Buckanovich, R. J., Yang, Y. Y., Musunuru, K., Zhong, R., Darnell, R. B., and Burley, S. K. (1999). Crystal structures of Nova-1 and Nova-2 K-homology RNA-binding domains, *Structure Fold Des* 7, 191-203.
- Lewis, H. A., Musunuru, K., Jensen, K. B., Edo, C., Chen, H., Darnell, R. B., and Burley, S. K. (2000). Sequence-specific RNA binding by a Nova KH domain: implications for paraneoplastic disease and the fragile X syndrome, *Cell* 100, 323-32.
- Liang, P., and Pardee, A. B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction, *Science* 257, 967-71.
- Lin, C. L., Bristol, L. A., Jin, L., Dykes-Hoberg, M., Crawford, T., Clawson, L., and Rothstein, J. D. (1998). Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis, *Neuron* 20, 589-602.
- Lin, Z., Haus, S., Edgerton, J., and Lipscombe, D. (1997). Identification of functionally distinct isoforms of the N-type Ca²⁺ channel in rat sympathetic ganglia and brain, *Neuron* 18, 153-66.

- Lisbin, M. J., Qiu, J., and White, K. (2001). The neuron-specific RNA-binding protein ELAV regulates neuroglial alternative splicing in neurons and binds directly to its pre-mRNA, *Genes Dev* 15, 2546-61.
- Lisitsyn, N., and Wigler, M. (1993). Cloning the differences between two complex genomes, *Science* 259, 946-51.
- Litman, P., Barg, J., Rindzoonski, L., and Ginzburg, I. (1993). Subcellular localization of tau mRNA in differentiating neuronal cell culture: implications for neuronal polarity, *Neuron* 10, 627-38.
- Liu, Q., and Dreyfuss, G. (1996). A novel nuclear structure containing the survival of motor neurons protein, *EMBO J* 15, 3555-65.
- Liu, Q., Fischer, U., Wang, F., and Dreyfuss, G. (1997). The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins, *Cell* 90, 1013-21.
- Lopez, A. J. (1998). Alternative splicing of pre-mRNA: developmental consequences and mechanisms of regulation, *Annu Rev Genet* 32, 279-305.
- Lorson, C. L., and Androphy, E. J. (2000). An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN, *Hum Mol Genet* 9, 259-65.
- Lorson, C. L., Hahnen, E., Androphy, E. J., and Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy, *Proc Natl Acad Sci U S A* 96, 6307-11.
- Lorson, C. L., Strasswimmer, J., Yao, J. M., Baleja, J. D., Hahnen, E., Wirth, B., Le, T., Burghes, A. H., and Androphy, E. J. (1998). SMN oligomerization defect correlates with spinal muscular atrophy severity, *Nat Genet* 19, 63-6.
- Lou, H., Helfman, D. M., Gagel, R. F., and Berget, S. M. (1999). Polypyrimidine tract-binding protein positively regulates inclusion of an alternative 3'-terminal exon, *Mol Cell Biol* 19, 78-85.

- Luo, M. J., and Reed, R. (1999). Splicing is required for rapid and efficient mRNA export in metazoans, *Proc Natl Acad Sci U S A* 96, 14937-42.
- Luque, F. A., Furneaux, H. M., Ferziger, R., Rosenblum, M. K., Wray, S. H., Schold, S. C., Jr., Glantz, M. J., Jaeckle, K. A., Biran, H., Lesser, M., and et al. (1991). Anti-Ri: an antibody associated with paraneoplastic opsoclonus and breast cancer, *Ann Neurol* 29, 241-51.
- Lynch, K. W., and Maniatis, T. (1996). Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila* doublesex splicing enhancer, *Genes Dev* 10, 2089-101.
- Macdonald, R. L. (1995). Ethanol, gamma-aminobutyrate type A receptors, and protein kinase C phosphorylation, *Proc Natl Acad Sci U S A* 92, 3633-5.
- Mankodi, A., Logigian, E., Callahan, L., McClain, C., White, R., Henderson, D., Krym, M., and Thornton, C. A. (2000). Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat, *Science* 289, 1769-73.
- Manley, G. T., Smitt, P. S., Dalmau, J., and Posner, J. B. (1995). Hu antigens: reactivity with Hu antibodies, tumor expression, and major immunogenic sites, *Ann Neurol* 38, 102-10.
- Markovtsov, V., Nikolic, J. M., Goldman, J. A., Turck, C. W., Chou, M. Y., and Black, D. L. (2000). Cooperative assembly of an hnRNP complex induced by a tissue-specific homolog of polypyrimidine tract binding protein, *Mol Cell Biol* 20, 7463-79.
- Martin, K. C., Casadio, A., Zhu, H., E, Y., Rose, J. C., Chen, M., Bailey, C. H., and Kandel, E. R. (1997). Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage, *Cell* 91, 927-38.
- Matsumoto, K., Wassarman, K. M., and Wolffe, A. P. (1998). Nuclear history of a pre-mRNA determines the translational activity of cytoplasmic mRNA, *EMBO J* 17, 2107-21.

