

1996

# The Paraneoplastic Antigen Nova-1 is a Neuron Specific and Sequence Specific RNA Binding Protein

Ronald J. Buckanovich

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The Paraneoplastic Antigen Nova-1 is a  
Neuron Specific and Sequence Specific RNA  
Binding Protein.

The Dissertation of Ronald J. Buckanovich

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Submitted December 6, 1996

Accepted December 20th, 1996

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*The impalpable sustenance of me from all things at all hours of the  
day,  
The simple, compact well-join'd scheme, myself disintegrated,  
everyone disintegrated yet part of the scheme, . . .*

W.W.



## Acknowledgments

There are numerous people to whom I owe great thanks for their help, support and or contributions. Foremost, this work would not have been possible without the training, support or scientific contributions, both intellectual and material, of Robert Darnell. I have been extremely fortunate in that being the first student in this lab has allowed me to develop a special scientific and personal relationship with Dr. Darnell. I would like to thank all the members of the lab for their contributions, assistance, discussions, and reading of manuscripts. In particular, I would like to thank members of the “Nova club”, Yolanda Yang for help in constructing the Nova-1 fusion protein and developing filter binding assays, Ru Zhong who isolated the Nova-1 genomic clones, Jennifer Darnell who assisted in cloning pcDNA Nova-1, and honorary member James Okano for materials for in situ hybridization . I owe a special gift of gratitude to Lori Newman who has been an integral part of my life both inside and outside the lab.

There are also many members of the Rockefeller scientific community to whom I am grateful. People who have helped me with various experiments include Bas van Steensel, Monica Boyle, Pepe Reyes, John Hannish, and Lyle Najita. I would also like to thank Magda Konarska for numerous intellectual discussions. In addition, I am indebted to my thesis committee members, Titia de Lange, Steve DiNardo, Nathaniel Heintz, and Adrian Krainer, who have generously given their valuable time and counsel.

Finally, it would be an injustice not to thank my family. I would like to thank my parents who have supported all of my endeavors from athletic to academic, and my sisters, niece and nephew, whose love has helped me to keep all things in the proper perspective. In addition, I would like to thank my grandmother and aunt, whose care packages have kept me well fed.



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## Abbreviations

CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
IHC	Immunohistochemistry
ISH	In situ hybridization
PND	Paraneoplastic Neurologic Disease.
PCbD	Paraneoplastic cerebellar degeneration
PCD	Programmed cell death .
PEM/SN	Paraneoplastic encephalomyelopathy-sensory neuropathy
POMA	Paraneoplastic opsoclonus-myoclonus
RT-PCR	Reverse transcription polymerase chain reaction.



## Abstract

We have identified a cDNA encoding a protein that is recognized by sera from (7/7) patients with paraneoplastic opsoclonus-myoclonus ataxia (POMA). The gene, Nova-1, encodes a novel nuclear RNA binding protein (RBP) that is extremely highly conserved (99% amino acid identity) between human and mouse. Nova-1 is normally expressed only within neurons of the central nervous system (CNS). Both Nova-1 protein and RNA are restricted to a subset of CNS neurons which correlate with the specific neurologic symptoms seen in POMA. Nova-1 contains three KH type RNA binding motifs, and binds RNA *in vitro* via these KH motifs. Mutations within the core of the KH motif abolish specific RNA binding. Utilizing RNA selection, we have identified an *in vitro* RNA ligand for Nova-1 consisting of three UCAUY repeats in the loop of a stem loop structure. We have identified a similar sequence upstream of the alternatively spliced exons (3A and 3B) of the Glycine receptor  $\alpha 2A$  (glyR $\alpha 2A$ ), and downstream of the alternatively spliced exon H of Nova-1, and demonstrate that Nova-1 binds these RNAs with high affinity (Kd ~2 nM). We have also shown that Nova-1 binds both the glyR $\alpha 2A$  and Nova-1 pre-mRNAs *in vivo*. Nova-1 binding to the target sequence in the glyR $\alpha 2A$  RNA in tissue culture cells stimulates splicing to exon 3A suggesting that Nova-1 functions in regulating alternative splicing in mouse brain. Moreover, the demonstration that paraneoplastic antibodies can prevent Nova-1 from binding to glyR $\alpha 2A$  RNA *in vitro* suggests a potential mechanism for the neurologic symptoms of POMA.



## Chapter I. Introduction

### Paraneoplastic Neurologic Diseases

The paraneoplastic neurologic diseases (PNDs) are considered cancer associated autoimmune diseases of the nervous system. PNDs are believed to arise when peripheral tumors express neuronal antigens inducing an immune response that suppresses tumor growth (Anderson et al., 1988b; Dalmau et al., 1990; Dalmau et al., 1991; Darnell and DeAngelis, 1993) but leads to neuronal dysfunction and/or death (Hall, 1974; Shnider and Manalo, 1979; Dalmau, et al., 1990; Posner and Furneaux, 1990). Each of the specific PNDs is associated with specific tumor types and high titers of specific anti-neuronal antibodies which are present in both serum and cerebrospinal fluid (CSF). A simple model of PND pathogenesis can be constructed based on our understanding of the Lambert-Eaton myasthenic syndrome (LEMS), a paraneoplastic syndrome involving the neuromuscular junction. In LEMS, small cell lung tumors induce an antibody response against presynaptic motor neuron  $Ca^{++}$  channels, leading to reduced  $Ca^{++}$  influx, reduced neurotransmitter release, and motor weakness (Lang et al., 1981; Lennon et al., 1995). These antibodies can reproduce motor weakness when passively transferred to animals (Lang, et al., 1981). These observations have suggested a model for PND pathogenesis in which ectopic expression of neuronal protein in tumors elicits an immune response which causes neurologic disease (Darnell, 1996). Unlike LEMS, passive transfer of PND antibodies does not lead to neurologic symptoms (Graus et al., 1991; Smitt et al., 1995) suggesting that the pathogenesis may be more complex.

PND antisera provide a unique opportunity to identify neuron-specific genes that are expressed in the affected regions of the brain. For example, the Yo antibody, found in patients with paraneoplastic cerebellar degeneration (PCbD) and breast or ovarian tumors (Peterson et al., 1992), was used to identify a novel neuronal leucine zipper protein expressed specifically in cerebellar Purkinje neurons and isolated brainstem nuclei (Dropcho et al., 1987; Sakai et



al., 1990; Fathallah-Shaykh et al., 1991; Corradi et al., 1996). Similarly the onconeural antigen recoverin, targeted in small cell lung cancer associated paraneoplastic blindness (Polans et al., 1991; Thirkill et al., 1992), is a  $\text{Ca}^{++}$  binding protein involved in cGMP gated receptor signaling and is expressed specifically in the photoreceptor (Dizhoor et al., 1991; Polans, et al., 1991; Thirkill, et al., 1992; Gray-Keller et al., 1993; Kawamura et al., 1993).

In addition to identifying neuronal antigens which are expressed in discrete brain regions, these disorders also provide a unique opportunity to identify neuronal/tumor antigens which are likely to be involved in important cellular functions. The specific expression of particular onconeural antigens by specific tumors, the presence of a potent anti-tumor immune response, and the association of the onconeural antigens with neurologic deficits suggests that the onconeural proteins perform important functions in tumors and in neurons (Darnell, 1996). Functional analysis of identified PND antigens lends support to this hypothesis. PND target antigens play critical roles in neuromuscular signaling (LEMS), and in signal transduction in the photoreceptor (recoverin). Also, the recently identified  $\beta$ -NAP protein, a novel paraneoplastic cerebellar degeneration antigen, is a neuronal vesicle coat protein which has been proposed to play a role in vesicle transport between the trans golgi network in the neuronal soma and the axon terminus (Newman et al., 1995). In addition, the Hu family of RNA binding proteins (HuC/PLE21, HuD and Hel-N1) were identified as target antigens in paraneoplastic encephalomyelopathy/sensory neuropathy (PEM/SN) (Szabo et al., 1991; Levine et al., 1993; Sakai et al., 1993), a spectrum of neurologic disorders associated with small cell lung cancer (Dalmau, et al., 1991). The Hu proteins contain three RNA recognition motifs and are the human homologues of the *Drosophila* elav protein (Szabo, et al., 1991). elav is a pan-neuronal RNA binding protein that is essential for neurogenesis (Campos et al., 1985; Robinow et al., 1988; Yao et al., 1993). Based upon elav's homology with the alternative splicing factor sex-lethal, it has been suggested that elav regulates alternative





splicing in neurons (Yao, et al., 1993). By analogy, the Hu antigens may be important for neuronal alternative splicing and neurogenesis (Szabo, et al., 1991; Levine, et al., 1993; Gao et al., 1994b).

### Paraneoplastic Opsoclonus-Myoclonus Ataxia (POMA)

Paraneoplastic Opsoclonus-Myoclonus Ataxia (POMA) is a disorder in which abnormal motor control of the eyes, trunk and limbs develop primarily in women with breast cancer, although POMA has also been reported in patients with small cell lung cancer and fallopian carcinomas. (Digre, 1986; Anderson et al., 1988a; Budde-Steffen et al., 1988; Luque et al., 1991). Opsoclonus is clinically considered to be a loss of tonic inhibitory control by omnipause neurons in the pontine paramedian reticular formation due to dysfunction of the omnipause neurons or the efferent midbrain pathways descending from the superior colliculus to the omnipause neurons (Anderson, et al., 1988a). Similarly myoclonus can be attributed to dysregulation of inhibitory interneurons that regulate pathways descending upon motor neurons of the spinal cord (Darnell, 1994). Ataxia is a deficit attributed to abnormal function of midline cerebellar pathways. One patient with opsoclonus, myoclonus, and ataxia progressively developed encephalomyelitis and rigidity, suggesting that in some cases POMA may be associated with more widespread neuronal involvement (Casado et al., 1994).

POMA is distinguished from other PNDs by the presence of specific antineuronal antibodies, termed Ri (Luque, et al., 1991) or POMA (Buckanovich et al., 1996), in patient's sera and CSF. These antibodies specifically recognize antigens in paraneoplastic tumors (Luque, et al., 1991) and predominantly nuclear neuronal antigens of  $M_r$  ~53-58 and ~74-80kDa (Luque, et al., 1991; Budde-Steffen, et al., 1988). While the neurologic symptoms of POMA are discrete and therefore suggest discrete expression of the target antigens, immunohistochemical studies with POMA serum have detected the expression of target antigen in all CNS neurons (Luque, et al., 1991; Graus et al., 1993). This discrepancy may be explained if there are quantitative or



qualitative differences in the antigens expressed in the affected regions of brain. The potential for diversity of POMA target antigens is suggested by the multiplicity of POMA reactive antigens identified on Western blot analysis (Budde-Steffen et al., 1988; Buckanovich et al., 1993). POMA is also distinguished from the other PNDs in that the symptoms in POMA can be remitting and relapsing. 5/10 cases of confirmed POMA positive patients had improvement of neurologic symptoms (Dropcho and Payne, 1986; Luque, et al., 1991; Casado, et al., 1994; Darnell, 1994). Consistent with this, while the pathology in PCbD and PEM/SN shows significant neuronal dropout, and inflammatory infiltrates in gray matter (Posner and Furneaux, 1990; Jean et al., 1994; Corradi et al., 1996), the pathology of POMA brains is minimal with little evidence for specific neuronal death (Anderson, et al., 1988a). However, inflammatory infiltrates in regions of the brainstem and cerebellum, as well as Purkinje and brainstem neuronal dropout have been reported in some POMA patients (Anderson, et al., 1988a; Hormigo et al., 1994).

### Role of the immune system in PNDs

While the pathogenesis of the PNDs remains unclear, the presence of conspicuously high levels of antibody in patient's sera and CSF (normally devoid of any antibody) indicates that these disorders may be antibody mediated. Supporting this hypothesis, there is active synthesis of anti-onconeural antibodies in the CSF of PND patients, indicating that B cells invade the nervous system (Graus et al., 1988; Furneaux et al., 1990; Hormigo, et al., 1994). Furthermore, neurologic symptoms are reversible in Stiff Man's Syndrome (SMS-Howard, 1963), an autoimmune disorder associated with auto-antibodies to either the enzyme glutamic acid decarboxylase, or the synaptic vesicle protein synaptophysin (Solimena et al., 1988; Darnell et al., 1993; DeCamilli et al., 1993; Folli et al., 1993; David et al., 1994). Neurologic symptoms are also reversible in some POMA patients (Digre, 1986; Dropcho and Payne,



1986; Posner and Furneaux, 1990; Darnell, 1994), and rarely after treatment, in some PEM/SN and PCbD patients (Posner and Furneaux, 1990). This reversibility of symptoms is consistent with antibody mediated disruption of protein function (and potentially inconsistent with the idea that symptoms are attributable to a cytotoxic cellular response and cell death). For example, in LEMS, antibodies have been demonstrated to be pathologic, and the neurologic symptoms of LEMS can be reversed with the removal of auto-antibodies (Newsom-Davis and Murray, 1984; Newsom-Davis, 1990).

However, unlike in LEMS, PND antigens are intracellular (Budde-Steffen, et al., 1988; Darnell et al., 1991; Luque, et al., 1991; Dalmau et al., 1992; Corradi, et al., 1996; Darnell, 1996) and passive transfer of antibodies to animals does not reproduce symptoms (Graus, et al., 1991). Similarly, animals injected with PND antigens produce antibodies, but do not develop neurologic disease (Smitt, et al., 1995; Buckanovich and Darnell, unpublished observations). While these attempts to create animal models may be flawed (dose, delivery of antigen, lack of proper co-stimulators or potentially the inability of the immune response to cross the blood brain barrier under these conditions (Posner and Dalmau, 1995)) they suggest that the antibody response alone may not be sufficient as a causative agent for PND pathogenesis, and suggest the possibility of a cellular immune response.

At autopsy, the presence of cytotoxic CD8<sup>+</sup> T cells (T<sub>C</sub>), CD4<sup>+</sup> helper T cells (T<sub>H</sub>), and B cells, as well as neuronal dropout has been reported within the affected regions of the brains of most PND patients (Posner and Furneaux, 1990). Complement components and natural killer cells (NK) are minimal or absent (Jean et al., 1994). These observations suggest that these disorders may be mediated in part by a cellular, rather than humoral immune response. This idea is consistent with the established role for T cells in anti-tumor immunity. When anti-tumor immune responses are induced experimentally, in most cases T<sub>C</sub> lymphocytes appear to be the critical mediators, though in some situations T<sub>H</sub> cells are also required (for review



see Boon et.al.). Thus T cells may play an important role in the initial recognition and establishment of an immune response against the onconeural antigens in PND tumors. T<sub>C</sub> and T<sub>H</sub> cells are thought to require major histocompatibility complex (MHC) and co-stimulator proteins for antigen recognition (Hart and Fabry, 1995). Interestingly, the expression of MHC-I on tumors has been correlated with the presence of anti-Hu antibodies in SCLC patients; 17/20 tumors of Hu antibody positive patients expressed both MHC-I and Hu protein, whereas only 4/30 tumors of Hu antibody negative patients expressed both MHC-I and Hu protein (Dalmau et al., 1995). The presence of additional co-stimulators, such as CD 80 and CD 86 (Schwartz, 1992; Azuma et al., 1993; Freeman et al., 1993; Townsend and Allison, 1993), may help to explain the 3/20 patients who have Hu antibody but do not express MHC-I. Similarly, the absence of specific co-stimulators may explain the absence of an Hu antibody response in the 4/30 patients with Hu antigen positive, MHC-I positive tumors. While MHC-I and MHC-II proteins may be present on tumor cells, neither are thought to be expressed in normal adult neurons (Lampson, 1987; Lampson et al., 1994), thus complicating the hypothesis that PND pathogenesis is solely T cell mediated. There is no experimental evidence for or against the role of T cells in PND pathogenesis.

Regardless of the mechanism of disease, the PND immune response, while detrimental in terms of the nervous system, is clearly beneficial in restricting tumor growth. A majority of PND patients come to medical attention because of their neurologic disease, unaware that they have an associated cancer (Posner and Furneaux, 1990; Darnell, 1994; Darnell, 1996). Paraneoplastic tumors are generally well contained and often difficult to locate due to their limited size, sometimes only detectable with microscopic examination at autopsy. ~90% of PCbD patients and ~80% of POMA patients have limited or undetectable tumors (Dropcho and Payne, 1986; Luque, et al., 1991; Casado, et al., 1994). Similarly ~95% of PEM/SN small cell lung cancer patients, have cancers which are limited to a single nodule, while 60-70% of SCLC patients





without Hu antibodies have widely metastatic disease (Dalmau, et al., 1990; Graus, et al., 1993). The presence of a PND is correlated with a better response to therapy and survival (Graus et al., 1996). There are reported cases of pathologically malignant neoplasms being eliminated without treatment in association with the onset of neurologic disease (Darnell and DeAngelis, 1993; Zaheer et al., 1993). Interestingly, there exists a subset (~15%) of small cell lung cancer patients who harbor low titers of anti-Hu antibodies but do not have neurologic deficits. These patients have moderately limited tumors; 7/7 of these patients present with tumor limited to the chest (Dalmau, et al., 1990). Thus there appears to be an inverse correlation between tumor burden, and the level of the immune response against PND antigens.

### RNA Binding Domains (RBDs)

RNA binding proteins (RBPs) are critical regulators of gene expression at every post-transcriptional level including splicing, transport, localization, translation, and stability (Bandzuilis et al., 1989; Dreyfuss et al., 1993; Morris D.R., 1993; Pinol-Roma and Dreyfuss, 1993). Numerous conserved RNA binding motifs have been identified in proteins with divergent functions (for review see Burd and Dreyfuss, 1994b). These motifs can occur in single or multiple copies in a protein.

By far the most abundant and best characterized of the RBDs is the RNA recognition motif (RRM) also known as the ribonucleoprotein-consensus sequence (RNP-CS). This is a large motif of 80-100 amino acids with two internal consensus sequences, RNP1 and RNP2. The RRM is found in numerous protein including the snRNA U1 binding protein U1A, hnRNPs A and C, poly A binding protein and several SR proteins including SF2 and U2AF (Dreyfuss et al., 1993; Gorlach et al., 1993; Burd and Dreyfuss, 1994; Ruskin et al., 1988; Krainer et al., 1990). Structural analysis of an RRM of U1A reveals that it contains a four stranded Beta sheet, supported by two alpha helices (Scherly et al., 1990). RNP1 and RNP2 lie side by side within the Beta sheet.



RNA appears to bind to the middle two beta strands and to a loop region on one side of the  $\beta$  sheet.

A second type of RBD is the KH (hnRNP K homology) motif. Determination of the solution structure of a KH module from the vigilin protein suggested that the motif forms a stable  $\beta(\alpha)_2(\beta)_2\alpha$  fold with a potential RNA binding surface in the loop between the two alpha helices (Morelli et al., 1995; Musco et al., 1996). The KH domain and proteins which utilize this motif will be described in depth below.

Another RBD is the arginine rich motif (ARM). This motif has a relatively poorly defined consensus and has been found mostly in viral and bacteriophage proteins. The best characterized of these is the HIV Rev protein (Zapp and Green, 1989; Heaphy et al., 1990; Kjems et al., 1991; Jensen et al., 1994). The RGG box represents yet another RBD, first identified in the hnRNP U protein (Kiledjian and Dreyfuss, 1992). Other proteins with RGG boxes include hnRNPs A1 and K as well as FMR-1 (Dreyfuss, et al., 1993; Siomi et al., 1993a). The RGG box consist of 6 to 18 RGG repeats separated by aromatic amino acids. The double stranded RNA binding domain is a poorly understood RBD, that has been identified in proteins such as the Drosophila staufen protein, DAI kinase, and double stranded RNA adenosine deaminases-RNA editing enzymes (Melcher et al., 1996).

Finally, the Zinc finger represents a well documented nucleic acid binding domain which can bind to both DNA and RNA. Zn fingers have been well characterized in many transcription factors including Gal4, GLI, WT-1 and transcription factor IIIA (TFIIIA;Engelke, 1980; Honda, 1980; Corton and Johnston, 1989; Pavletich and Pabo, 1993). However, several proteins including TFIIIA, HIV nucleocapsid proteins and eIF-2, also utilize the zinc finger motif as an RBD (Picard, 1979; Dannull et al., 1994; Flynn et al., 1994; Komatsu and Tozawa, 1994). Interestingly, the nine Zn fingers of TFIIIA are utilized to bind both DNA (regulating 5S RNA transcription) and RNA (regulating 5S RNA transport



and storage). While Zn fingers 1-3 represent a minimal DNA binding domain, and Zn fingers 4-7 represent a minimal RNA binding domain, all of the fingers appear to be involved in both RNA and DNA binding (Theunissen et al., 1992; Clemens et al., 1993; Hansen et al., 1993).

### Functions of RNA Binding Proteins in Neurons

Most previously described RBPs, such as the hnRNP's, snRNP's, splicing factors such as SC35, and ribosomal proteins are thought to be ubiquitously expressed, general cellular factors (Dreyfuss, et al., 1993; Kamma et al., 1995). Although it has been suggested that these ubiquitous proteins may be expressed at different levels in different tissues, few tissue specific RNA binding proteins have been described. The nervous system has several unique needs which may require tissue specific neuronal RBPs (nRBPs). These include tissue specific RNA splicing, RNA editing, localization, and stability.

The CNS has extremely complex gene expression. It is estimated that 30-60% of human genes are expressed in the CNS (Adams et al., 1991). The majority of these RNAs are neuronal in origin (vs. glia) and analysis suggests that 40-65% of the mRNAs expressed in brain are brain specific (Stamm et al., 1994). Of the neuronally expressed genes identified thus far there are numerous examples of regulation of gene expression at the level of alternative splicing (Stamm, et al., 1994). Alternatively spliced gene products demonstrate different expression patterns, between neural and non-neural tissues, within the CNS, during development, and in an activity dependent manner. Both the alpha-tropomyosin exon 9c, and the clathrin en exon, demonstrate developmental regulation within brain; exon included splice forms appear at different times and abundance relative to the exon excluded forms (Stamm et al., 1992). Neural activity also appears to regulate the splice decision. Increasing neuronal activity appears to increase splicing of the exon included form of clathrin (Stamm, et al., 1992). It is likely that specific sequences within the



alternatively spliced hnRNAs and specific RBP recognition of these sequences will be required to regulate the complex patterns of neuronal alternative splicing. Numerous genes, including c-src, clathrin, tropomyosin and others demonstrate tissue specific splicing within the CNS. Two potential mechanisms for tissue specific splicing have been suggested. One mechanism utilizes tissue specific factors which bind to specific target sequences in hnRNA to regulate alternative splicing. For example, the neuron specific 'en' exon of clathrin is not utilized in non-neural tissues because of poor consensus splice donor and acceptor sites. Gel shift analysis with brain and non-brain extracts suggests that neuron specific factors will be required to enhance the utilization of these sites (Stamm, et al., 1992). An alternative mechanism involves tissue specific differences in ratios of ubiquitous factors (Caceres et al., 1994). For example, the KH type RBP KSR is expressed in both neural and non-neural cells, and together with the ubiquitous hnRNP F appears necessary for activation of the neuron specific c-src exon (Min et al., 1996).

Another level of regulation of gene expression likely to require nRBPs is RNA editing. Members of the glutamate receptor family undergo RNA editing; this editing requires specific intronic sequences which form specific double stranded RNA structures (Higuchi et al., 1993). The recently isolated RED1 gene encodes a double stranded RNA adenosine deaminase which edits the Q/R site in GluR B *in vitro* (Melcher et al., 1996). While RED1 expression is not neuron specific, Northern blot analysis suggests neuron specific forms may exist. It has been proposed that there is likely a gene family of deaminases with distinct substrate specificities.

Neurons are also distinct from many cell types in that they are polarized cells with specific cytoplasmic compartments. While the predominance of cytoplasmic RNA is restricted to the cell soma, some RNAs, including mRNAs for MAP2, the IP3 receptor type II, and the alpha subunit of calcium





dependent calmodulin kinase, have been localized to the dendritic compartment (Kleiman et al., 1990; Steward and Banker, 1992) (Mayford et al., 1996). Electron microscopy studies have identified polyribosomes in post-synaptic dendritic spines, suggesting that localized protein synthesis may be important for neuronal plasticity. Some evidence also exist for the presence of tRNAs, ribosomal RNAs and mRNAs in axons (Van Minnen et al., 1994). There are two proposed models for RNA localization in a polarized cell (Steward and Banker, 1992). One model is based upon the mechanism for targeting transmembrane proteins to the endoplasmic reticulum. RNAs to be localized are partially translated, producing a signal peptide. The signal peptide is bound by 'signal recognition' protein which suppresses translation and then targets the RNA to the proper cellular compartment via protein:protein interactions. No experimental evidence exists for this model. A second model relies upon specific sequences in an RNAs 5' or 3' UTR which act as a 'zip code' (Kislauskis and Singer, 1992). The localization sequences in the RNA would be identified by RNA binding proteins which would transport the RNA to the proper cellular location.

Support for this model first came from studies in *Drosophila*. Early in *Drosophila* development, the egg is a single multi-nucleated polarized cell. Over twenty different developmentally expressed *Drosophila* RNAs demonstrate specific localization (Ding and Lipshitz, 1993). Localization of these RNAs and the proteins they encode is important in defining the *Drosophila* body axis. Some of these RNAs are localized via specific signals in their 3'UTR which are recognized by specific RBPs. *bcd* RNA encodes the anterior determining morphogen in *Drosophila*, and is localized to the anterior pole. The *exu* and *eap* gene products have been implicated in forming ribonucleoprotein particles with *bcd* mRNA and transporting the RNA along microtubules to the anterior pole (Wang and Hazelrigg, 1994; MacDonald et al., 1995). *Staufen* encodes an RBP considered to be important for binding and maintaining the localization of *bcd* mRNA (Ding and Lipshitz,



1993). *oskar* and *nanos* RNAs are localized at the posterior pole and are important in defining posterior development (Gavis and Lehmann, 1992; Kim-Ha et al., 1995). *oskar* encodes an RBP which can bind to its own 3'UTR and stabilize the localization (Rongo et al., 1995). *nanos* encodes an RBP which translationally inhibits the anterior determining proteins, *hunchback* and *bcd*, from being expressed in the posterior compartments. Similarly, *bcd* can act as an RBP to translationally inhibit expression of the caudal mRNA in the anterior compartment.

Based upon these studies and others, it is predicted that specific RNA signals and n-RBPs will play an important role in localizing, stabilizing and translationally regulating RNAs in different neuronal compartments. Consistent with this prediction, transgenic experiments utilizing a reporter gene and sequences from the alpha CamKIIa mRNA demonstrated that the 3' UTR of alpha CamKII mediates the dendritic localization of this mRNA (Mayford et al., 1996). One candidate to mediate this RNA localization is the recently identified nRBP, the testis brain RBP (TB-RBP). TB-RBP binds specific structures in the 3'UTR of protamine-1 mRNA to regulate its translation and stability (Han et al., 1995a). TB-RBP has also been shown to bind neuronal microtubules suggesting that it might be involved in RNA transportation and localization (Han et al., 1995b).

### Neuronal RNA Binding Proteins (nRBPs)

The first identified family of nRBPs consist of RRM containing proteins homologous to the *Drosophila* alternative splicing factor *sxl*. Members of this family are the *Drosophila* RBPs *elav*, RBP-9 and couch potato (*cpo*), and their mammalian homologues, the human and mouse Hu/HelN-1/MelN-1 proteins (Szabo, et al., 1991; Kim and Baker, 1993; Levine, et al., 1993; King, 1994; Abe et al., 1996) and the *elr* protein in *Xenopus laevis* (Good, 1995). These proteins have three RRMs, and have multiple splice variants. *elav* (embryonic lethal abnormal vision), is a pan-neuronal marker which because



of its homology to *sxl*, has been proposed to be involved in the establishment of neuron specific splicing patterns (Robinow, et al., 1988; Yao, et al., 1993). Developmental loss of function of *elav* leads to embryonic lethality, while temperature sensitive mutations lead to behaviorally defective adult flies (Campos, et al., 1985). Similarly, *cpo* was identified as an embryonic lethal mutation. Hypomorphic *cpo* mutations lead to abnormal function of the *Drosophila* sensory organs and abnormal phototaxis, geotaxis and flight behavior (Bellen et al., 1992). These observations suggests that nRBPs are important for both neuronal development and function in the adult. However, since the n-RBPs characterized to date demonstrate widespread or pan-neuronal expression, it is unlikely that these RBPs regulate regional development, differentiation, or function.

The Hu/Hel-N1 family of proteins were originally identified as antigenic targets in PEM/SN (Szabo, et al., 1991; Levine, et al., 1993; King, 1994; Okano and Darnell, 1996). Hu antigenicity can be detected in both the nucleus and cytoplasm of neurons just after they exit the cell cycle, and remains detectable in all mature neurons. In-situ hybridization studies using Hel-N1 coding sequences suggested that Hel-N1 is a pan-neuronal marker (Gao et al., 1994a). However, because of the extremely high level nucleic acid identity found between family members, distinguishing the expression patterns of the different Hu family members will likely require extensive in-situ hybridization studies using highly gene specific probes (e.g. UTR). Such studies are being performed and suggest that individual members of the Hu gene family may show regional and developmental specific expression (Okano and Darnell, 1996). The function of the Hu protein remains unclear. RNA selection performed using Hel-N1 identified U rich elements (Levine, et al., 1993). *In vitro* binding studies have shown that Hu family members can bind to AU sequences in the 3' UTRs of short lived mRNAs, such as *Id*, *c-fos* and *IL-6 in vitro*, thus implicating Hu proteins in regulating mRNA stability (Levine, et al., 1993; Gao, et al., 1994b; King et al., 1994; Ma et al., 1996). However,



RNA selection performed in our laboratory with multiple Hu family members identified distinct, complex RNA sequences (Okano and Darnell unpublished data) indicating that the function of the Hu proteins may be more complex than binding to AU rich elements. Based upon the homology of Hu proteins with the *Drosophila* elav and sxl proteins, it is believed that Hu proteins will be important for neuronal development, and may be involved in neuronal splicing.

A second family of RRM containing neuronal RNA binding proteins (n-RBPs) has recently been identified. This family is made up of the *Drosophila* and mouse musashi proteins (msi-Nakamura et al., 1994; Sakakibara et al., 1996) and the *Xenopus-laevis* nrp-1 protein (Richter et al., 1990). These proteins are analogous to the hnRNP A/B proteins. Mouse msi and nrp-1 are expressed in neural precursors in the ventricular zone of the developing neural tube in a pattern complementary to the Hu family of proteins. *Drosophila* msi is expressed in both the central and peripheral nervous systems of *Drosophila*, and is required for development of the adult external sensory organs.

### KH type RNA Binding Proteins

The KH type RBDs consist of a core of 36 primarily hydrophobic amino acid residues interspersed with essential charged residues. Three KH motifs are present in, and were first described and defined in hnRNP K. hnRNP K was originally identified with a group of proteins which co-purified with nuclear poly A+ RNA (Matunis et al., 1992). hnRNP K was defined as an RBP *in vitro* based on its ability to bind ribohomopolymers. hnRNP K preferentially binds poly rC *in vitro* and this observation has led to the suggestion that hnRNP K may bind to polypyrimidine tracts *in vivo* (Matunis, et al., 1992). Mutational analysis of the hnRNP K protein has demonstrated that the KH motifs are necessary for RNA binding *in vitro*. All three KH domains are required for full RNA binding activity (Siomi et al., 1994). Small deletions of the first or third KH domains, or mutation of the three KH domains reduced RNA





binding activity 70-95%. Mutation of the RGG box in hnRNP K had no effect on RNA binding. hnRNP K also avidly binds to single stranded DNA. Similar to RNA binding, mutation of the KH motifs abrogates ssDNA binding.

hnRNP K localizes predominantly to the nucleus, and is expressed in numerous tissues. Western blot analysis suggests that hnRNP K is expressed at highest levels in thymus, testis, spleen, lung and liver (Bustelo et al., 1995), while immunofluorescence suggests that the highest levels are in the brain and testis (Kamma et al., 1995). hnRNP K has been implicated in signal transduction via its potential protein interacting partners. Purification of the tyrosine kinase c-src from mitotic cells co-purified hnRNP K (Fumagalli et al., 1994). Also, yeast two-hybrid analysis with the vav proto-oncogene, an SH2-SH3 protein implicated in tyrosine kinase signal transduction in hematopoietic cells, identified hnRNP K as a binding partner (Bustelo et al., 1995). These two observations suggest a role for hnRNP K in mitogenic, tyrosine kinase mediated signal transduction. Consistent with this, hnRNP K has subsequently been identified as an up-regulated nuclear tumor antigen (Dejgaard et al., 1994).

Interestingly, despite being originally identified as an RBP, functional studies have suggested that hnRNP K is a transcription factor. *In vitro* the hnRNP K protein is capable of binding to the CT enhancer element in the promoter region of c-myc (Takimoto et al., 1993; Michelotti et al., 1995). Co-transfection of hnRNP K and various reporter plasmids suggested that hnRNP K activates transcription in a CT element dependent manner. Also, addition of hnRNP K stimulated RNA polymerase II activity in an *in vitro* transcription assay. Consistent with this hnRNP K binds TBP *in vitro* and co-immunoprecipitates with TBP from stably transfected cell lines (Tomonaga and Levens, 1995). Finally, yeast two-hybrid analysis with a portion of hnRNP K as the bait identified a putative transcriptional repressor protein zik1 (Denisenko et al., 1996). The *in vivo* relevance of this interaction is uncertain.



FMR-1 encodes a KH type RBP and was identified as the affected gene in the fragile X syndrome (Pieretti et al., 1991; DeBouille et al., 1993). Loss of FMR1 protein function, as a result of either hypomethylation and transcriptional down regulation, or point mutation, leads to a constellation of abnormalities including mental retardation, macro-orchidism and infertility. Similar to hnRNP K, FMR-1 was identified and defined as an RBP based on the presence of two KH domains, an RGG box, and its ability to bind ribohomopolymers (preferably rG and rU) *in vitro* (DeBouille, et al., 1993; Siomi, et al., 1994). The exact domains of FMR-1 required to bind RNA remain somewhat unclear. Deletion of the RGG box from FMR-1, leaving both KH domains intact, completely eliminated RNA binding activity *in vitro*. However, a point mutation of a conserved amino acid (I367=>N) of the second KH motif of FMR-1 in a single patient resulted in extreme mental retardation, suggesting a loss of function of FMR-1 protein and a critical role for the KH domains. The I367=>N mutation of FMR-1 reduces the *in vitro* binding affinity of FMR-1 for rU but not rG homopolymers. NMR spectroscopy suggests that this mutation disrupts the KH fold (Musco, et al., 1996).

The function of the FMR-1 protein is unknown. FMR1 is reported to be widely expressed throughout the developing embryo and adult, with expression in nearly all brain regions and in many clinically unaffected organs (Devys et al., 1993). Unlike hnRNP K, FMR-1 is predominantly cytoplasmic (Devys et al., 1993), although a role for FMR-1 in the nucleus has been proposed (Fridell et al., 1996). Yeast two-hybrid analysis with FMR-1 identifies FMR1 related proteins FXR1 and FXR2 as potential binding partners (Siomi, et al., 1996). Cell fractionation studies have shown that FMR-1 and the FXR proteins colocalize with the ribosomal 60S subunit (Khandjian et al., 1995; Siomi et al., 1996). Moreover, FMR-1 and the FXR proteins co-immunoprecipitated with proteins of the 60S ribosomal subunit, and thus it has been suggested that the FMR family of proteins may function in mRNA translation or stability.



While the functions of hnRNP K and FMR-1 remain unclear several KH proteins have been implicated in regulating alternative splicing. Genetic analysis identified the yeast MER1 protein as required for the proper meiosis specific splicing of the yeast MER2 transcript (Engbrecht and Roeder, 1990; Engbrecht et al., 1991; Nandabalan and Roeder, 1995). The MER-1 protein has a single KH motif and RNA footprinting suggests MER-1 is capable of binding to MER2 RNA in the region of the 5' exon/intron boundary *in vitro*. A second KH protein, Drosophila PSI, harbors three KH motifs and is required for the somatic tissue specific splice inhibition of the P element transposase transcript (Siebel et al., 1995). PSI functions in somatic tissues by binding to a 5' pseudo-splice donor and stabilizing U1 snRNP binding, preventing U1 binding to the correct 5' splice site. With U1 bound to the pseudo-splice junction, splicing does not occur, the intronic sequence is retained leading to a premature stop codon and an inactive transposase in somatic cells. Similarly, the KH RBP KSR appears to be necessary for the tissue specific splicing of the c-src N1 exon. KSR hnRNP F and 4 other proteins appear to interact to form a splice enhancer complex which leads to inclusion of c-src N1 exon in neurons (Min, et al., 1996).

Other roles for KH proteins include RNA localization and stability. The Drosophila Bicaudal-C (Bic-C) protein contains five KH motifs. Mutations of the Bic-C KH domains lead to mis-localization of oskar RNA in developing embryos, leading to the proposal that Bic-C may function to localize RNA in Drosophila embryos (Mahone et al., 1995). The alpha-complex proteins alpha-CP1 and alpha-CP2 are KH containing proteins recently identified as part of an alpha globin mRNP complex in erythrocytes. These proteins are believed to be involved in forming a sequence specific mRNP complex essential for maintaining the long half life of alpha globin mRNA (Kiledjian et al., 1995).



### Specific Aims.

The goal of the work presented here was to identify and characterize a gene encoding the target antigen for the recently described PND POMA. Identification of the genes encoding target antigens allows one to test the current model of PND pathogenesis (Darnell, 1996). This model proposes that it is the ectopic expression of immune privileged proteins in peripheral tumors that induces the autoimmune response which leads to neurologic disease. This model predicts that target antigens in general are expressed in immunologically privileged sites, and that target antigens are expressed in the neurologically affected regions of brain. In the case of POMA, a neurologic disease of the motor system, it is predicted that in addition to being restricted to immuno-privileged tissues, target antigens are expressed in the motor nervous system.

The second major aim was to determine the potential function of the POMA antigen in neurons and tumors. Functional analysis of bona-fide target antigens may provide insights into basic neurobiology. For example, identification of Recoverin, the target antigen of cancer associated retinopathy, has led to a greater understanding of cGMP mediated/ $\text{Ca}^{++}$  regulated photo-signal transduction in photoreceptor cells (Dizhoor, et al., 1991; Gray-Keller, et al., 1993). Knowledge about the role of a protein in brain can potentially lead to understanding the role of that protein in tumors. For example, ectopic expression of recoverin may lead to abnormal regulation of cGMP mediated signal transduction in small cell lung cancers.