- Mattox, W., and Baker, B. S. (1991). Autoregulation of the splicing of transcripts from the transformer-2 gene of *Drosophila*, *Genes Dev* 5, 786-96.
- Mehta, A., Kinter, M. T., Sherman, N. E., and Driscoll, D. M. (2000). Molecular cloning of apobec-1 complementation factor, a novel RNA- binding protein involved in the editing of apolipoprotein B mRNA, *Mol Cell Biol* 20, 1846-54.
- Melki, J., Abdelhak, S., Sheth, P., Bachelot, M. F., Burlet, P., Marcadet, A., Aicardi, J., Barois, A., Carriere, J. P., Fardeau, M., and et al. (1990). Gene for chronic proximal spinal muscular atrophies maps to chromosome 5q, *Nature* 344, 767-8.
- Meyer, T., Fromm, A., Munch, C., Schwalenstocker, B., Fray, A. E., Ince, P. G., Stamm, S., Gron, G., Ludolph, A. C., and Shaw, P. J. (1999). The RNA of the glutamate transporter EAAT2 is variably spliced in amyotrophic lateral sclerosis and normal individuals, *J Neurol Sci* 170, 45-50.
- Michael, W. M., Eder, P. S., and Dreyfuss, G. (1997). The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein, *EMBO J* 16, 3587-98.
- Miller, R. D., and Riblet, R. (1995). Improved phenol emulsion DNA reassociation technique (PERT) using thermal cycling, *Nucleic Acids Res* 23, 2339-40.
- Milner, J. J., Cecchini, E., and Dominy, P. J. (1995). A kinetic model for subtractive hybridization, *Nucleic Acids Res* 23, 176-87.
- Min, H., Turck, C. W., Nikolic, J. M., and Black, D. L. (1997). A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer, *Genes Dev* 11, 1023-36.
- Missler, M., and Sudhof, T. C. (1998). Neurexins: three genes and 1001 products, *Trends Genet* 14, 20-6.
- Misteli, T. (2001). Protein dynamics: implications for nuclear architecture and gene expression, *Science* 291, 843-7.

- Misteli, T., Caceres, J. F., and Spector, D. L. (1997). The dynamics of a pre-mRNA splicing factor in living cells, *Nature* 387, 523-7.
- Misteli, T., and Spector, D. L. (1996). Serine/threonine phosphatase 1 modulates the subnuclear distribution of pre-mRNA splicing factors, *Mol Biol Cell* 7, 1559-72.
- Modafferi, E. F., and Black, D. L. (1997). A complex intronic splicing enhancer from the c-src pre-mRNA activates inclusion of a heterologous exon, *Mol Cell Biol* 17, 6537-45.
- Muller, S., Matunis, M. J., and Dejean, A. (1998). Conjugation with the ubiquitin-related modifier SUMO-1 regulates the partitioning of PML within the nucleus, *Embo J* 17, 61-70.
- Mulligan, G. J., Guo, W., Wormsley, S., and Helfman, D. M. (1992). Polypyrimidine tract binding protein interacts with sequences involved in alternative splicing of beta-tropomyosin pre-mRNA, *J Biol Chem* 267, 25480-7.
- Musunuru, K., and Darnell, R. B. (2001). Paraneoplastic neurologic disease antigens: RNA-binding proteins and signaling proteins in neuronal degeneration, *Annu Rev Neurosci* 24, 239-62.
- Nakayama, A., Odajima, T., Murakami, H., Mori, N., and Takahashi, M. (2001). Characterization of two promoters that regulate alternative transcripts in the microtubule-associated protein (MAP) 1A gene, *Biochim Biophys Acta* 1518, 260-6.
- Nayler, O., Hartmann, A. M., and Stamm, S. (2000). The ER repeat protein YT521-B localizes to a novel subnuclear compartment, *J Cell Biol* 150, 949-62.
- Newman, L. S., McKeever, M. O., Okano, H. J., and Darnell, R. B. (1995). Beta-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein, *Cell* 82, 773-83.
- Okabe, S., Miwa, A., and Okado, H. (1999). Alternative splicing of the C-terminal domain regulates cell surface expression of the NMDA receptor NR1 subunit, *J Neurosci* 19, 7781-92.