Finally, the last objective was to combine descriptive and functional studies of the target antigen in an attempt to explain the role of this protein in the initiation and symptomatology of POMA. For example, in paraneoplastic cerebellar degeneration (PCbD), the target antigen CDR 62 is expressed specifically in Purkinje neurons and limited brainstem nuclei, suggesting a cause for the specificity of symptoms in PCbD (Fathallah-Shaykh, et al., 1991).





CDR 62 is a leucine zipper protein and functional studies suggests that CDR 62 interacts with c-myc and may inhibit c-myc mediated transcription (Corradi and Darnell, personal communication). Aberrant regulation of an oncogene can clearly lead to tumorigenesis in peripheral tissues, and may induce programmed cell death in neurons. Taken together these observations suggest that aberrant regulation of c-myc may play a role in PCbD tumorigenesis and may also lead to Purkinje cell apoptosis.

We have used POMA sera to identify a gene, Nova-1, which encodes an antigenic target of POMA sera. Nova-1 expression is consistent with the predictions for target antigen expression; Nova-1 is restricted to the CNS and is expressed predominantly in the subcortical motor systems. Functional analyses of Nova-1 demonstrate that Nova-1 is a neuronal RNA binding protein both *in vitro* and *in vivo*. Specifically, Nova-1 binds Nova-1 and glycine receptor alpha 2A pre-mRNAs in a sequence specific manner and may regulate alternative splicing of these pre-mRNAs. Finally, the *in vitro* RNA binding function of Nova-1 can be inhibited by POMA antibodies, suggesting that autoantibody mediated disruption of Nova-1 function may lead to the neurologic disease *in vivo*.



## Chapter II. Materials and Methods

**Cloning and Sequencing of Nova-1 cDNA's:** Recombinant phage were plated at a density of  $10^5$  pfu per 150mm plate of E. coli XLI-Blue; cDNA libraries from different areas of the brain of a single individual (cerebellum, frontal cortex, brainstem and hippocampus, Stratagene) and from a small cell lung cancer cell line were screened. After incubation for 6 hours at 37°C, plates were overlaid with filters soaked in IPTG (10mM) and incubated for a further 12 hours at 37°C. The filters were removed and incubated with anti Ri sera (1:500 dilution in 5% Milk in PBS) for 4 hours at room temperature. Filters were washed in TBST (50mM Tris pH7.5, 100mM NaCl, 0.2% Triton) and incubated with  $^{125}\text{I}$  Protein A. After washing again with TBST, filters were exposed to XAR5 film at -70°C with a lightning plus intensifying screen. Clones yielding positive signals were purified by several rounds of antibody screening until 100% of the plaques gave positive signals. The lRi8 clone was subcloned into the pBluescript (pBS) vector using the phagemid rescue protocol (Short et al., 1988). The full length cerebellar cDNA sequence was obtained by using a 240bp EcoR1-Bam 5' fragment of pRi8 as a random prime template, to generate  $^{32}\text{P}$ -labeled probe according to the manufacturer's protocol (Boehringer Mannheim). The full length cDNA from cerebellum generated an EcoR1-Bam 5' fragment of 800bp; both probes were used to screen duplicate filters from a number of libraries to generate Ri cDNA's from different areas of brain and tumor. cDNA's shown in Figure 2 were sequenced on both strands using the dideoxy termination method using a Sequenase kit (USB) and a variety of vector based and internal oligonucleotide primers. Sequence analysis was facilitated by the use of the MacVector sequence analysis program (IBI).

**pRi8 Fusion Protein Analysis:** Plasmid clone pRi8 was grown to an OD of 0.6 and induced by adding IPTG. Cell lysates were prepared in 2% SDS, and resolved by 8% SDS-PAGE and transferred to nitrocellulose essentially as



previously described (Darnell et al., 1991). Filters were blocked in 5% milk in PBS, incubated with antisera at the stated dilutions in 5% milk in PBS, washed 3 times in TBST, incubated with  $^{125}\text{I}$  Protein A, washed 3 times in TBST, and exposed to XAR film.

**$\lambda$  Affinity Purification of POMA Antisera:**  $\lambda\text{Ri8}$  or  $\lambda\text{Yo}$  clones were grown to confluence on a 150mm plate of E coli XL1-blue (Stratagene); filters were prepared and incubated with Ri antiserum as described in Figure 3. After extensive washing in TBST (see Figure 3), filters were incubated in 0.2M glycine pH2.5 with agitation for 5 minutes at room temperature. The glycine solution was removed (3mls) and immediately brought to neutral pH with 1M Tris pH9.5. Western blots of purified human Purkinje neurons were prepared and probed with antibody as described above; 50 $\mu\text{g}$  Purkinje neuronal extracts, prepared as described (Darnell et al., 1991), were run in each lane.

**NFP Affinity purification of Antibodies.** NFP was covalently coupled to cyanogen bromide 4B sepharose (Pharmacia) as per manufacturers protocol. 10 mls of POMA antiserum was spun at 40,000xg to remove precipitates, and the supernatant was incubated with 2 mls of NFP-CNBr Sepharose overnight in RIPA buffer (150 mM NaCl, 50mM Tris pH 7.4, 0.1% SDS, 0.1% NP 40, 0.5% deoxycholate). Sepharose was washed 5x 50mls in RIPA, column eluted with 4 mls 0.2M glycine pH 2.5, neutralized with 1M Tris pH 9.5 and dialyzed against phosphate-buffered saline (PBS).

**RT-PCR Analysis:** 1 $\mu\text{g}$  total RNA from whole embryos (E.8, E10, E12), embryo heads (E.14, E.18) or adult brain, (obtained using the method of Chomcynski and Sacchi, 1987) were denatured at 70°C for 10 minutes in the presence of 2.5mM random hexamers (Boehringer Mannheim), and incubated in the presence or absence of 100 Units of reverse transcriptase (Superscript, BRL) in a 20 $\mu\text{l}$  reaction containing 1mM of each deoxynucleoside triphosphate (dNTP's) for 10 minutes at room temperature followed by 40 minutes at 42°C.



Reactions were terminated by incubation at 95°C for 5 minutes. One tenth of each RT reaction product was added to a PCR reaction containing 50mM Tris pH 9.5, 1.5mM MgCl<sub>2</sub>, 20mM Ammonium sulfate, 1 unit of AmpliTaq-Polymerase (Perkin-Elmer Cetus) 2.5mCi deoxycytidine 5' (α-<sup>32</sup>P) triphosphate, and 0.5mM each of a 5' primer Ri17 and 3' primer Ri22. PCR analysis was performed in 50ml final volume with the following cycle: 5 minutes at 94°C; 35 cycles of 45 seconds at 51°C, 1 minute at 72°C, 45 seconds at 94°C; 10 minutes at 72°C. Twenty percent of the PCR reaction was run on 10% PAGE and the products analyzed by autoradiography. PCR products were analyzed by cloning into the TA cloning vector (InVitrogen) according to the manufacturer's directions, and sequencing as described.

**Northern blot analysis:** 2.0μg of polyA<sup>+</sup> RNA from the indicated tissues were run on a 1.0% formaldehyde containing agarose gel, and transferred to nitrocellulose (MTN blot, obtained from Clontech). Probe was prepared by random prime nick translation of a 1380bp 5' fragment of plasmid pRi8, labeled to a specific activity of 10<sup>9</sup> CPM/μg, and hybridized in 40% formamide at 42°C for 24 hours; stringent washes were in 0.1XSSC/0.1% SDS at 55°C, and XAR film exposure for 72 hours.

**Immunohistochemistry** Slides were fixed for 10 minutes in 75% ethanol 20% acetic acid, washed 3 times in PBS, incubated in 0.3% H<sub>2</sub>O<sub>2</sub>/PBS and rewashed 3 times with PBS. Blocking was performed using avidin and biotin blocking solutions (Vector Labs) with 5% normal goat serum. After washing 3 times in PBS slides were incubated with primary antibody (λ affinity purified and λ mock purified POMA sera were used undiluted; NFP affinity purified antibody was diluted 1:100; anti-Hu sera or normal human sera were diluted 1:500 in 2% BSA/PBS) for 2 hours at room temperature. Antibody was removed, slides were washed 3 times in PBS and incubated overnight at 4°C with 1:2000 biotinylated goat anti-human antibody in PBS. After washing, slides were treated with avidin-biotin peroxidase complex (Vectastain ABC





complex, Vector Labs) for 45 minutes, washed in PBS and developed with 0.1% diaminobenzidine-HCl (Hsu et al., 1981), counterstained in Harris modified hematoxylin, dehydrated and mounted in Permount (Fischer Scientific).

**Protein Alignments.** KH domain alignment was made using the Geneworks (Intelligenetics Inc.) and the MACAW programs.

**Immunofluorescence.** 12  $\mu$ m frozen mouse brain sections were fixed in acetone/methanol (1:1) for 2 minutes, washed 3X in 0.1% Triton X100 in PBS, blocked in 10% normal goat serum and 50mM glycine in PBS, washed 3X and incubated with primary antibodies overnight (dilutions used were rabbit GFAP 1:300, affinity purified POMA antibodies 1:50). Slides were washed 3X in 0.25% gelatin-PBS, incubated with secondary antibodies (FITC anti-rabbit, and rhodamine anti-human, Vector Laboratories) for 45 minutes and washed 3X in gelatin-PBS.

***In situ* hybridization** Sense and anti-sense RNA's from bp 2399-4129 (3' UTR) or 590-1453 (3' coding region), or bp -110 to -20 (5'UTR) of the mouse Nova-1 c-DNA, were *in-vitro* transcribed with T7 RNA polymerase and  $^{35}$ S UTP (used for embryonic sections) or  $^{33}$ P UTP (used for adult sections) to a specific activity  $2 \times 10^9$  cpm/ $\mu$ g. *In situ* hybridization was performed with fresh frozen 12  $\mu$ m sections using a method adapted from previous descriptions ([newman]) and a hybridization temperature of 60°C or 50°C for 5' UTR. Slides were dipped in Kodak NTB2 emulsion. Embryonic sections were exposed in the dark for 3-4 days, adult sections and 5' UTR hybridized sections were exposed for 10-14 days, developed, and counterstained with Cresyl-violet or hematoxylin.

**Fusion Proteins.** A cDNA encoding the full length Nova protein (NFP), PCR generated fragments harboring the Nova-1 KH1 domain (N1) or KH3 domain (N3), or a gene encoding a control protein (Yo; Fathallah-Shaykh et al., 1991) were cloned into pET21B (Novagen) such that each was in an open reading



frame encoding the T7 epitope at the N-terminus and a Histidine tag at the C-terminus. All clones were sequenced in their entirety to ensure fidelity of the PCR reaction. The N3<sup>L21N</sup> protein was constructed by site directed oligo based mutagenesis. The N1<sup>I14T</sup> fusion protein was isolated from a PCR AUU->ACU mutation. Fusion proteins were purified by nickel chelation chromatography.

**Determination of Active Fusion Protein.** Protein was incubated with an excess of ribohomopolymer agarose, or ssDNA agarose for 30 min at room temperature (B1). Agarose was washed 5 times and bound protein eluted with 2X sample buffer. Supernatant from B1 was incubated with a second batch of ribohomopolymers for 30 minutes (B2). B2 agarose was washed 5 times and bound protein was eluted with 2X sample buffer. The supernatant from B2 was frozen and lyophilized then resuspended in sample buffer. Protein present in the B1, B2 and supernatant fractions were estimated by western blot analysis and densitometry.

**Ribohomopolymer and ssDNA agarose binding assays.** 500ng of NFP or equimolar amounts of N1 and N3 fusion proteins were incubated with ribohomopolymer agarose or single-stranded DNA agarose (Sigma) in 1ml of RSB binding buffer (Swanson and Dreyfuss, 1988) containing the indicated NaCl concentrations and 1mg/ml heparin (Sigma). Agarose was washed 5 times in binding buffer and bound protein was analyzed by SDS-PAGE and Western blot using a T7 monoclonal antibody (Novagen) and enhanced-chemiluminescence (Amersham). To control for protein loading, an amount equivalent to 20% of the fusion protein used in each binding assay was loaded (+). Binding for quantitative analysis was performed in 1 ml of 250 mM NaCl-RSB, heparin and the indicated amounts of protein. Western blots were quantitated by densitometry.

**Northwestern analysis.** Equimolar amounts of indicated fusion proteins were run on SDS-PAGE, and transferred to nitrocellulose. Protein blots were



blocked overnight in binding buffer (10mM Tris pH 7.4, 50mM NaCl, 1mM EDTA, 2X Denhardt's, 100µg/ml tRNA), probed with a <sup>32</sup>P end-labeled polyriboguanosine probe, washed 4 times for 15 minutes in binding buffer, and exposed to film. The filter was washed twice for 30 minutes at 55°C in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl) and then reprobed with T7 antibody.

**Binding to brain RNA.** NFP or control fusion protein (50 µg) was incubated with 10 µg of total mouse brain RNA prepared as previously described (Chomcynski and Sacchi, 1987) in RNA binding buffer (Levine et al., 1993). Proteins were then immunoprecipitated in the same buffer with the indicated antibodies, washed 5 times with binding buffer, and bound RNA was phenol extracted, DNase digested and ethanol precipitated. RNA was reverse transcribed and PCR amplified in the presence of <sup>32</sup>P α-dCTP as previously described ([Buckanovich, 1993 #181]), using either Nova-1, actin, or NAP specific primers. To ensure RNA integrity, total brain RNA was also amplified (+ Control). As a control for DNA contamination each RNA sample was analyzed in duplicate with or without (+ or -) reverse transcriptase. Ten percent of each PCR product was analyzed by non-denaturing PAGE and the gel was dried and exposed to film.

**RNA/Protein Filter binding assay** (chapter IV). 100ng of fusion protein was incubated with the indicated concentrations of affinity purified antibody and BSA for 30 minute at room temperature in 100µl of Binding buffer (250mM LiCl, 20 mM Tris pH 7.4, 10mM MgCl<sub>2</sub>). 100ng of <sup>32</sup>P end-labeled rG ribohomopolymer was added and incubated for 25 minutes at room temperature. Samples were vacuum filtered through 0.2µM nitrocellulose, washed with 1 volume of cold binding buffer and analyzed in a scintillation counter.



**RNA selection.** An oligonucleotide harboring a 52 bp random sequence surrounded by primer binding sites (GGG AGA ATT CCG ACC AGA AG N(52) TAT GTG CGT CTA CAT GGA TCC TCA; Famulok and Szostak, 1992) was synthesized on an ABI DNA synthesizer to yield 2 mg DNA with an estimated complexity of  $\sim 3 \times 10^{16}$  sequences; the oligonucleotide was characterized and PCR amplified using forward and reverse oligonucleotide primers as described (Ellington and Szostak, 1990; Famulok and Szostak, 1992). Following PCR amplification the sequences of 24 random clones from this pool were determined; each clone was unique, and the overall base composition showed a slight excess of purines (24% A; 33% G) over pyrimidines (21% C; 20% U), and a random distribution of di-nucleotide frequencies (data not shown). Two pool equivalents of library DNA (110 $\mu$ g,  $\sim 2.1 \times 10^{14}$  molecules) were transcribed *in vitro* with T7 RNA polymerase and [ $\alpha$ - $^{32}$ P]UTP as described (Ellington and Szostak, 1990). RNA was purified from denaturing acrylamide gels, heated to 70°C for 5 minutes and applied to a pre-column containing an irrelevant histidine tagged fusion protein to adsorb non-specifically bound RNAs. The eluate was applied to a histidine-tagged Nova-1 nickel affinity column in high salt buffer (0.5 M LiCl, 20mM Tris-HCl, pH 7.6, 1mM MgCl<sub>2</sub>, 60mM imidazole; (see Ellington and Szostak, 1990; Green et al., 1991). Following extensive washing, protein and RNA were co-eluted by the addition of 1.0 M imidazole in high salt buffer. Protein-bound RNA was extracted in phenol/0.5% SDS at 50°C, and ethanol precipitated with LiCl and glycogen.

**Filter binding assays.** Filter binding was performed as previously described (Buckanovich et al., 1996). Briefly, the indicated concentration of fusion proteins were incubated with 50 fmol of *in vitro* transcribed  $^{32}$ P labeled RNA in 100 $\mu$ l of binding buffer (0.5 M LiCl, 20 mM Tris-HCl pH 7.4, 1 mM MgCl<sub>2</sub> and 1mg tRNA/ml) for 30 minutes, filtered through nitrocellulose, washed





with 1 volume of binding buffer, and retained counts determined in a scintillation counter.

**Gel shift assays.** Gel shift assays were performed with 50 ng, or otherwise indicated amounts, of fusion protein in 10  $\mu$ l of protein buffer (1 mg BSA/ml 0.5 M LiCl, 20mM Tris-HCl, pH 7.6, 1 mM MgCl<sub>2</sub>). 100 fmol of RNA was heated to 80°C for 5 minutes, cooled to room temperature and then added to RNA buffer (0.5 M LiCl, 20 mM Tris-HCl, pH 7.6, 1 mM MgCl<sub>2</sub>, 1 mg tRNA/ml) in a final volume of 10 $\mu$ l. Protein and RNA samples were mixed and allowed to equilibrate for 30 minutes at room temperature. For supershift experiments, antibody was then added and incubated an additional 30 minutes at room temperature. 2% of each sample was resolved with a 0.5 M Tris-glycine 4% PAGE, dried and exposed to film.

**Immunoprecipitation.** Adult mouse brains were homogenized with a Dounce in NET-Triton (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.1% triton-X 100, protease inhibitors). Nuclei were collected by centrifugation and resuspended in 1 ml NET-Triton, sonicated, spun in a microfuge, and the supernatant was precleared with Protein-A Sepharose for 30 minutes at 4°C, followed by incubation with the indicated antibodies and Protein-A Sepharose. After washing 5 times with cold RIPA buffer (150 mM NaCl, 50 mM Tris 7.4, 0.05% SDS, 1% nonidet P-40, 0.5% deoxycholate), samples were resuspended in DNase buffer (50 mM Tris-HCl pH 7.4, 6 mM MgCl<sub>2</sub>, 3 mM CaCl<sub>2</sub>) with 20 units of DNase and 20 units of RNase inhibitor at 37°C for 1 hour, extracted with phenol-chloroform at 55°C, extracted with chloroform and precipitated with ethanol and sodium acetate. Reverse transcription and PCR was performed as previously described. All PCR reactions, with the exception of the PCR reaction to clathrin, were performed using 3 cycle PCR (1 minute at 94°C, 30 seconds at 58°C, 45 seconds at 72°C). PCR to detect clathrin DNA was performed using 3 cycle PCR 1 minute at 94°C, 30 seconds at 54°C, 45 seconds at 72°C. Primers used were as follows: GlyR $\alpha$ 2A



AAAATACTAGTGGGAAGTTATCATGCA, CATGGTGGTTTCTGTGACTGATC.Nova-1, GCGAATTCTCCAGATCGCATCAAACAA,ACTGAAGGCTCCAAAGTCTTC Clathrin TTAACCCTGTGCCTGCCTGTCTTG, GCTGCTGGTAGAACGCTTTGTCAG, HuD GGACATGAATTACTTGCCAT, CATAGGCCATATTAAGCA, HeIN1 ATTGCTGTAACCAATCTA, ATTCCATCGATCGTCA TT, brain specific Na<sup>+</sup> channel, GGAATTCTGGAACTGGCTGGATTT CAGTG, TGC GGAATTTATCATGG CACC.

### Splicing Assays

Transfections. RAC cells were grown to 80-90% confluence in 60 mM dishes. 0.5 µg pJΩ GlyRα2A, 0.25 µg pCMV β-galactosidase, and 3 µg pCDNA3 vector or the indicate amounts of pCDNA Nova-1 DNA + pCDNA vector to equal 3 µg total were added to 300µl serum free DMEM and mixed with 300 µl DMEM containing 10 µl lipofectamine (Gibco-BRL), and allowed to incubate for 35 min at room temperature. 2.5 mls serum free DMEM were added to each DNA/lipofectamine mixture and the mixture was added to cells which had been washed once in warm serum free media. After 5 hrs, 3 mls of DMEM/1mM L-Gln/20%FCS was added to each transfection.

Harvesting cells. After 30 hours of transfection cells were trypsinized with 0.5 mls trypsin-EDTA, then trypsin was inactivated with 1ml DMEM 10% FCS. 0.70 mls of cell suspension from each transfection were used for RNA isolation, and 0.7 mls of cells from each transfection were used to make protein extract for β-galactosidase assays and western blot analysis.

RNA isolation was performed according to Current Protocols rapid isolation of RNA protocol. RNA was resuspended in 100 µl DEPC H<sub>2</sub>O at 80C for 10 min, desalted using S200 spin columns (Pharmacia), and digested with 25 units DNase, 40 units BamH1 for 1 hr at 37C, phenol-chloroform, extracted, and ethanol precipitated.



RT-PCR was performed as described. RT was determined to be linear with RNA input between 0.3 and 5 µg RNA. PCR was determined to be linear with input between 22 and 30 cycles. RNA input for RT reactions was normalized for transfection efficiency as determined by B-galactosidase activity for each transfection.

β-galactosidase activity was determined using 40% of isolated cell pellet for each assay. Assays were performed for 20-30 min at 37°C as per manufacturer's directions (Stratagene).

### Constructs used.

#### Primer list

CGCGTCGACGGGACCCATACGGGGGTTC	NFP Forward
GCACGGCCGGATTGGCAGCCCGAACTC	NFP reverse
CGGCCGCCCTCTCAGTAGTACCTGG	Pr 3' KH1
GTCGACGTAGTTGAAATAGCAGTTCCAG	pr 5' KH3
CGGGGTACCTCAATCCTCTTGATCTTCTGGATG	3' KH2 proex
GGGAATTCCATATGCAGTACTTTCTAAAGGTTCTCATACCT	5' KH1 proex
GGCAAAGGAGGGAAAACCAACGTT	Delta 21 primer 1(psp 14061)
CACAACGTTGGTTTTCCCTCCTTTGCC	Delta 21 rc
GCGAATTCTCCAGATCGCATCAAACAA	FRi5'
AGTAATACGACTCACTATAGGGAGAATTCCGACCAGAAG	5' Selex primer
ATTAACCCTCACTAAAGGGAACAAAAG	3' Selex primer
TAGACGCACATAAGCGCGTTAGACCCTTGATGAG	stem mutant primer
AAGACGTGAGTCAGCATGATAAG	SB2 T1
TTCTTCTCTTCAAGGGAGTCAGTGGG	SB2 T2-3



CCTTAATTAAAATTATTATTAGACTGCAAAGAAAAAAA	Gly mutant bttm pr #2
CTAATAATAATTTTAATTAAGGTTACATCGCTAACCAC	Gly mutant top pr #2
AGTAATACGACTCACTATAGGGAGCTTTCTGCAAAGACCATGAC	Gly t7-E2
CATGGTGGTTTCTGTGACTGATC	Gly Ex 3A
GGGAAGTTATCATGCAGTTCTGG	Gly 5'3A
AAAATACTAGTGGGAAGTTATCATGCA	Gly st insert
AAGCTTGTGGTTCAAATGCTCTGTATTAG	5' Gly E2
GGATCCGTGCAAAGTATTCCTAACACC	3' Gly E2
CCCATATCCCATTTCCTTCACAC	3' Gly 3B
CCGCTGTGAAAGTATGGTGTAGATGAG	D3 Reverse
AGTGTGTAGAGTAGAGGAGTATACTGTACCTGATTGGCTC D3	Reverse mutant
AGTAATACGACTCACTATAGGGAGACATTGCCATCTTCCCC	t7 Eh primer
CCGCTGTGAAAGTATGGTGTAGATGAG	D3 reverse
AGTGTGTAGAGTAGAGGAGTATACTGTACCTGATTGGCTC	D3 rev mt

### Synthesis of Constructs Used

#### Fusion Proteins.

NFP. PCR synthesized using NFP forward and NFP reverse primers and mouse Ri17 as template. PCR product was Ta cloned, digested with Sal 1 and Eag1 and cloned into Sal/Eag digested pet 21b.

N3. PCR synthesized using 5'KH3 and NFP reverse primers and mouse Ri17 as template and cloned as described in NFP construction.

N3L21N. PCR synthesized as two fragments; primer pair 5' KH3 and delta 21, and primer pair delta 21 rc and NFP reverse were used to amplify from mRi17. The resulting fragments contain a Psp14061 restriction site which





creates a point mutation. Resulting PCR fragments were digested with Sal/Eag 1/ and Psp14061 and ligated into Sal/Eag digested pet21b.

N1. PCR synthesized using NFP forward and 3' KH1 primers and mouse Ri17 as template and cloned as described in NFP construction.

N1-2. PCR synthesized using 5'KH1 and 3'KH2 proex primers and mouse Ri17 as template. The PCR product was digested with Nde and KpnI and cloned into Nde1 Kpn 1 digest proex vector.

#### Selex mutants.

SB2 Stem mutant. PCR synthesized using 5' Selex and stem mutant primers using SB2 as template. PCR products were gel-purified (Qiagen) and directly transcribed using T7 RNA polymerase.

SB2 A=>U. PCR synthesized as two fragments using cloned SB2 (Ta vector) as a template and the primer pairs 5' Selex- SB2T1, and SB2T2-M13. Individual PCR fragments were purified, blunt end ligated and the ligated products were used as template to PCR amplify using the 5' selex and 3' Selex primers. PCR product was Ta cloned and sequenced.

#### Glycine Receptor a2 clones.

GlyR E2. PCR cloned using primers 5'E2 and 3'E2 and mouse genomic DNA as template. PCR product was Ta cloned.

GlyR E 3a-3b. PCR cloned from genomic DNA using primers 5'Gly3a and 3'Gly 3B. PCR product was cloned using PCRscript.

Ta Gly R stem insert. PCR cloned from GlyR E 3a-3b using primers 5'stem insert and 3'Gly 3B. PCR product was cloned using PCRscript.



Ta Gly E2-3B. GlyR E 3a-3b was digested with Bam H1 and SacI, insert was gel purified and cloned into BamH1/SacI digested Ta GlyRE2.

pJOmega GlyRa2. Ta GlyE2-3B was digested with Xba1 and SacI, insert was gel purified and ligated into Xba-SacI digested pJ3Omega vector.

pJOmega GlyRst stem insert. Ta Gly R stem insert was digested with Xba1 and SacI, insert was gel purified and ligated into Xba-SacI digested pJ3Omega vector.

pJomega Gly mt (B46). PCR synthesized as two fragments using GlyRE2-3B as template and primer pairs 5'E2 -Gly Bottom mutant#2, and Gly mutant bottom primer-3'E3b. PCR product were EtoH ppt, digested with Pac I, gel purified, and ligated. Ligation was used as template to amplify with 5'Gly E2 and 3'Gly 3B primers. PCR product was Ta cloned. Ta clone was EcoRI digested, insert was purified and cloned into EcoRI digested pJomega.

#### GlyR clones used for FBA.

Ta Gly 3A-B or Ta Gly mt B46 were linearized using AluI, treated with proteinase K and transcribed with T7 RNA Polymerase.

In addition PCR products, synthesized with T7-E2 and Ex3A primers using either wild type or mutant glyreceptor constructs as template, were gel purified and transcribed using T7 RNA Polymerase.

#### Nova-1 Genomic DNA clones .

For FBA mouse genomic DNA was amplified with T7-EH and either D3 reverse or D3 reverse mutant primers. PCR product was gel purified and transcribed using Ty RNA Polymerase.



### pCDNA Eukaryotic expression clones.

pCDNA Nova-1. mmtvRi (a kind gift from Jennifer Darnell) was digested with XhoI and ligated into XhoI digested CIP treated pCDNA-3.

pCDNA Nova-1 pt mutant (Psp2). PCR synthesized as two fragments using primer pairs Fri5'-delta21, and delta 21 rc and NFP reverse were used to amplify from mRi17. The resulting fragments contain a Psp14061 restriction site which creates a point mutation. Resulting PCR fragments were digested with PflmI/BstEII and Psp14061 and ligated into PflmI/BstEII digested pCDNA Nova-1.

pCDNA Nova-1 Deletion mutant (Psp18). Purified as a result of an aberrant ligation in the preparation of pCDNA Nova-1 pt mt. Creates a stop codon just distal to KH2.



### **Chapter III. -**

#### **Identification and Expression of Nova-1, a Target Antigen of POMA.**

The association of specific PND antigens with specific auto-immune neurologic disease and the recruitment of specific antigens by specific tumor types, suggests that PND antigens may perform an important function in brain and in tumors. Moreover, the model of PND pathogenesis suggests that peripheral tumors elicit an immune response when they ectopically express immunologically privileged antigens. This model thus predicts that normally target antigen expression is restricted to immunologically privileged sites (e.g. neurons). Thus isolating genes which encode PND target antigens is a method to identify potentially important neuronal genes. Moreover identification of genes encoding PND target antigens allows one to test the prediction of the model for PND pathogenesis, and begin to determine the functional significance of these proteins in brain and tumors.

Using autoantibodies from PND patients sera, it has been possible to identify clone, and characterize genes encoding PND target antigens. In the case of the PND PEM/SN, autoantibodies identified the Hu family of neuron specific RNA binding proteins (Szabo, et al., 1991). These proteins have been implicated in regulating neuronal alternative splicing and mRNA stability (Levine, et al., 1993; Gao, et al., 1994b; Ma, et al., 1996). In PCbD, autoantibodies identified CDR 62, a novel leucine zipper protein which is expressed primarily in cerebellar Purkinje cells and the testis. CDR 62 has been implicated in the regulation of c-myc activity. Currently characterized PND target antigens all have expression limited to immune privileged tissues, including primarily the nervous system but also the germ cells of the testis and ovaries (Newman, et al., 1995; Corradi, et al., 1996; Darnell, 1996; Okano and Darnell, 1996).





A novel anti-neuronal antibody (Ri) was discovered in 7/8 patients with paraneoplastic opsoclonus, myoclonus, and ataxia (POMA) (Luque, et al., 1991). We used sera from one of these patients to screen a human cerebellar cDNA expression library and identified a gene, termed Nova-1, which encodes a 54 Kd, 510 amino acid target antigen, containing three putative KH type RNA binding domains. Immunohistochemistry using affinity purified Ri antisera, northern blot analysis, and in-situ hybridization with Nova-1 specific probes, reveal that Nova-1 is expressed only in the nervous system. A detailed developmental study of Nova protein and Nova-1 RNA expression demonstrates that both Nova antigens and Nova-1 RNA are first detected in ~E10 embryos and are maintained throughout development and adulthood. Expression is restricted to neurons of the central nervous system at all stages. While the Nova family of antigens are expressed in all CNS neurons, Nova-1 protein and RNA expression are restricted to a subset of subcortical neurons throughout development and adulthood. Thus, not only is Nova antigen expression restricted to the CNS throughout development and adulthood as predicted by the current model for PND pathogenesis, but the specific restriction of Nova-1 protein and RNA to neurons of the subcortical CNS is consistent with the focal neurologic deficits of PND patients.

Identification and Characterization of a cDNA Clone Encoding a POMA Target Antigen We screened a human cerebellar cDNA expression library with POMA antiserum (clinically termed Ri) (Luque, et al., 1991) from one patient with POMA, yielding three identical isolates (by restriction analysis) from  $10^6$  plaques. One, termed  $\lambda$ Ri8, was subcloned by phagemid rescue and the plasmid (pRi8) used to make fusion protein in E. coli. Figure 1 shows a Western blot of the Ri8 fusion protein probed with POMA antiserum. POMA antiserum recognized a fusion protein of  $M_r$  40 kDa (Figure 1A, "Anti-Ri Sera": lanes 1-6, titration of POMA antiserum from one patient), but normal human serum or disease controls do not (Figure 1A, "Non-Ri Sera": lanes 7-



15, sera from 9 different patients). The pRi8 fusion protein reacts at similar titers (1 $\mu$ g/ml IgG or less) with sera from 7 of 7 adult patients tested with POMA (Figure 1B). These results demonstrate that POMA antisera recognize the recombinant clone pRi8 with high sensitivity and specificity.

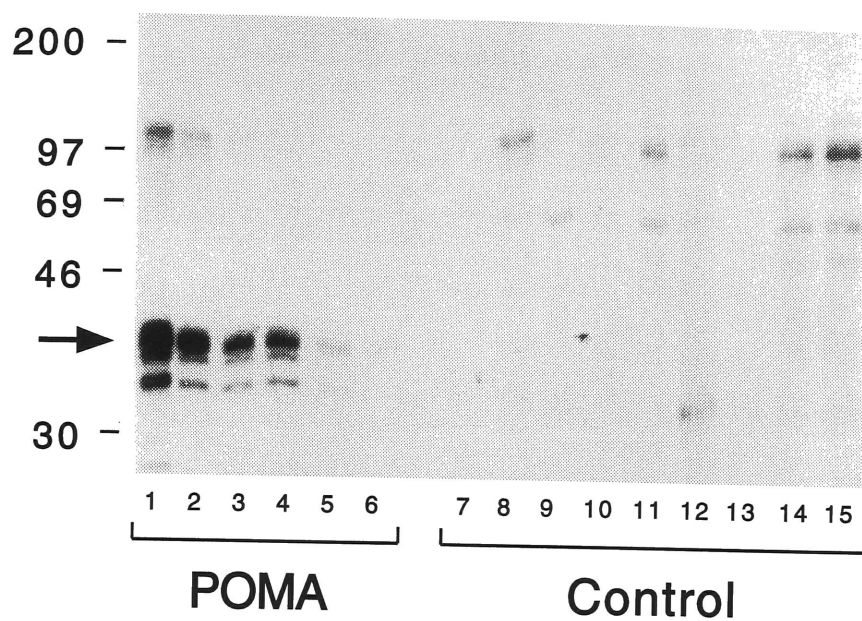
$\lambda$ Ri8 fusion protein was used to affinity purify antibody from POMA antiserum. Native POMA antiserum was incubated with nitrocellulose filters confluent with either  $\lambda$ Ri8 or, as a negative control, an irrelevant lambda clone. After washing the filters extensively, specifically bound (affinity purified) antibody was eluted at low pH and used to probe Western blots. Figure 2 shows a Western blot of human Purkinje neuronal extracts; native POMA antiserum binds to a major antigen of 55kDa and a minor antigen of higher  $M_r$  (as previously described, Budde-Steffen, et al., 1988; Luque, et al., 1991; Figure 2, lane 3;POMA). Normal human serum gives no signal (Figure 2, lane 4; NHS). POMA antiserum affinity purified with the  $\lambda$ Ri8 fusion protein bound antigens of identical  $M_r$  to those recognized by native POMA antiserum (Figure 2, lane 1 versus lane 3), while POMA antiserum mock purified with an irrelevant lambda clone showed no detectable binding on Western blots (Figure 2, lane 2). Affinity purified POMA antibody also recognized nuclear neuronal antigens by immunohistochemistry on adult human and mouse brain tissues, while mock purified antibody did not (data not shown, and Figure 8-11). Finally we constructed a full length fusion protein which was used to generate polyclonal rabbit antiserum. Both native and affinity purified rabbit anti-Nova-1 antibody, revealed a western blot and immunohistochemical pattern identical to POMA sera. These data demonstrate that the cloned pRi8 fusion cDNA encodes a neuronal antigen recognized by POMA sera.

Figure 1. POMA antiserum recognizes the pRi8 fusion protein.

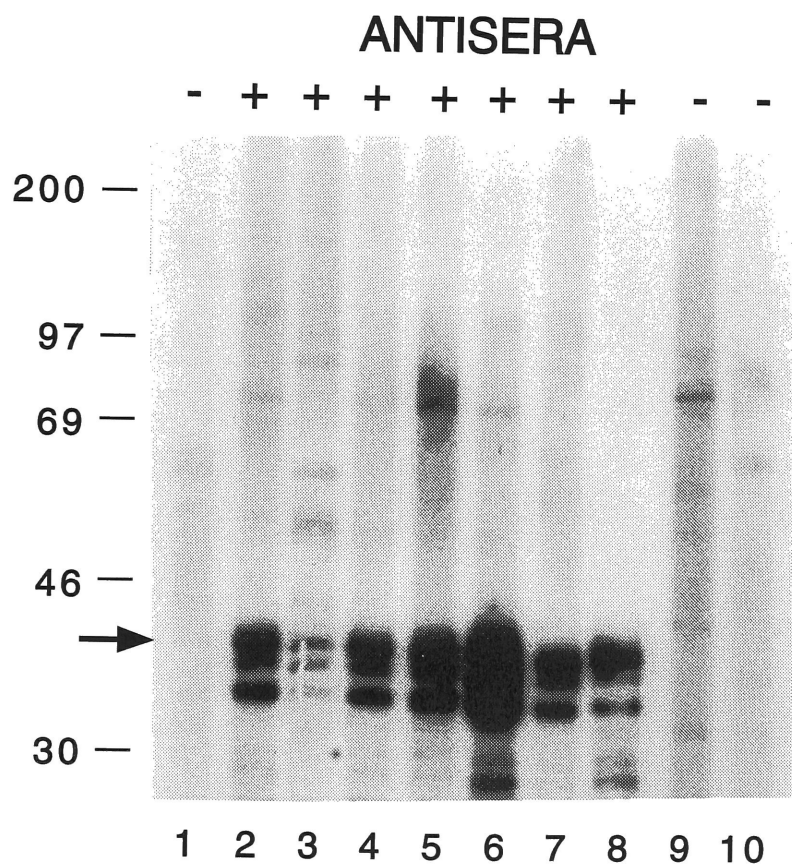
A. Western blot analysis with protein produced from *E. coli* containing plasmid pRi8 and antisera from either a patient with POMA (POMA lanes 1-6, showing two-fold serial dilutions ranging from 1:250 in lane 1 to 1:8000 in lane 6) or a number of normal sera and sera from unrelated neurologic disease [lane 7, serum from a patient with Sjogren's syndrome that reacts with a 40kDa neuronal antigen (unpublished data), 1:250 dilution; lane 8, serum from a patient with renal cell cancer, without a paraneoplastic syndrome, with reactivity to an unidentified 80kDa neuronal antigen (unpublished data), 1:250 dilution; lane 9 and 10, two different normal human sera, 1:125 dilution; lane 11, 14, 15, sera from a patient with atypical cerebellar degeneration, no malignancy (unpublished data), 1:250, 1:125 and 1:62.5 dilutions, respectively; lanes 12 and 13, sera from two children with neuroblastoma and paraneoplastic opsoclonus/myoclonus, 1:250 dilution]. Sera from patients with paraneoplastic Hu (Szabo et al., 1991), Yo (Fathallah-Shaykh et al., 1991) or Nb (Darnell et al., 1991) antibodies are not reactive with the Ri fusion protein, nor are over 20 sera from normal human individuals (data not shown). POMA antisera reacts with a fusion protein of  $M_r$  40kDa (indicated by the arrow), and some smaller molecular weight species whose intensity varies between preparations and are likely to be proteolytic fragments; there is non-specific reactivity at high serum concentrations with high molecular weight *E coli* fragments (lanes 1, 8 and 15).