Okano, H. J., and Darnell, R. B. (1997). A hierarchy of Hu RNA binding proteins in developing and adult neurons, *J Neurosci* *17*, 3024-37.

Oleynikov, Y., and Singer, R. H. (1998). RNA localization: different zipcodes, same postman?, *Trends Cell Biol* *8*, 381-3.

Orr, H. T., and Zoghbi, H. Y. (2001). SCA1 molecular genetics: a history of a 13 year collaboration against glutamines, *Hum Mol Genet* *10*, 2307-11.

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

Pellizzoni, L., Charroux, B., and Dreyfuss, G. (1999). SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins, *Proc Natl Acad Sci U S A* *96*, 11167-72.

Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998). A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing, *Cell* *95*, 615-24.

Petersen-Mahrt, S. K., Estmer, C., Ohrmalm, C., Matthews, D. A., Russell, W. C., and Akusjarvi, G. (1999). The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation, *EMBO J* *18*, 1014-24.

Philips, A. V., Timchenko, L. T., and Cooper, T. A. (1998). Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy, *Science* *280*, 737-41.

Picetti, R., Saiardi, A., Abdel Samad, T., Bozzi, Y., Baik, J. H., and Borrelli, E. (1997). Dopamine D2 receptors in signal transduction and behavior, *Crit Rev Neurobiol* *11*, 121-42.

Pieretti, M., Zhang, F. P., Fu, Y. H., Warren, S. T., Oostra, B. A., Caskey, C. T., and Nelson, D. L. (1991). Absence of expression of the FMR-1 gene in fragile X syndrome, *Cell* *66*, 817-22.

Polans, A. S., Buczylo, J., Crabb, J., and Palczewski, K. (1991). A photoreceptor calcium binding protein is recognized by autoantibodies obtained from patients with cancer-associated retinopathy, *J Cell Biol* 112, 981-9.

Polydorides, A. D., Okano, H. J., Yang, Y. Y., Stefani, G., and Darnell, R. B. (2000). A brain-enriched polypyrimidine tract-binding protein antagonizes the ability of Nova to regulate neuron-specific alternative splicing, *Proc Natl Acad Sci U S A* 97, 6350-5.

Poorkaj, P., Bird, T. D., Wijsman, E., Nemens, E., Garruto, R. M., Anderson, L., Andreadis, A., Wiederholt, W. C., Raskind, M., and Schellenberg, G. D. (1998). Tau is a candidate gene for chromosome 17 frontotemporal dementia [published erratum appears in *Ann Neurol* 1998 Sep;44(3):428], *Ann Neurol* 43, 815-25.

Quinlan, J. J., Firestone, L. L., and Homanics, G. E. (2000). Mice lacking the long splice variant of the gamma 2 subunit of the GABA(A) receptor are more sensitive to benzodiazepines, *Pharmacol Biochem Behav* 66, 371-4.

Racca, C., Gardiol, A., and Triller, A. (1998). Cell-specific dendritic localization of glycine receptor alpha subunit messenger RNAs, *Neuroscience* 84, 997-1012.

Rajendra, S., Lynch, J. W., and Schofield, P. R. (1997). The glycine receptor, *Pharmacol Ther* 73, 121-46.

Ramanathan, K., Michael, T. H., Jiang, G. J., Hiel, H., and Fuchs, P. A. (1999). A molecular mechanism for electrical tuning of cochlear hair cells, *Science* 283, 215-7.

Raska, I., Ochs, R. L., Andrade, L. E., Chan, E. K., Burlingame, R., Peebles, C., Gruol, D., and Tan, E. M. (1990). Association between the nucleolus and the coiled body, *J Struct Biol* 104, 120-7.