B. The pRi8 fusion protein reacts with sera or CSF from 7 POMA patients. A Western blot of pRi8 fusion protein with various sera: those labeled "+" are from patients with POMA and breast cancer (lanes 2-5, lane 7), small cell lung cancer (lane 8), or POMA with no identified tumor (lane 6, patient #6 in Luque et al., 1991), and those labeled "-" are from normal sera (lanes 1, 10) or a patient with atypical cerebellar degeneration without malignancy but with high titer anti *E coli* antibodies (used in lanes 11, 14 and 15 in (Figure 1A)). In each case, sera from patients with POMA showed significant immunoreactivity with the pRi8 fusion protein (band identified by arrow), while in no case did control sera show immunoreactivity with the fusion protein. Serum titers were 1:250 except in lanes 2 (1:1000), 4 (1:2000), 6 (1:4000), 7 (1:1000) and 8 (CSF was used, 1:50 dilution; no reactivity was seen with normal CSF at 1:10 dilution, data not shown).

**A.**



**B.**





Characterization of the Nova-1 cDNA. The full length brain cDNA, obtained from analysis of overlapping cDNA clones, consists of 3670 nucleotides and has a long open reading frame extending 510 amino acids ("B" in Figure 3A). We have named the gene Nova-1 to reflect its origin and localized expression as an onconeural ventral nervous system antigen (see below). pRi8, the clone encoding the 40kDa Nova-1 fusion protein, extends from base pair 497 to the 3' terminus of the brain sequence. The predicted protein encoded by this gene is 54kDa, corresponding to the size of the major antigen detected on Western blot with POMA sera (see Figure 2). There are two potential translation initiation codons in the 5' end without an in-frame upstream stop codon, and a near consensus polyadenylation signal 31bp before a stretch of polyadenosine in the 3' end of the cDNA. Strong and weak Kozak consensus sequences (Kozak, 1987; Kozak, 1989), respectively, bound the distal and proximal 5' methionines; either could act as an N-terminal amino acid of the Nova-1 protein. The sequence of 20 cDNA's from human cerebellar, hippocampal and brainstem libraries using the 5' most 800 base pair of the Nova-1 cDNA as probe yielded no new 5' sequences, although several alternatively spliced transcripts were identified (see below); PCR amplification of human cerebellar cDNA suggests that the putative 5' UTR may contain at Computer-assisted sequence analysis of the Nova-1 cDNA and predicted protein sequence revealed an internally repeated sequence of 111 base pair, encoding a 36 amino acid motif (Figure 3B). Searching for homologies to this element revealed significant homology with KH domains of hnRNP-K, FMR-1, the Drosophila alternative splicing factor PSI and Bicaudal-C proteins, the yeast alternative splicing factor MER-1, and others (Figure 3B; Burd and Dreyfuss, 1994a). The KH domains in the Nova-1 gene are approximately 64% homologous to each other, and 55-60% homologous to hnRNP K. We also identified homology with a segment of the pol gene product of multiple members the retrovirus family (data not shown). In each retrovirus, the

Figure 2. The  $\lambda$ Ri8 fusion protein affinity purifies antibody from POMA antisera with binding specificity identical to native POMA antisera. .

Antibody affinity purified from POMA serum with  $\lambda$ Ri8 or mock purified with an unrelated  $\lambda$  clone ( $\lambda$ Yo, encoding the Yo fusion protein; Fathallah-Shaykh et al., 1991) was used to probe a Western blot of purified human Purkinje neurons. Lane 3 shows the reactivity of native POMA antisera (1:500 dilution) with Purkinje neurons; the predominant antigen has an  $M_r$  of 55kDa; there is no reactivity with normal human serum (1:500 dilution, lane 4). Lane 1 shows reactivity of  $\lambda$ Ri8 affinity purified POMA antiserum (1:2 dilution of eluate) recognizes antigens that exactly co-migrate with those recognized by native POMA antiserum (lane 3); there is no reactivity of  $\lambda$ Yo mock purified POMA antiserum (1:2 dilution of eluate) with Purkinje cell antigens (lane 2). In addition, while the mouse and human Nova-1 genes are extremely highly conserved over the coding sequences (99.2% identity over 510 amino acids), the mouse sequence has a single nucleotide deletion in the putative 5' untranslated region (at base pair 40 in Figure 3A). These observations suggest that the brain cDNA sequence in Figure 3A encodes the full-length Nova-1 protein. least an additional 500bp (Darnell, unpublished data).



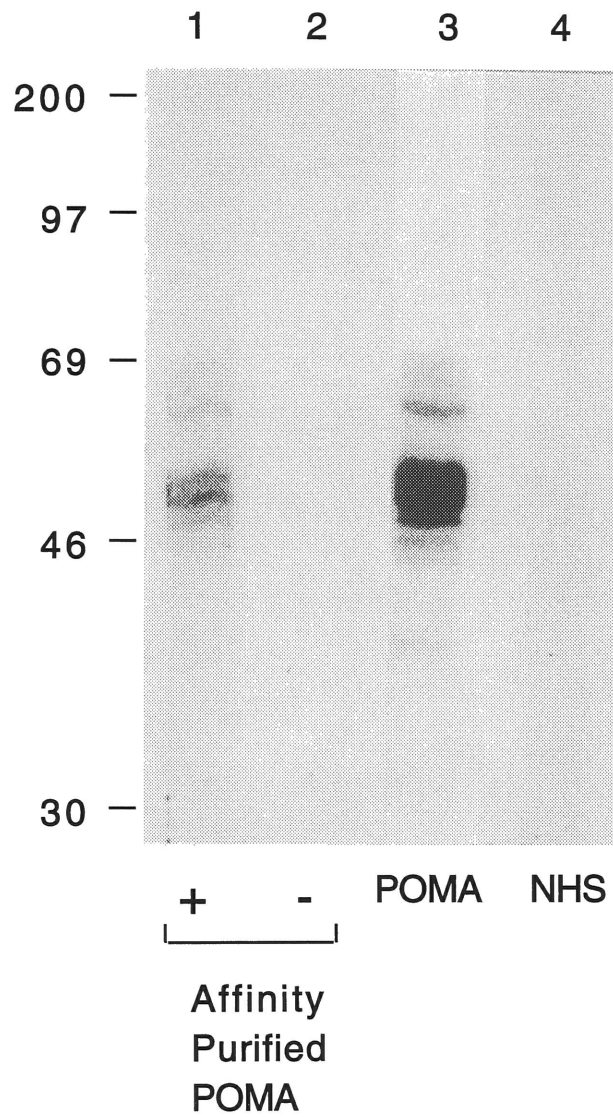






Figure 3. The primary sequence of Nova-1 encodes a 54kd protein with three KH domains.

(A) Sequence of the Nova-1 gene obtained from human brain and tumor cDNA's. The brain (human cerebellum) and tumor (small cell lung cancer cell line) cDNA clones are designated B and T. The deduced translation product is shown for the longest open reading frame in the brain cDNA, and for the corresponding reading frame in the tumor cDNA. The three KH domains within the Nova-1 brain sequence are boxed, and labeled as KH 1-3. The tumor cDNA diverges from brain cDNA exactly upstream of the start of KH 2 (base pair 589), and its open reading frame terminates 8 amino acids downstream. Dashes indicate gaps in the cDNA sequence, introduced to align the sequences; the tumor cDNA is lacking a duplicated 9 base pair motif exactly at the terminus of KH 1. Asterisks indicate translation termination codons, and underlined sequences indicate a series of AUUUA elements in the 3' untranslated region.

3500

[illegible]



homologous sequence encodes part of a viral protease gene; this sequence is conserved among many retroviruses, including mouse mammary tumor virus (Moore et al., 1987), simian immunodeficiency virus (Thayer et al., 1987), feline immunodeficiency virus (Phillips et al., 1990), HTLVIII, Visna and equine infectious anemia virus (Chiu et al., 1985; Stephens et al., 1986), but does not encode a protease consensus sequence (Moore, et al., 1987). The Nova-1 KH domains are 50-54% conserved to the retroviral sequence motif (except equine Infectious anemia virus which is 25% conserved).

The 3' untranslated region of the Nova-1 gene has a large number of AUUUA sequences (Figure 3A, underlined) found in a number of cellular proto-oncogene and cytokine mRNA's that are short lived and tightly regulated in their level of expression (Shaw and Kamen, 1986; Zubiaga et al., 1995a; Zubiaga et al., 1995b). The Nova-1 gene encodes no signal peptide, transmembrane domain, or obvious nuclear localization signal, despite the immunohistochemical localization of the Nova protein to the nucleus (see figure 6). An alanine-rich region present in the C-terminal end of the protein, just proximal to KH3, is weakly homologous (20-30% identity over 20-25 amino acids in this region) to similar alanine-rich regions present in a number of transcription factors (see discussion), identified using the Blast algorithm to search the NCBI transcription factor data base. Genomic Southern blot analysis using a probe from pRi8 yielded single bands or predicted doublets for 5/5 enzymes, indicating that Nova-1 is likely to be a single copy gene (data not shown).

#### Alternatively Processed Nova-1 Gene Transcripts In Brain and Tumor

POMA has primarily been reported to be associated with breast and small cell lung cancers (Digre, 1986; Budde-Steffen, et al., 1988), and antisera from POMA patients with either of these two cancers recognize the Nova-1 fusion protein (Figure 1B). To compare tumor and brain Nova-1 cDNA's, we screened a human small cell lung cancer cDNA library with a radiolabeled probe from

Figure 3 cont.

(B) Alignment of the 36 amino acid Nova-1 KH domains with Related Proteins.

A statistically significant consensus sequence is shown on top that encompasses the central 28 amino acids of the KH domain; within individual KH domains, amino acids identical to the consensus are indicated as dots, and amino acid homologies are shaded. A single amino acid (amino acid 21, \*) is a highly conserved hydrophobic amino acid in which an *in vivo* mutation (I367N) in the second FMR-1 KH domain leads to severe mental retardation. In addition, *in vitro* mutation of this amino acid leads to diminished RNA binding of FMR-1, hnRNPK (Siomi et al., 1994), and Nova-1 (see below)). Next to each gene the number of the KH domain shown is indicated;  $\Phi$  indicates a hydrophobic amino acid. Protein sequences were obtained from the following references: FMR KH domains 1-2, (Siomi et al., 1993); PSI KH domains 1-3, (Siebel et al., 1995); MER1, (Engebrecht and Roeder, 1990); hnRNP K KH domains 1-3, (Matunis et al., 1992); GAPp62, (Wong et al., 1992); Bicaudal-C KH domain 2 (Bic-C), (Mahone et al., 1995). Additional KH homologies are reviewed elsewhere (Burd and Dreyfuss, 1994).

C. The structure of the Nova-1 cDNA is similar to that of hnRNP K. The spacing of the three KH domains (Black boxes) of Nova-1 and hnRNP K. Amino acid positions are indicated by the numbers. Exon H of Nova-1 is indicated by the large hatched box. The second and third KH domains of Nova-1 are separated by a potential polyalanine hinge while the second and third KH domains of hnRNP K are separated by a potential poly (proline/glycine/arginine hinge) (thin hatched box).

D. Diagrammatic representation of the Exon H associated alternative splices identified in Nova-1. Thin line connects all potential splice donors and acceptors. Asterisks indicate stop codons. Exons G, H, and I have all been identified by genomic DNA analysis. D3 sequences lie directly adjacent to the splice donor of exon H. Identified splices include; Exon G-Exon I, Exon G-Exon H-Exon I, Exon G-Exon H-D1, Exon G-Exon H-D2, and Exon G-Exon H-D3.

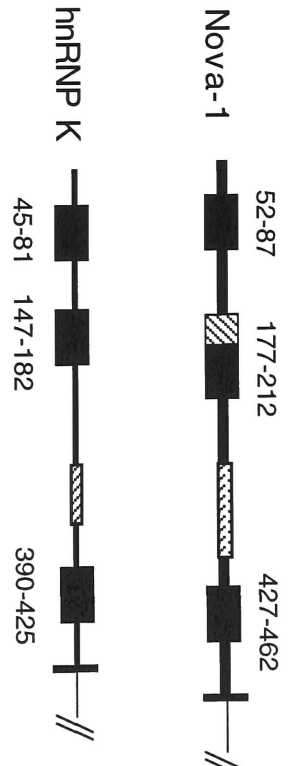
E. Mapping the epitope of Nova-1. POMA reactivity on western blot analysis (1:500 dilution) is indicated by a +. Block Boxes represent KH domains, hatched box represents exon H, amino acid positions relative to the full length Nova-1 without exon H are indicated. The clone containing amino acids 147-486 represents pRi8.



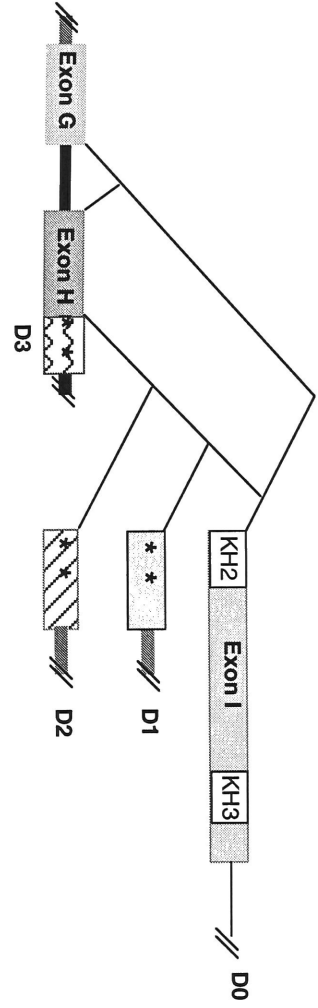
B

Consensus	Φ	VP	Φ	G	Φ	IGKG	* IKΦ	S rGA	-IΦ
Nova-1 KH1	LKVL	I	SYAA	SI	...	QT	VOLOKET	..	-T.KLSK
Nova-1 KH2	VKIL	..	NSTA	LI	...	AT	V.AVMEQS	..	-WVQLSQ
Nova-1 KH3	VEIA	..	ENLV	AIL	...	KT	LVEYQELT	..	-R.QISK
FMR 1	EQFI	..	REDLM	LA	..	AN	QQARKVP	V	TA.DLDE
FMR 2	DVIQ	..	RNLV	KV	..	N.KL	QEIVDKS	V	VRVRIEA
PSI 1	QEIMI	..	GAKV	LV	...	DT	QLOEKT	..	-KMTIIQ
PSI 2	TEVF	..	KIAY	VV	...	DM	RKIQTTC	C	-KLQFIQ
PSI 3	ITFL	..	ASKC	IV	..	ET	LINOQS	..	-HTEMDR
MER1	LEIKL	NKTQI		TFL	..	AK	ESLREKS	..	-S.KIIP
hnmpk 1	LRLI	QSKNA		AV	...	KN	ALRTDYN	..	-SVSVPD
hnmpk 2	LRLI	IHOSLA		GI	..	VK	ELRENTQT		-T.KLFO
hnmpk 3	TQVTI	..	KDLA	SI	...	QR	QIRHES	..	-S.KIDE
GAPp62	VKQY	PKFNFV		KIL	..	PO	RLQEET	..	-K.SVLG
Bic-C 2	MKMD	..	SYTDH	SYI	..	R	..	NN	-H.HFSN
	1			11			21		-31

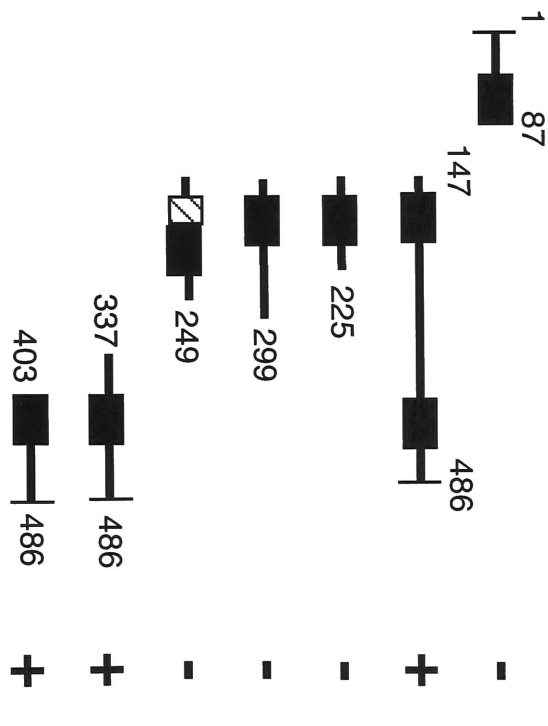
C



D



E



Construct      Antigenicity



the 5' end of the full length brain Nova-1 cDNA, and isolated a truncated form of the Nova-1 transcript ("T" in Figure 3A). Three features are of interest in the tumor Nova-1 cDNA. First, there is an in frame deletion of 9bp (nucleotide 321 in Figure 3A), precisely at the end of KH1. A similar 9bp alternatively spliced sequence has been reported in the neuronal synaptic protein neurexin and the *C. elegans* tumor suppressor KH domain protein *gld-1* (Ushkaryov et al., 1992; Jones and Schedl, 1995). Second, there is an in frame insertion of 72bp, precisely at the 5' boundary of KH2. This exon (see below), termed exon-H, encodes 24 amino acids and is rich in serine and proline residues; of the 6 serines in this sequence, 4 are next to prolines, suggesting that they may be phosphorylation sites of serine/threonine protein kinases (Kemp and Pearson, 1990). Finally, the tumor sequence diverges from the brain cDNA precisely after exon-H into a novel sequence encoding a termination codon 8 amino acids downstream. This suggests that a truncated form of the Nova-1 mRNA, derived from alternative splicing, is expressed in this small cell lung cancer cell line. Subsequently, exhaustive screening of cDNA libraries has identified transcripts including exon-H in human and mouse brain cDNA's (cerebellum, brainstem, hippocampus and frontal cortex), and truncated transcripts identical to the small cell lung cancer cDNA in human frontal cortex (termed D3 for deleted transcript#3), as well as a unique transcripts, also truncated after exon-H, in brainstem and cerebellum (termed D1 and D2, Figures 3D). RT-PCR analysis was performed to confirm the expression of these truncated transcripts in both brain and SCLC (data not shown).

Characterization of human and mouse genomic DNA revealed that sequences directly 3' of exon H are identical to the sequences in the SCLC cDNA isolate, suggesting that the D3 cDNA isolates are a result alternative 3' splice donors or a result of intronic retention. The in vivo relevance of this sequence remains unclear, however the fact that this sequence has been



isolated from several independent cDNA libraries suggests that it is less likely to be an artifact. Also, 33/35 of the initial nucleotides of the D3 sequence (adjacent to exon H) are identical between man and mouse suggesting that this sequence may have important functional or regulatory capacities (see chapter V). Characterization of a mouse genomic clone from the 3' terminus of Nova-1, revealed that the entire carboxy terminal encoding DNA sequence from KH2 through the 3'UTR, exist as one giant exon (Exon I). Genomic sequences encoding D1 and D2 have yet to be identified. These data indicate that Nova-1 has numerous splice forms. Differential splicing is likely to have a significant effect on Nova-1 function.

To determine whether POMA antisera recognize a specific sequence within Nova-1, we constructed a number of fusion proteins and tested them for reactivity with POMA antisera by western blot. The original fusion protein producing clone pRi8 recognized by POMA antibodies in expression cloning, encodes the second and third KH domains of Nova-1. Constructs including the N terminus and first KH domain, or only the second KH domain with or without the adjacent exon H were not recognized by POMA antisera (Fig. 3E). However a fusion protein consisting of the last 84 amino acids of Nova-1, including the third KH domain and carboxy terminus, was reactive with POMA antisera (Fig. 3E). Moreover, mutation of a single amino acid in the third KH domain of Nova-1 eliminates POMA reactivity (see below). Thus we conclude that the disease-related epitope of Nova-1 is encoded by the C-terminus of Nova-1 and is likely to map specifically to the third KH domain.