Reed, R., and Hurt, E. (2002). A Conserved mRNA Export Machinery Coupled to pre-mRNA Splicing, *Cell* 108, 523-31.

Reed, R., and Magni, K. (2001). A new view of mRNA export: separating the wheat from the chaff, *Nat Cell Biol* 3, E201-4.

- Robinow, S., Campos, A. R., Yao, K. M., and White, K. (1988). The elav gene product of *Drosophila*, required in neurons, has three RNP consensus motifs, *Science* 242, 1570-2.
- Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis, *Science* 234, 364-8.
- Ross, A. F., Oleynikov, Y., Kislauskis, E. H., Taneja, K. L., and Singer, R. H. (1997). Characterization of a beta-actin mRNA zipcode-binding protein, *Mol Cell Biol* 17, 2158-65.
- Rothstein, J. D., Martin, L., Levey, A. I., Dykes-Hoberg, M., Jin, L., Wu, D., Nash, N., and Kuncel, R. W. (1994). Localization of neuronal and glial glutamate transporters, *Neuron* 13, 713-25.
- Rothstein, J. D., Martin, L. J., and Kuncel, R. W. (1992). Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis, *N Engl J Med* 326, 1464-8.
- Rueter, S. M., Dawson, T. R., and Emeson, R. B. (1999). Regulation of alternative splicing by RNA editing, *Nature* 399, 75-80.
- Ryan, S. G., Buckwalter, M. S., Lynch, J. W., Handford, C. A., Segura, L., Shiang, R., Wasmuth, J. J., Camper, S. A., Schofield, P., and O'Connell, P. (1994). A missense mutation in the gene encoding the alpha 1 subunit of the inhibitory glycine receptor in the spasmodic mouse, *Nat Genet* 7, 131-5.
- Sakai, K., Mitchell, D. J., Tsukamoto, T., and Steinman, L. (1990). Isolation of a complementary DNA clone encoding an autoantigen recognized by an anti-neuronal cell antibody from a patient with paraneoplastic cerebellar degeneration, *Ann Neurol* 28, 692-8.
- Sargent, T. D., and Dawid, I. B. (1983). Differential gene expression in the gastrula of *Xenopus laevis*, *Science* 222, 135-9.

- Saul, B., Schmieden, V., Kling, C., Mulhardt, C., Gass, P., Kuhse, J., and Becker, C. M. (1994). Point mutation of glycine receptor alpha 1 subunit in the spasmodic mouse affects agonist responses, *FEBS Lett* 350, 71-6.
- Schmucker, D., Clemens, J. C., Shu, H., Worby, C. A., Xiao, J., Muda, M., Dixon, J. E., and Zipursky, S. L. (2000). *Drosophila* Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity, *Cell* 101, 671-84.
- Schneider, R., Kadowaki, T., and Tartakoff, A. M. (1995). mRNA transport in yeast: time to reinvestigate the functions of the nucleolus, *Mol Biol Cell* 6, 357-70.
- Shaw, P. J., Forrest, V., Ince, P. G., Richardson, J. P., and Wastell, H. J. (1995). CSF and plasma amino acid levels in motor neuron disease: elevation of CSF glutamate in a subset of patients, *Neurodegeneration* 4, 209-16.
- Shiang, R., Ryan, S. G., Zhu, Y. Z., Hahn, A. F., O'Connell, P., and Wasmuth, J. J. (1993). Mutations in the alpha 1 subunit of the inhibitory glycine receptor cause the dominant neurologic disorder, hyperekplexia, *Nat Genet* 5, 351-8.
- Shipston, M. J., Duncan, R. R., Clark, A. G., Antoni, F. A., and Tian, L. (1999). Molecular components of large conductance calcium-activated potassium (BK) channels in mouse pituitary corticotropes, *Mol Endocrinol* 13, 1728-37.
- Siddique, T., and Deng, H. X. (1996). Genetics of amyotrophic lateral sclerosis, *Hum Mol Genet* 5, 1465-70.
- Siebel, C. W., Admon, A., and Rio, D. C. (1995). Soma-specific expression and cloning of PSI, a negative regulator of P element pre-mRNA splicing, *Genes Dev* 9, 269-83.
- Sil, A., and Herskowitz, I. (1996). Identification of asymmetrically localized determinant, *Ash1p*, required for lineage-specific transcription of the yeast *HO* gene, *Cell* 84, 711-22.
- Singer, R. H., and Green, M. R. (1997). Compartmentalization of eukaryotic gene expression: causes and effects, *Cell* 91, 291-4.