## **Expression of Nova-1**

Timing of Expression. Western blot analysis of Nova antigens using affinity purified POMA antibody and mouse embryonic extracts from various time points revealed a single antigen of ~55Kd detectable at E.10, E.14, E18 and P.2



there are two distinct groups of POMA reactive antigens, one group of approximately three ~53-58 Kd proteins, and a second more abundant group of two ~74-80 Kda reactive antigens (Fig. 4). In the adult, there are at least seven distinct reactive protein species, with the 53-58 Kd species being the most abundant. In order to look specifically at the timing of Nova-1 expression, we utilized Nova-1 specific primers for RT-PCR analysis. Primers were chosen to bound the third KH motif of Nova-1, the region determined to be the antigenic epitope of Nova-1 protein. Total embryo RNA from various developmental stages was used as a template for RT-PCR amplification of Nova-1 RNA. An RT dependent Nova-1 specific product was detected at every time point tested after E.8 (Fig. 5). An unexpected product of approximately 900 bp, which appears to be upregulated at E.17.5, was also detected. However we have been unable to clone and characterize this product. The integrity of RNA from each time point was insured by performing RT-PCR analysis with actin primers (data not shown). Taken together these data demonstrate that Nova-1 expression begins at approximately E10 in the mouse, a time just after the neural tube has started to close and just prior to the beginning of synaptogenesis.

#### Protein Expression is Restricted to CNS Neurons.

Immunohistochemical analysis using POMA antisera revealed specific reactivity throughout the CNS (see below). Reactivity was present in the nucleus and cytoplasm (including dendritic processes) of cells with neuronal morphology (Figure 6A). To determine if Nova protein is restricted to neurons we labeled adult mouse brain sections with anti-glial fibrillary acidic protein (GFAP) and affinity purified POMA antibodies. Double-label immunofluorescence analysis revealed that GFAP and Nova antigen expression are mutually exclusive throughout the CNS (Figure 6C, and data not shown). To confirm that the cells recognized by POMA antibodies were neurons, we performed double labeling experiments with affinity purified

#### Figure 4. Timing of Nova Antigen Expression.

Western blot analysis of mouse extracts from various developmental timepoints using affinity purified rabbit anti-Nova-1 antibodies. Whole E.10 embryos, whole heads from E.14, E.18 and P.0, and isolated brain were used to make the extracts for the indicated developmental stages. Equal amounts of protein from each extract were analyzed by SDS-PAGE and western blotting with affinity purified rabbit anti-Nova-1 sera (1:100).



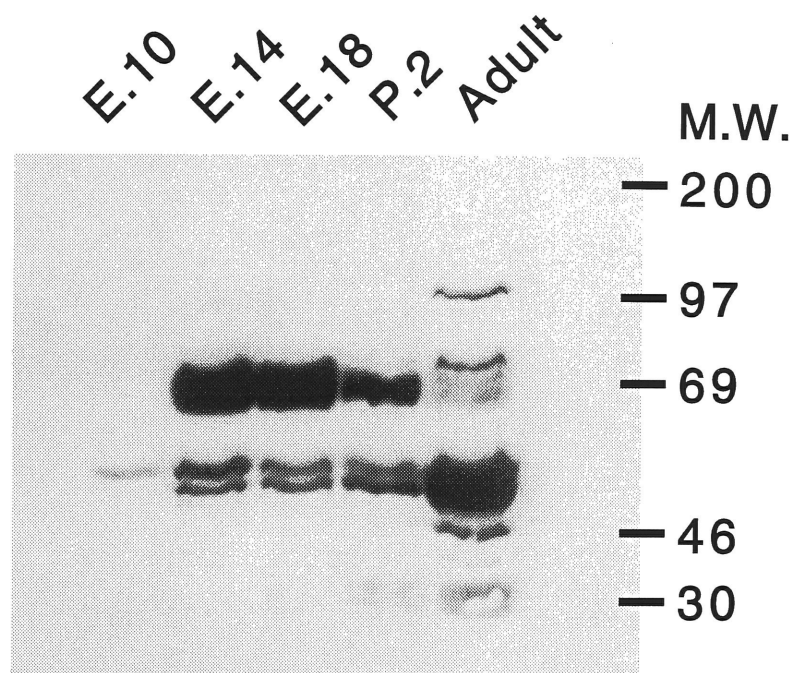






Figure 5. Timing of Nova-1 RNA Expression.

Reverse Transcription-Polymerase chain reaction using Nova-1 specific primers and total brain RNA from the indicated developmental stages. The arrow indicates the expected Nova-1 product which was cloned and sequenced. 100bp size markers are shown on the left. RT-PCR analysis with actin primers was performed to insure the integrity of the RNA (data not shown).

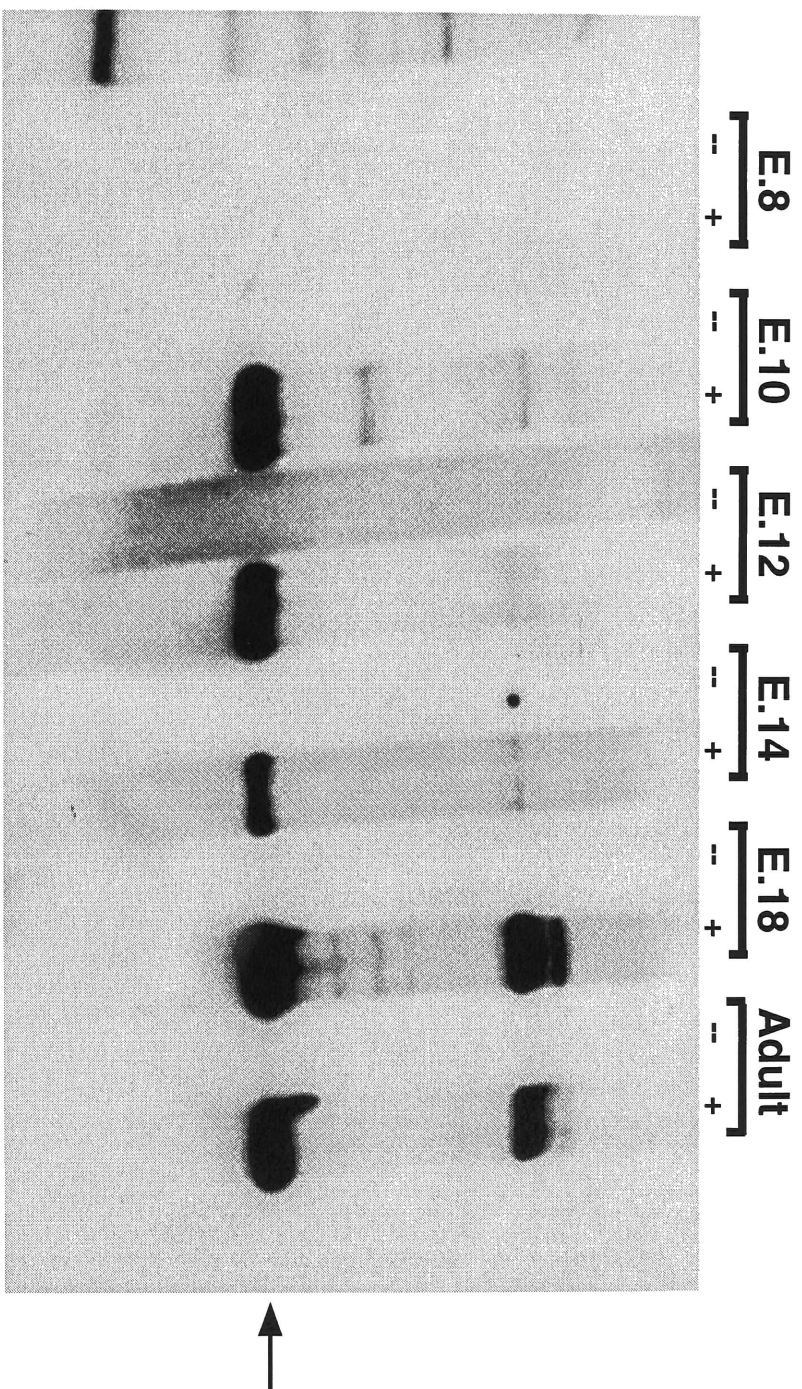






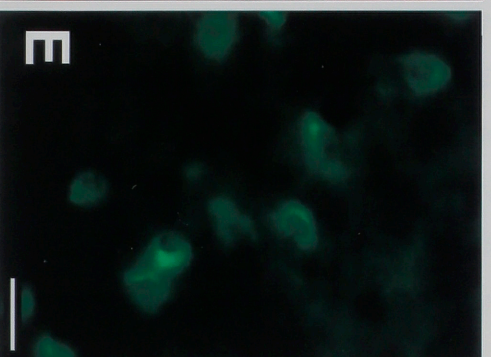
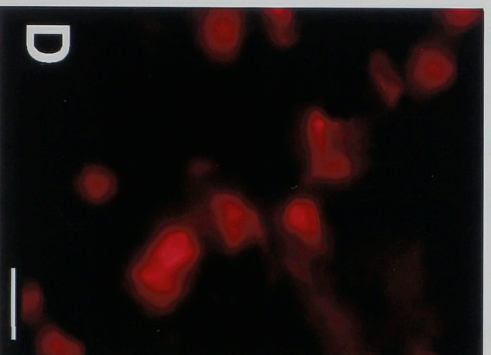
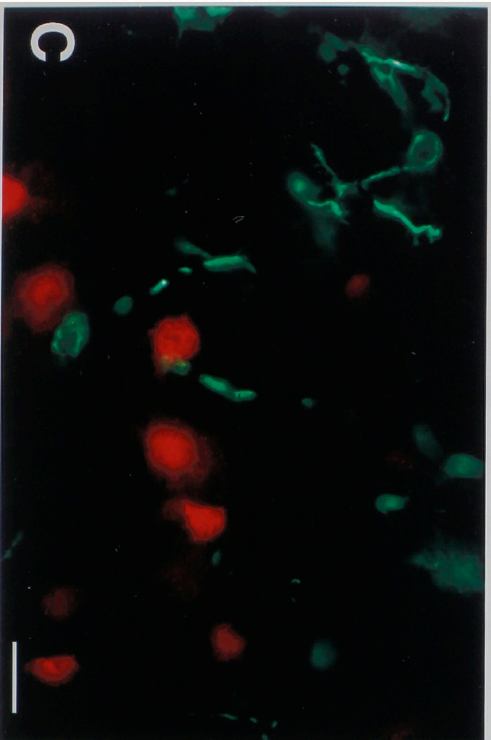
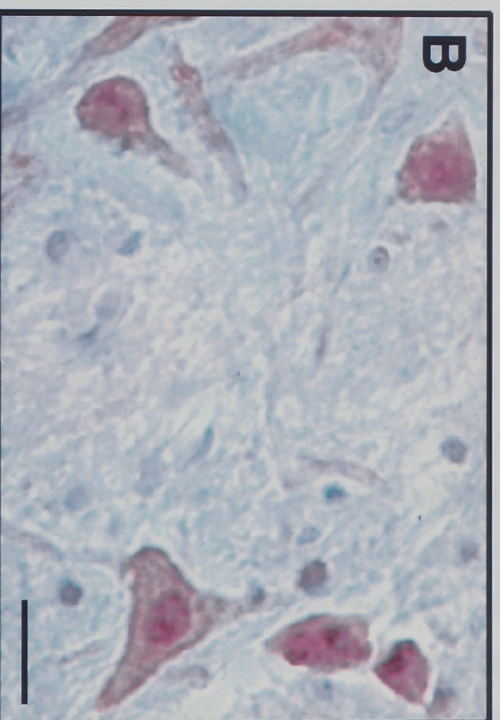
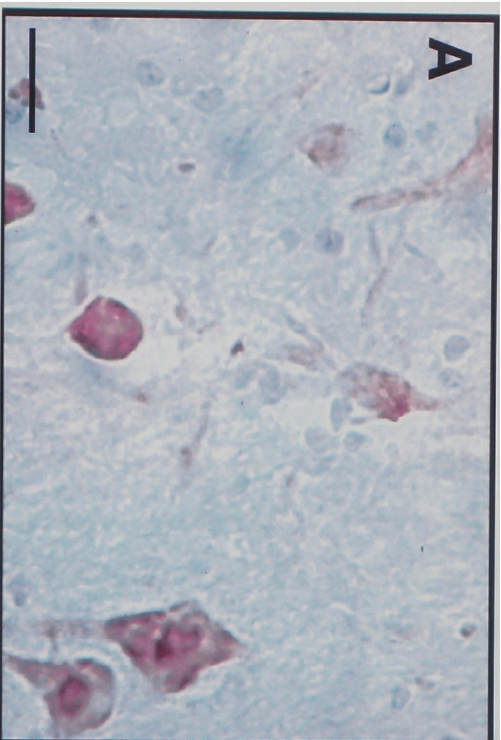
Figure 6. Nova-1 is Neuron Specific.

(A)&(B). Immunohistochemistry of adult brainstem neurons reacted with POMA antiserum (A) and the pan-neuronal reactive Hu antiserum (B). Strong reactivity is detected in the cytoplasm, nucleus, and processes of cells with neuronal morphology. Bar = 10  $\mu$ m.

(C) Immunofluorescence double exposure of GFAP (green) and Nova (red) in a section of adult mouse hindbrain demonstrating that GFAP and Nova reactivity are non-overlapping. GFAP reactivity is detected in the cytoplasm and processes of glial cells, while Nova reactivity is detected strongly in neuronal nuclei and more weakly in the neuronal cytoplasm. Bar = 10  $\mu$ m.

(D) and (E) Immunofluorescence exposure of NCAM (green) and Nova (red) in a section of adult mouse neocortex demonstrating that NCAM and Nova reactivity are overlapping. NCAM reactivity is detected in the neuronal cytoplasm while Nova reactivity is detected primarily in neuronal nuclei and more weakly in the neuronal cytoplasm. Bar = 10  $\mu$ m.







POMA antibodies and either anti-neural cell adhesion molecule (NCAM) or anti-neuron specific enolase (NSE) antibodies. Nova antigen is expressed in all cells recognized by NCAM (Figure 6D and 6E) and NSE (data not shown). These results, together with the demonstration that Nova protein expression is absent from white matter tracts, demonstrate that expression of the Nova antigen is neuron specific.

#### Restriction of a Nova antigen to a subset of Neurons

The focal oculomotor neurologic dysfunction seen in POMA patients is not explained by the pan-neuronal expression pattern detected with POMA antisera (Fig. 7B). Use of affinity purified antibody (POMA or rabbit anti-Nova-1) and varied fixation conditions uncovered a pattern of Nova antigenicity restricted to a subset of neurons within the CNS of adult mouse (Fig 7A). There was strong expression throughout the midbrain particularly in the regions of the tegmentum and superior colliculus. There was very strong expression in the brainstem, and pons (data not shown) as well as in cerebellar granular cells and deep cerebellar nuclei (Fig 7D). Expression was absent in the neocortex, medial thalamus and inferior colliculus (Fig. 7A and C).

Analysis of developmental expression first detected reactivity at E.10 where there was reactivity in the ventral most neurons of the spinal cord and a few isolated ventral brainstem neurons (Fig. 8 Ai, see also Fig 14). At E12 and E14 antigenicity was strong in the regions of the brainstem, mesencephalon, and ventral spinal cord. Expression was absent from dorsal midbrain and the neocortex. Expression was also absent from cells of the ventricular zone, suggesting that Nova-1 is only expressed in post-mitotic neurons (Fig. 8 B,C and data not shown). Analysis of E.18 embryos using these conditions demonstrated that Nova antigen is restricted to neural tissues (Fig. 9A).

Figure 7. Immunohistochemical Detection of Nova Antigen with a limited pattern of Expression.

(A) Immunohistochemistry using affinity purified anti-Nova-1 antibodies, acetic acid-ethanol fixation and adult mouse brain axial section. Anti-Nova reactivity (reddish-brown) is restricted to regions of the mesencephalon and cerebellum. No reactivity is detected in the neocortex (N).

(i) Higher magnification of the midbrain region boxed in (A). Anti-Nova reactivity is apparent in the superior colliculus (SC), presubiculum (PS) and cerebellar granule cells (CbGC), but not in the inferior colliculus (IC) or hippocampus (H).

(ii) Higher magnification of the cerebellar region boxed in (A) demonstrating Anti-Nova reactivity in the deep cerebellar nucleus (DCbN) and cerebellar granule cells (CbGC).

(B) Immunohistochemistry using anti-Nova-1 antibodies, paraformaldehyde fixation, in an adult brain axial section (serial section ~100  $\mu$ M ventral of the section in (A)). Anti-Nova reactivity is detected in all neurons.



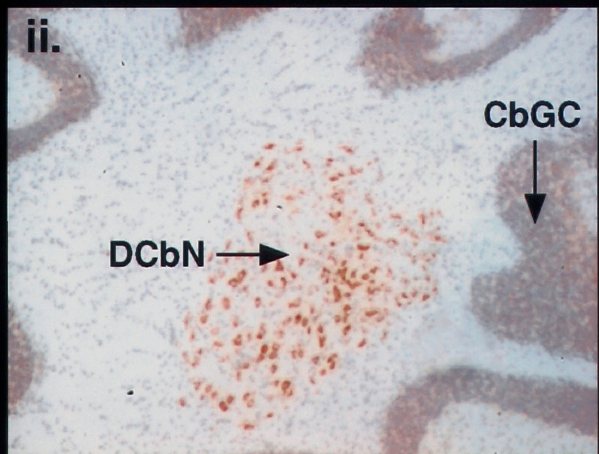
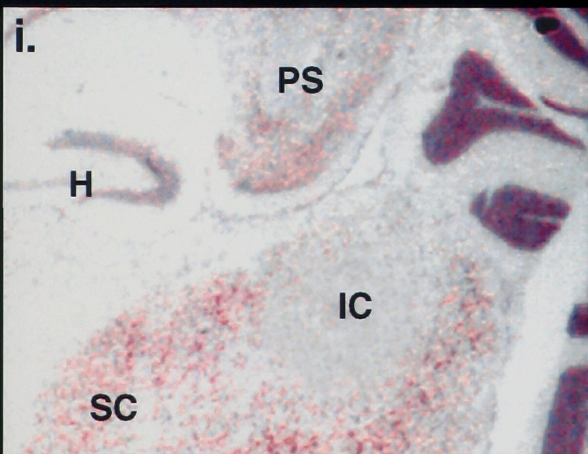
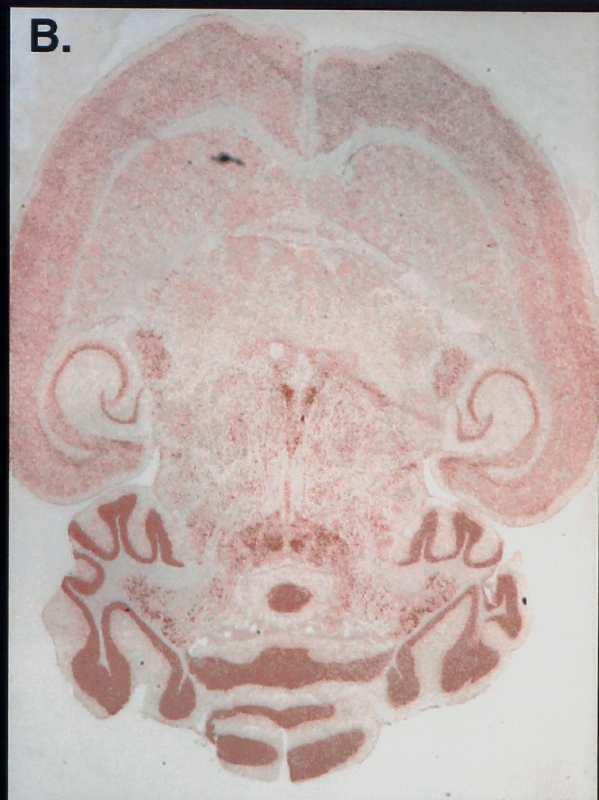
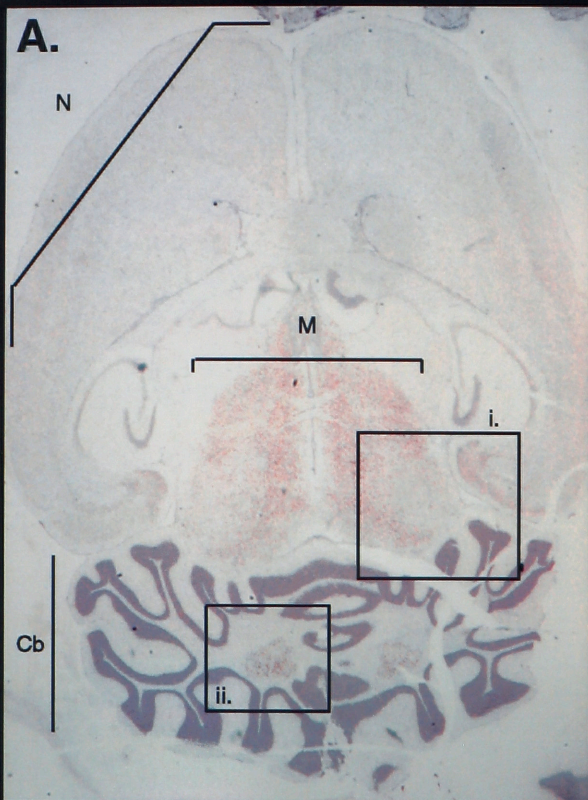






Figure 8. Restricted expression pattern of a Nova antigen at E.10 and E.12.

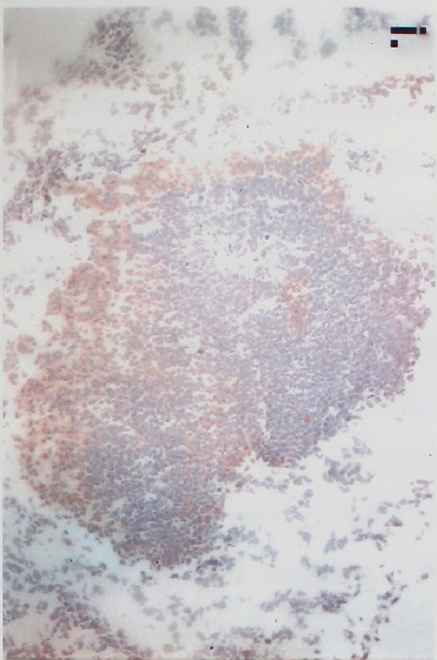
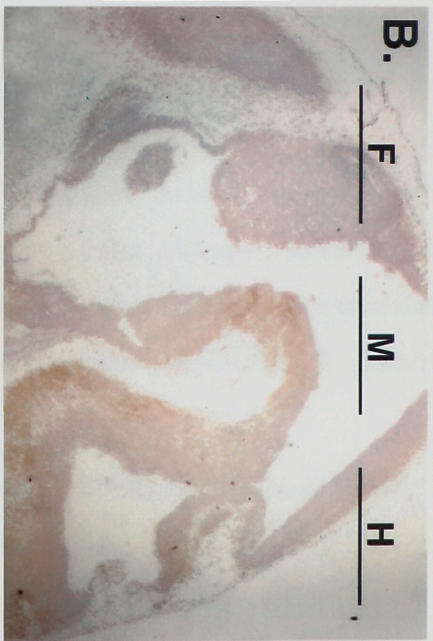
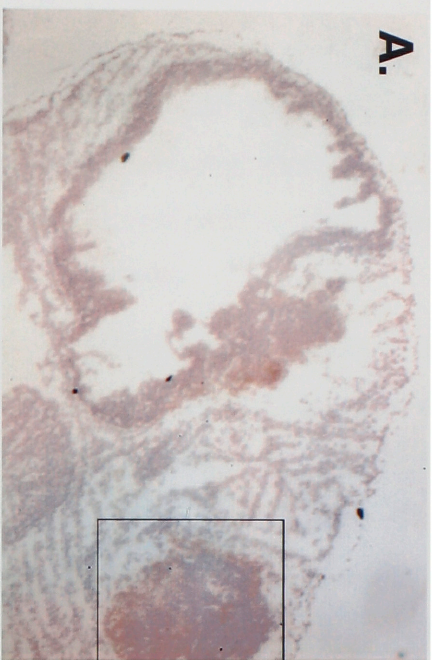
(A) Affinity anti-Nova antibodies reacted with a sagittal section of an E.10 mouse.

(i) Higher magnification of the region boxed in A. Anti-Nova reactivity can be detected in the ventral most neurons of the hindbrain.

(B) High magnification of affinity anti-Nova antibodies reacted with a sagittal section of an E.12 mouse head. Nova reactivity is present in the ventral hindbrain (H) and midbrain (M), but no reactivity is seen in the forebrain (F).

(C). Affinity anti-Nova antibodies reacted with an axial section of an E.12 mouse brain, showing nova reactivity restricted to the developing hindbrain (H) and midbrain (M).







Moreover, expression is restricted to neurons of the CNS; no reactivity is detected in neurons of peripheral nervous system, although under these conditions neurons are readily detected by the pan-neuronal anti-Hu antibody (Fig. 9A and C). Within the CNS, antigenicity is prominent throughout the brainstem, and midbrain, but is absent in the neocortex and thalamus (Fig. 9G). In the spinal column, reactivity appeared to be restricted to the ventral half of the spinal cord (Fig 9E vs F- further detailed below).

Analysis of P0 mice showed a similar pattern of expression, as seen in the adult. Nova antigen was undetectable in neocortex, medial thalamus and habenula, however reactivity was detectable in the lateral thalamus and lateral habenula, midbrain, and cerebellum (Fig. 10A, i, and ii). At P0 cerebellar development is still ongoing. Nova antigen was detected in the post-mitotic inner granule cell layer, but was absent in the pre-mitotic external granule cell layer (Fig. 10 iii).

Expression pattern of Nova-1 RNA Northern blot analysis with polyA+ RNA and a Nova-1 3' coding region probe, revealed a 4.7kb brain-specific transcript (Figure 11). Thus, similar to Nova-1 protein, Nova-1 transcripts are brain-specific. The discrepancy between the size of the cloned Nova-1 cDNA (3.7kb) and the transcript detected (4.7kb) is likely to be due to the presence of additional 5' untranslated sequence in the mature mRNA (R. Darnell, unpublished data).

In order to assess specifically where Nova-1 RNA is expressed in the developing brain we performed in-situ hybridization using several different probes. One probe consisting of ~800 base pairs of the coding region from the carboxy-terminus of Nova-1, and a second encompassing ~1500 base pairs of the 3' UTR. Both probes yielded an identical sub-cortical expression pattern similar to that determined using 'stringent' IHC. ISH of sagittal sections of

Figure 9. Restricted expression of a Nova antigen in the E18.5 mouse. \_

(A) Immunohistochemistry using affinity purified POMA antibodies in E.18 mouse sagittal sections. Reactivity is restricted to the central nervous system, expressed predominantly in the ventral midbrain and spinal cord. (B). Immunohistochemistry using mock affinity purified antibodies in serial section to A. (C). Affinity purified anti-Nova antibodies reacted with E18 cervical spinal cord, showing staining of the ventral cord and an absence of reactivity with peripheral nervous system (see D).(D). Hu antiserum reacted with a section serial to C, staining all central and peripheral neurons. (E). Affinity purified anti-Nova antibodies reacted with a sagittal section of E18 spinal cord; reactivity is primarily in the ventral spinal cord. (F). Pan-neuronal Hu antiserum reacted with a section serial to E, staining all neurons. (G). Affinity anti-Nova antibodies reacted with an axial section of an E.18 mouse brain, showing lack of reactivity in the telencephalon, and ventral but not dorsal reactivity in the midbrain. (H). Hu antiserum reacted with a section serial to G stained all neurons. (vsc) Ventral spinal cord; (dsc) Dorsal spinal cord; (vmb) ventral midbrain; (dmb) Dorsal midbrain; (li) Liver; (si) Small Intestine; (scg) Superior cervical ganglion; (vag) root of the Vagus nerve; (tele) Telencephalon. Size bars: 2mm A and B; 425mm, C and D; 100mm E and F; 1mm G and H.



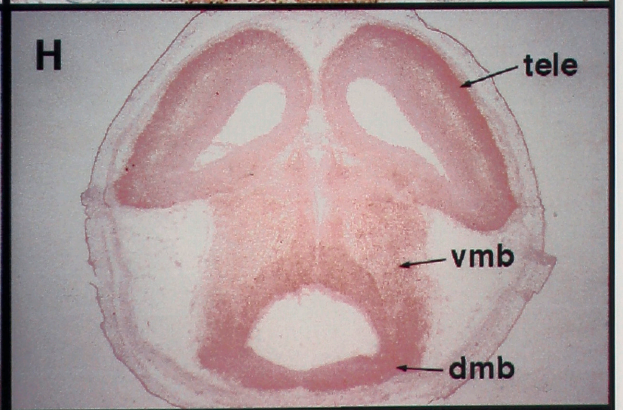
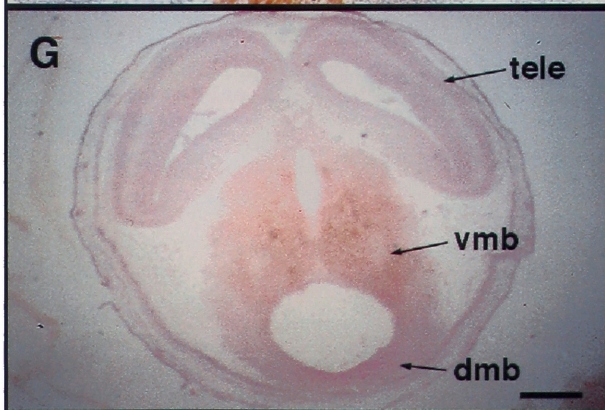
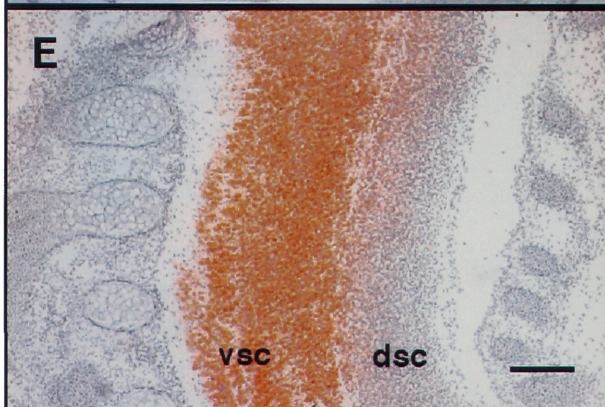
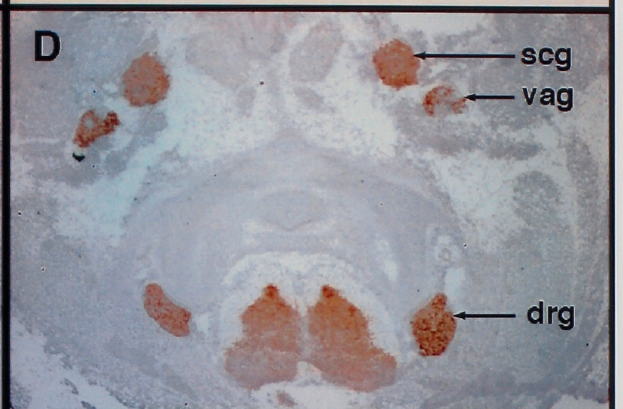
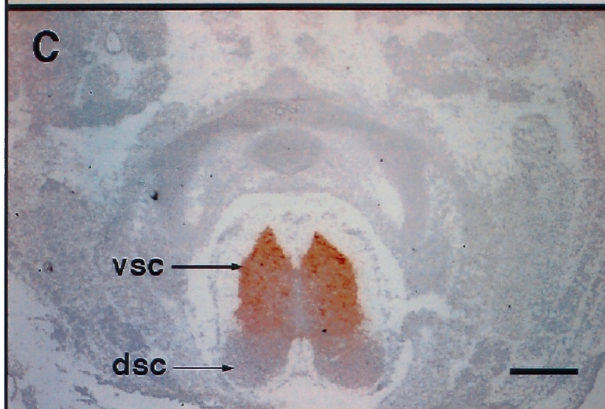
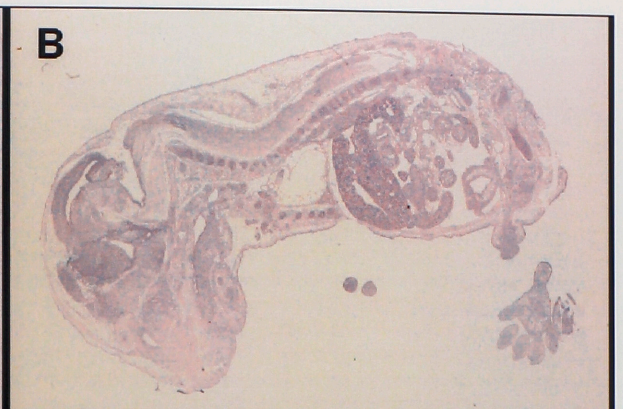
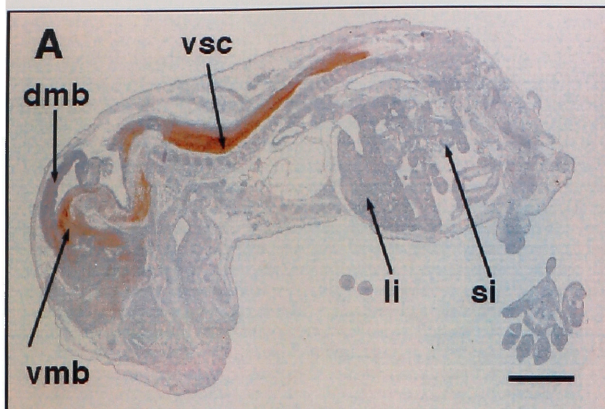






Figure 10. Restricted of Expression Nova antigen in P0 mouse brain.

(A). Anti-Nova immnohistochemisry in an axial section of a P0 mouse brain.

(i) Higher magnification of diencephalic region boxed in (A) demonstrating specific anti-Nova reactivity in the septal nuclei (S) and lateral habenula (H<sub>L</sub>)but not the medial habenula (H<sub>M</sub>).

(ii) Higher magnification of the thalamic region boxed in (A) demonstarng specific anti-Nova reactivity in the lateral thalamus (T<sub>L</sub>) but not the medial thalamus (T<sub>M</sub>).

(iii) Higher magnification of the cerebellar region boxed in (A) demonstrating specific anti-Nova reactivity in the deep cerebellar nuclei (DCbN) and in the post-mitotic internal granule layer (IGL) but not the mitotic external granule layer (EGL).



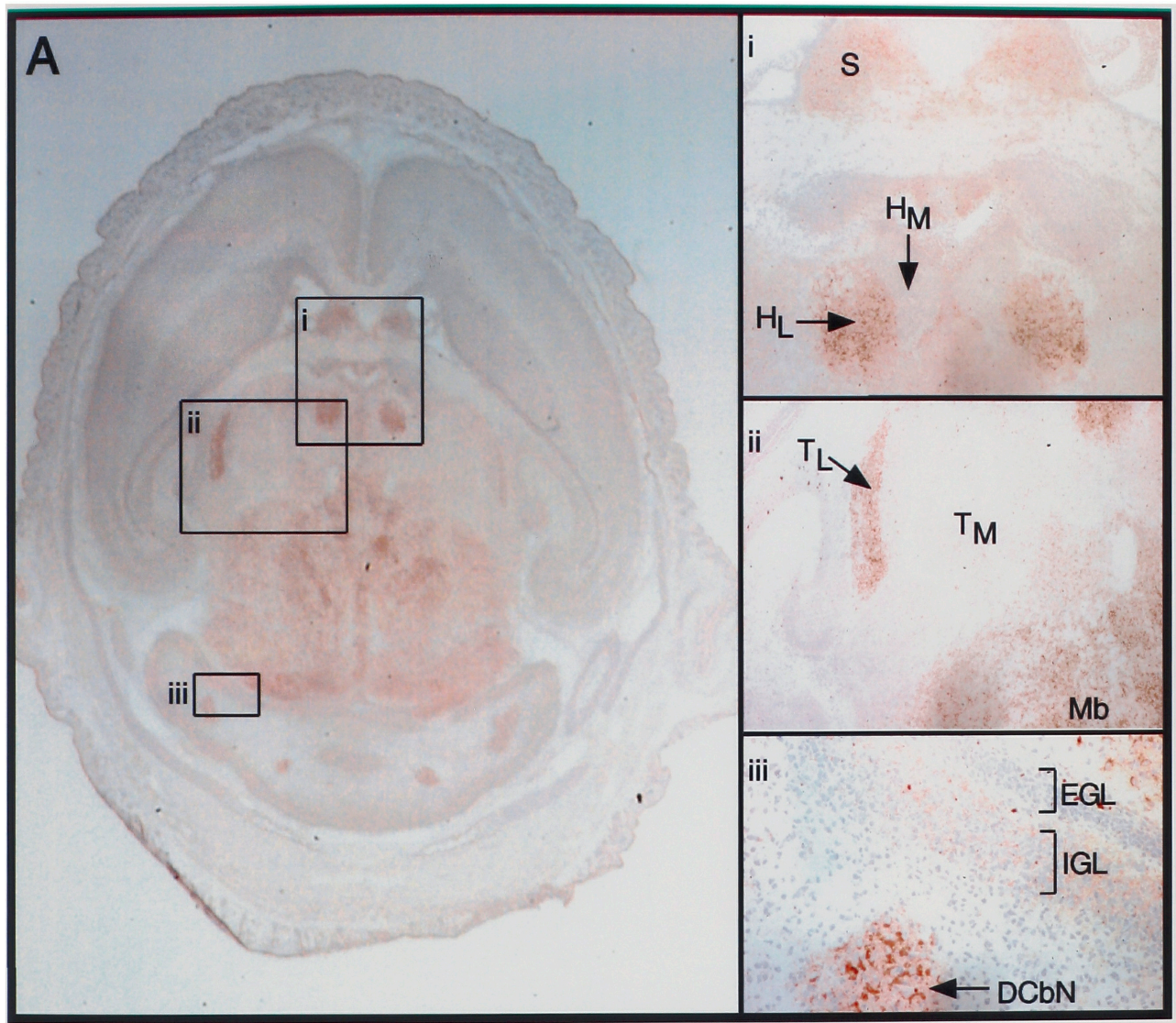
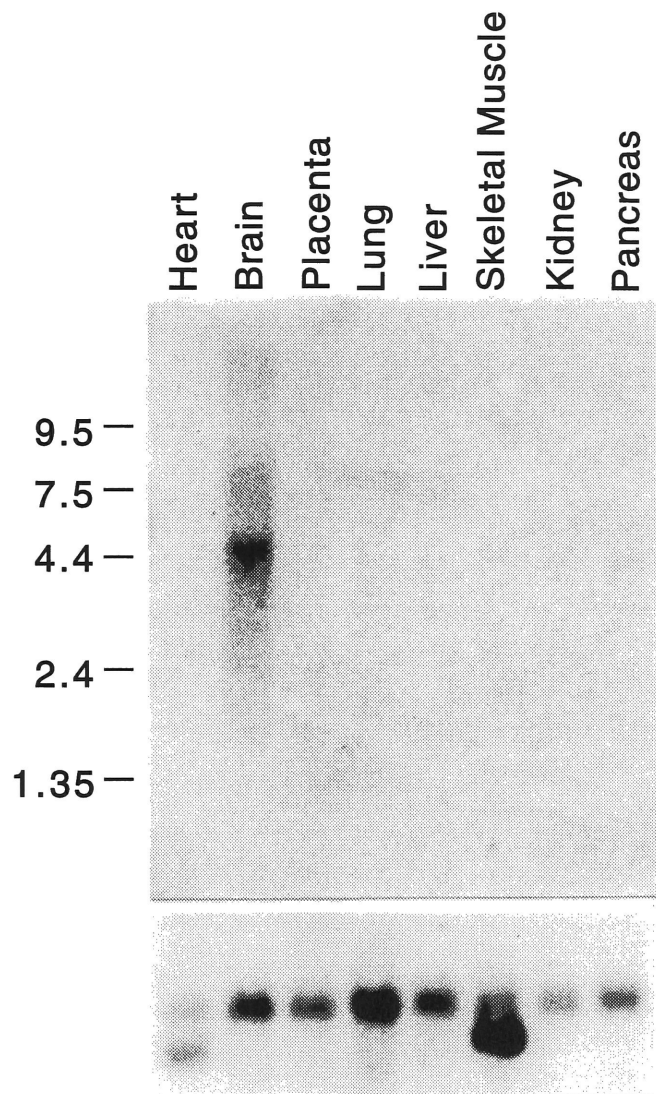






Figure 11. Northern blot analysis of Nova-1 expression. \_

2.0 µg of polyA<sup>+</sup> RNA obtained from the indicated tissues were run on a 1.0% formaldehyde containing agarose gel, transferred to nitrocellulose and probed with an Nova-1 probe derived from a 1380bp 5' fragment of plasmid pRi8. An approximately 4.7 Kb RNA was detected only in brain. The blot was then boiled in ddH<sub>2</sub>O and re-probed with a β-actin cDNA as a positive control.