- Sive, H. L., and St John, T. (1988). A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction, *Nucleic Acids Res* 16, 10937.
- Smith, C. W., and Valcarcel, J. (2000). Alternative pre-mRNA splicing: the logic of combinatorial control, *Trends Biochem Sci* 25, 381-8.
- Solimena, M., Folli, F., Denis-Donini, S., Comi, G. C., Pozza, G., De Camilli, P., and Vicari, A. M. (1988). Autoantibodies to glutamic acid decarboxylase in a patient with stiff-man syndrome, epilepsy, and type I diabetes mellitus, *N Engl J Med* 318, 1012-20.
- Sommer, B., Kohler, M., Sprengel, R., and Seeburg, P. H. (1991). RNA editing in brain controls a determinant of ion flow in glutamate-gated channels, *Cell* 67, 11-9.
- Spector, D. L. (1993). Macromolecular domains within the cell nucleus, *Annu Rev Cell Biol* 9, 265-315.
- Spector, D. L. (2001). Nuclear domains, *J Cell Sci* 114, 2891-3.
- Spector, D. L., Fu, X. D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus, *Embo J* 10, 3467-81.
- Spillantini, M. G., Murrell, J. R., Goedert, M., Farlow, M. R., Klug, A., and Ghetti, B. (1998). Mutation in the tau gene in familial multiple system tauopathy with presenile dementia, *Proc Natl Acad Sci U S A* 95, 7737-41.
- Staley, J. P., and Guthrie, C. (1998). Mechanical devices of the spliceosome: motors, clocks, springs, and things, *Cell* 92, 315-26.
- Stamm, S., Zhang, M. Q., Marr, T. G., and Helfman, D. M. (1994). A sequence compilation and comparison of exons that are alternatively spliced in neurons, *Nucleic Acids Res* 22, 1515-26.
- Steward, O., and Worley, P. F. (2001). Selective targeting of newly synthesized Arc mRNA to active synapses requires NMDA receptor activation, *Neuron* 30, 227-40.

Stutz, F., Bachi, A., Doerks, T., Braun, I. C., Seraphin, B., Wilm, M., Bork, P., and Izaurralde, E. (2000). REF, an evolutionary conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export, *Rna* 6, 638-50.

Sugino, H., Hamada, S., Yasuda, R., Tuji, A., Matsuda, Y., Fujita, M., and Yagi, T. (2000). Genomic organization of the family of CNR cadherin genes in mice and humans, *Genomics* 63, 75-87.

Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Wong, E., Henson, J., Posner, J. B., and Furneaux, H. M. (1991). HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and Sex-lethal, *Cell* 67, 325-33.

Takimoto, M., Tomonaga, T., Matunis, M., Avigan, M., Krutzsch, H., Dreyfuss, G., and Levens, D. (1993). Specific binding of heterogeneous ribonucleoprotein particle protein K to the human c-myc promoter, in vitro, *J Biol Chem* 268, 18249-58.

Taneja, K. L., McCurrach, M., Schalling, M., Housman, D., and Singer, R. H. (1995). Foci of trinucleotide repeat transcripts in nuclei of myotonic dystrophy cells and tissues, *J Cell Biol* 128, 995-1002.

Tani, T., Derby, R. J., Hiraoka, Y., and Spector, D. L. (1995). Nucleolar accumulation of poly (A)+ RNA in heat-shocked yeast cells: implication of nucleolar involvement in mRNA transport, *Mol Biol Cell* 6, 1515-34.

Thirkill, C. E., Tait, R. C., Tyler, N. K., Roth, A. M., and Keltner, J. L. (1992). The cancer-associated retinopathy antigen is a recoverin-like protein, *Invest Ophthalmol Vis Sci* 33, 2768-72.

Tian, M., and Maniatis, T. (1994). A splicing enhancer exhibits both constitutive and regulated activities, *Genes Dev* 8, 1703-12.

Timchenko, L. T., Miller, J. W., Timchenko, N. A., DeVore, D. R., Datar, K. V., Lin, L., Roberts, R., Caskey, C. T., and Swanson, M. S. (1996). Identification of a (CUG)_n triplet

repeat RNA-binding protein and its expression in myotonic dystrophy, *Nucleic Acids Res* 24, 4407-14.

Tingley, W. G., Roche, K. W., Thompson, A. K., and Huganir, R. L. (1993). Regulation of NMDA receptor phosphorylation by alternative splicing of the C-terminal domain, *Nature* 364, 70-3.

Valcarcel, J., Singh, R., Zamore, P. D., and Green, M. R. (1993). The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA, *Nature* 362, 171-5.

Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995). Serial analysis of gene expression, *Science* 270, 484-7.

Vezzani, A., Speciale, C., Della Vedova, F., Tamburin, M., and Benatti, L. (1995). Alternative splicing at the C-terminal but not at the N-terminal domain of the NMDA receptor NR1 is altered in the kindled hippocampus, *Eur J Neurosci* 7, 2513-7.

Visa, N., Izaurralde, E., Ferreira, J., Daneholt, B., and Mattaj, I. W. (1996). A nuclear cap-binding complex binds Balbiani ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export, *J Cell Biol* 133, 5-14.

von Stein, O. D., Thies, W. G., and Hofmann, M. (1997). A high throughput screening for rarely transcribed differentially expressed genes, *Nucleic Acids Res* 25, 2598-602.

Wafford, K. A., Bain, C. J., Whiting, P. J., and Kemp, J. A. (1993). Functional comparison of the role of gamma subunits in recombinant human gamma-aminobutyric acidA/benzodiazepine receptors, *Mol Pharmacol* 44, 437-42.

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

- Wan, J. S., and Erlander, M. G. (1997). Cloning differentially expressed genes by using differential display and subtractive hybridization, *Methods Mol Biol* 85, 45-68.
- Wang, L., Miura, M., Bergeron, L., Zhu, H., and Yuan, J. (1994). Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death, *Cell* 78, 739-50.
- Wang, Z., and Brown, D. D. (1991). A gene expression screen, *Proc Natl Acad Sci U S A* 88, 11505-9.
- Wang, Z., and Grabowski, P. J. (1996). Cell- and stage-specific splicing events resolved in specialized neurons of the rat cerebellum, *Rna* 2, 1241-53.
- Wei, N., Lin, C. Q., Modafferi, E. F., Gomes, W. A., and Black, D. L. (1997). A unique intronic splicing enhancer controls the inclusion of the agrin Y exon, *Rna* 3, 1275-88.
- Whiting, P., McKernan, R. M., and Iversen, L. L. (1990). Another mechanism for creating diversity in gamma-aminobutyrate type A receptors: RNA splicing directs expression of two forms of gamma 2 phosphorylation site, *Proc Natl Acad Sci U S A* 87, 9966-70.
- Wu, Q., and Maniatis, T. (1999). A striking organization of a large family of human neural cadherin-like cell adhesion genes, *Cell* 97, 779-90.
- Xiao, S. H., and Manley, J. L. (1997). Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing, *Genes Dev* 11, 334-44.
- Xie, J., and Black, D. L. (2001). A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels, *Nature* 410, 936-9.
- Xie, J., and McCobb, D. P. (1998). Control of alternative splicing of potassium channels by stress hormones, *Science* 280, 443-6.

Yang, Y. Y., Yin, G. L., and Darnell, R. B. (1998). The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia, *Proc Natl Acad Sci U S A* 95, 13254-9.

Zandomeni, R., and Weinmann, R. (1984). Inhibitory effect of 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole on a protein kinase, *J Biol Chem* 259, 14804-11.

Zeng, J., Gorski, R. A., and Hamer, D. (1994). Differential cDNA cloning by enzymatic degrading subtraction (EDS), *Nucleic Acids Res* 22, 4381-5.

Zhang, L., Ashiya, M., Sherman, T. G., and Grabowski, P. J. (1996). Essential nucleotides direct neuron-specific splicing of gamma 2 pre- mRNA, *RNA* 2, 682-98.

Zhang, L., Liu, W., and Grabowski, P. J. (1999). Coordinate repression of a trio of neuron-specific splicing events by the splicing regulator PTB, *RNA* 5, 117-30.

Zhou, Z., Luo, M. J., Straesser, K., Katahira, J., Hurt, E., and Reed, R. (2000). The protein Aly links pre-messenger-RNA splicing to nuclear export in metazoans, *Nature* 407, 401-5.

138700B 155
11-18-02 13220 TH



THE LIBRARY



19010000495158