E.14 mouse revealed Nova-1 RNA expression restricted to the subcortical CNS and ventral spinal cord (Fig 12). Axial sections of E.12 and E.18 heads as well as sagittal sections of P0 and P8 brains reveal the clear absence of Nova-1 RNA from the telencephalon and medial thalamus (Fig 12 B,C,D and E). Strong reactivity exists in the midbrain, pons and deep cerebellar nuclei. Analysis of the P.8 cerebellum, which continues to develop postnatally, shows that Nova-1 is expressed early in the deep cerebellar nuclei and Purkinje neurons (data not shown). Interestingly, while protein was only detectable in the IGL, (see Fig. 10), Nova-1 mRNA was present in both the external and internal granular cell layers at P0 and P8 (data not shown and Fig. 12E). It is unclear if this is due to a lag between transcription and translation of Nova-1, or due to a post-transcriptional regulatory event. Finally, in the adult, the expression pattern of Nova-1 mRNA persisted and continued to be restricted to the subcortical CNS. Axial sections of adult brain revealed expression in the Superior colliculi, septal nuclei, mesencephalon, cerebellum and pons. Expression of Nova-1 RNA is absent from the neocortex, medial thalamus, and inferior colliculi. Coronal sections reveal Nova-1 expression in the amygdala and hypothalamus (Fig. 13). A detailed analysis of Nova-1 expression using both IHC and ISH is summarized in table 1.

#### Expression in the spinal cord.

The domain of expression of Nova antigen and Nova-1 mRNA in the ventral regions of the spinal cord expanded with increasing developmental age. To study this we performed a detailed analysis of spinal cord axial and sagittal sections. Expression of Nova-1 was first detected in the ventral most neurons (~five cell widths) of the caudal spinal cord of E.10 embryos (Fig 14). By E.12 expression expanded to include the ventral 1/3-1/2 of the spinal cord. Comparison of Nova-1 expression with the pattern of islet protein (a marker of motor neurons and the intermediolateral column neurons), indicated that

Figure 12. In-situ hybridization (ISH) reveals that Nova-1 mRNA in developing mouse brain is restricted to the subcortical CNS.

Nova-1 ISH in an E.14 mouse embryo sagittal section (A), E.12 axial section (B), E.18 axial section (C), P0 sagittal section (D), and P8 sagittal section (E). Expression is predominantly in the ventral spinal cord, throughout the cerebellum (Cb), pons (P), midbrain (M), in the hypothalamus (H) and basal ganglia (BG). Expression is absent from the dorsal thalamus (T) and neocortex (N). The insert in (E) shows the expression of Nova-1 mRNA in both the internal and external granule cell layers (IGL, EGL). Expression is absent from the dorsal thalamus and neocortex. Probes from bp 590-1453 Nova-1 coding region and the last 1400 bp of the Nova-1 3' UTR were used and gave similar results.



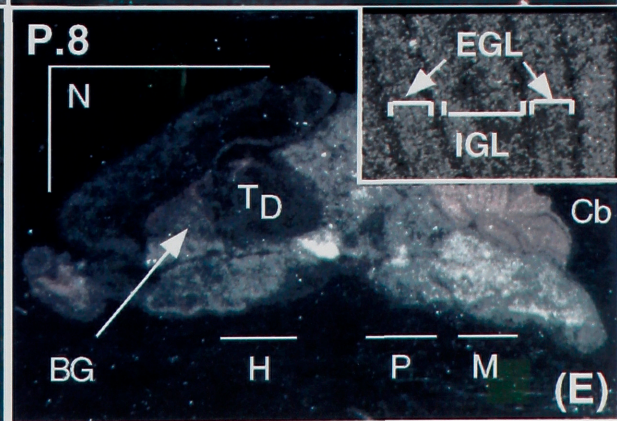
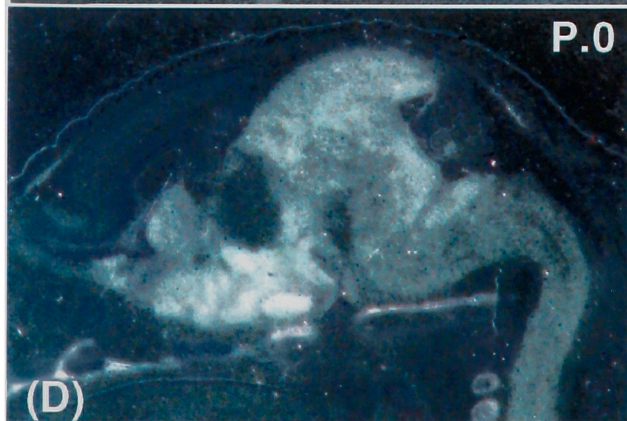
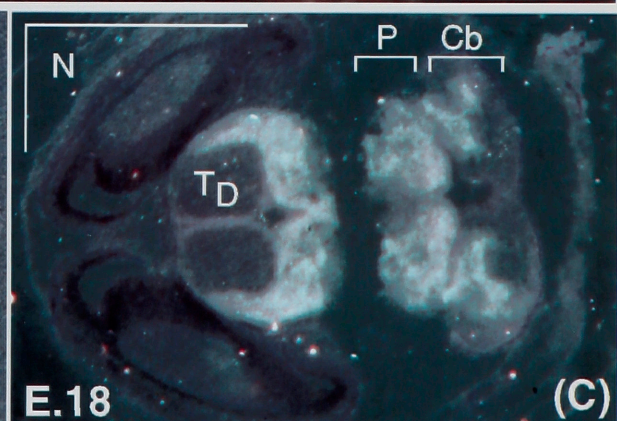
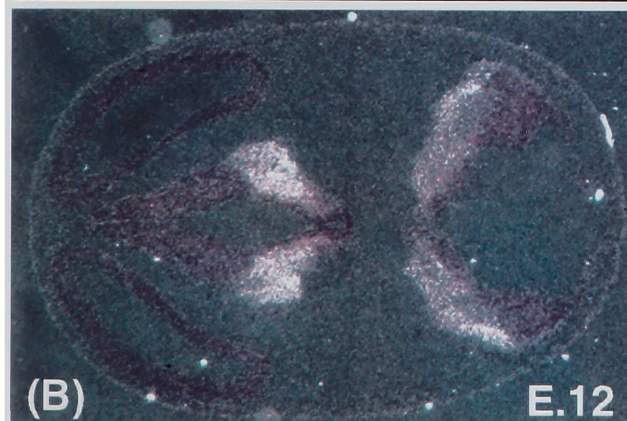
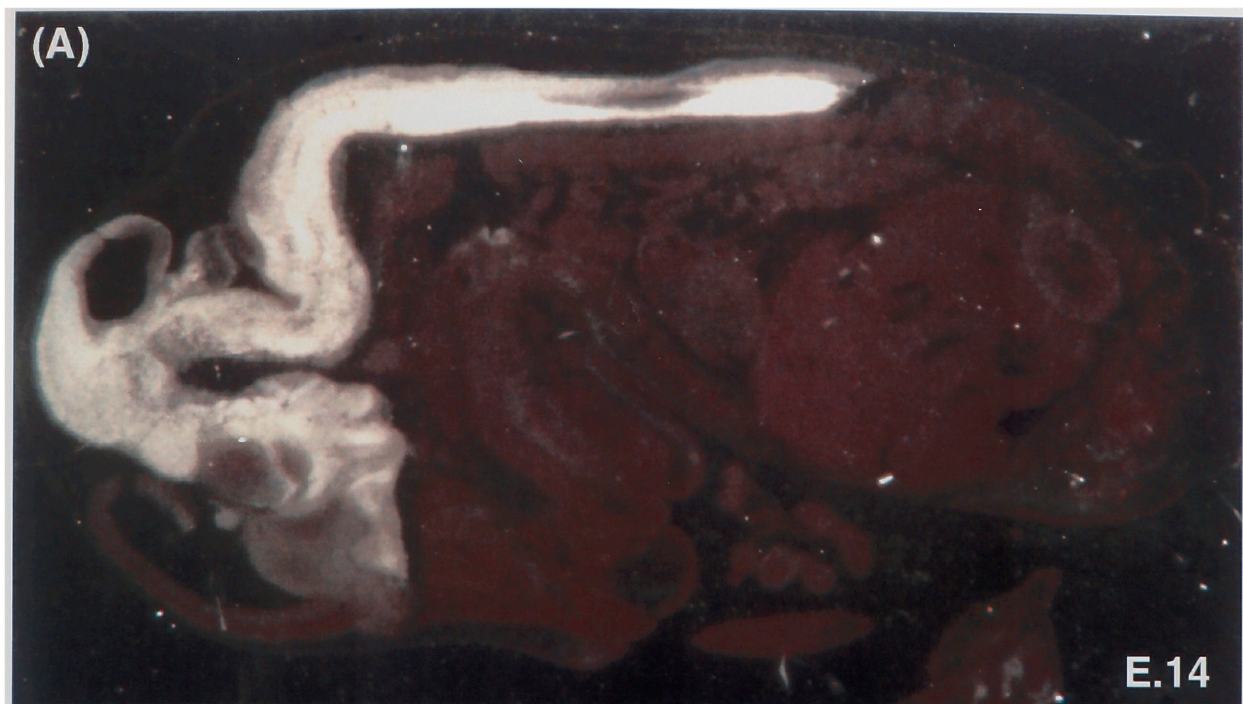






Figure 13. Nova-1 mRNA is expressed in a restricted pattern in the adult.

(A&B) ISH in axial sections of adult mouse brain. Expression is detected in the superior colliculus (S.C.), septal nuclei (Sp.N.), mesencephalon (M), and pontine nuclei (P.N.). Expression is absent from the neocortex (N), dorsal thalamus (T), and inferior colliculus (I.C.).

(C). ISH in an adult brain frontal section revealing expression in the amygdala (A) and hypothalamus (H).

(D). Higher magnification Nova-1 ISH revealing expression in the cerebellar granule cells (CbGC) and deep cerebellar nuclues (DCbN).

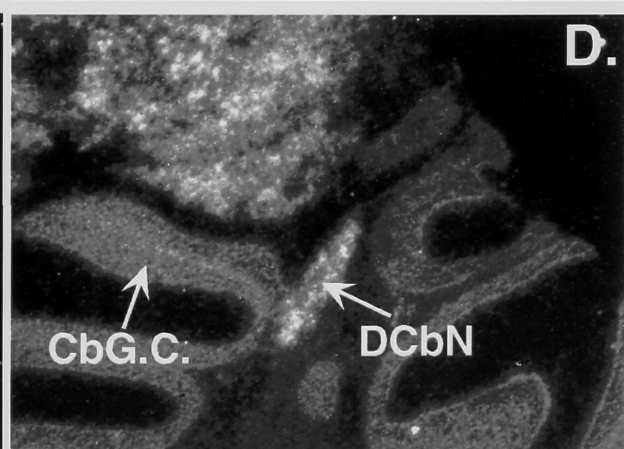
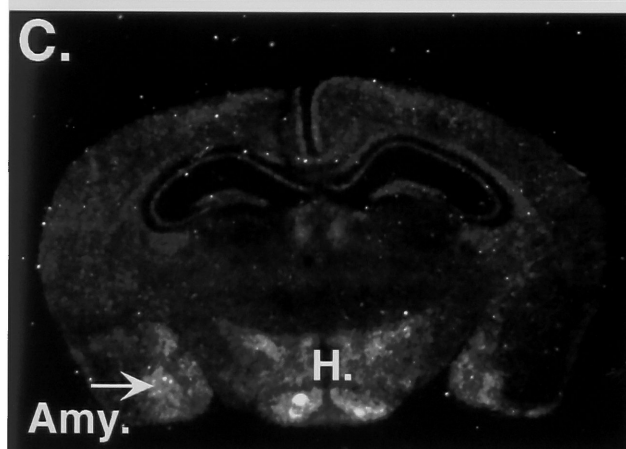






Table 1. A summary of Postnatal Nova-1 Expression.

Expression of Nova-1 as determined by *in situ* hybridization and immunohistochemistry in P0 and adult mouse brain axial sections. Symbols indicate, +++ Strong signal; ++ Average signal; + weak signal; +/- very weak; - undetectable signal.



Telencephalon	Neocortex	-
	Olfactory Bulb	-
	Olfactory epithelium	-
	Septal Nuclei	++
	Caudate/Putamen	+
	Amygdala	++
Diencephalon	Medial habenula	-
	Lateral habenula	+++
	Thalamus:	
	Dorsal medial	-
	VPL/VPM	-
	Reticular nuclei	+++
	LGN	+++
Mesencephalon	Hypothalamus	++
	Superior Colliculi	+++
	Inferior Colliculi	+ / -
	Tegmentum	+++
Metencephalon	Pons	
	Medial	+++
	Lateral	+
	Tegmentum	+++
	Cerebellum	
	Purkinje Cells	+
	Granule Cells	+++
Rhombenceph.	Deep Nuclei	+++
	Medulla	+++
	Spinal cord	
	Dorsal	+
	Ventral	+++





Figure 14. Nova-1 expression in the ventral spinal cord expands with development.

(A). High magnification of anti-Nova IHC in sagittal section of an E.10 mouse spinal cord revealing expression in ventral most cells. The ventral side of the spinal cord is oriented toward the left side of the page (V).

(B-D). Nova-1 IHC in mouse axial spinal cord sections at the indicated developmental stages. Dorsal spinal cord (D) is oriented toward the top of the page and ventral (V) is oriented toward the bottom of the page.

(E). Hu IHC in a P.1 mouse axial spinal cord section labeling all neurons.

(F-I). Nova-1 ISH in an axial mouse spinal cord sections at the indicated developmental stages.

(J). HuC ISH labelling all neurons in an E.12 axial mouse spinal cord serial section to F.

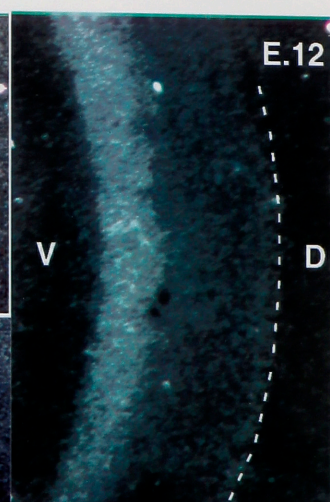
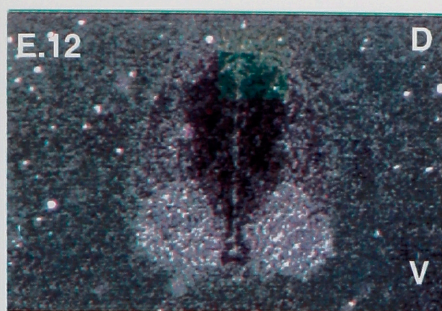
(K&L). Nova-1 ISH in mouse sagittal spinal cord sections at the indicated developmental stages. Arrows indicate the spinal cord borders. Dorsal and ventral are as indicated.

(M). HuC ISH in a mouse E.12 sagittal section serial to K.

E.10

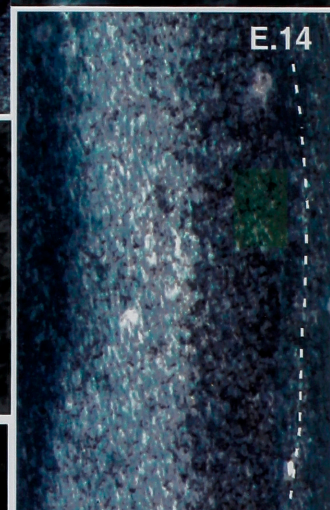
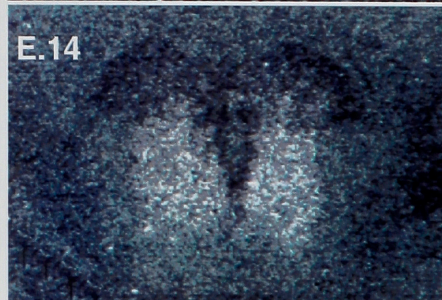
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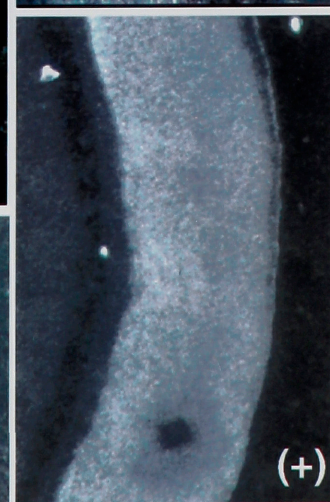
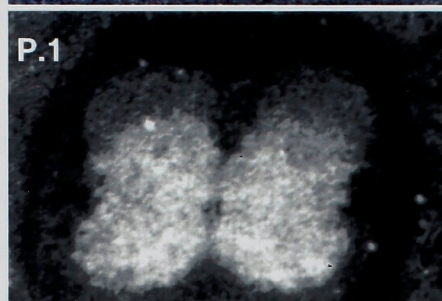


E.14

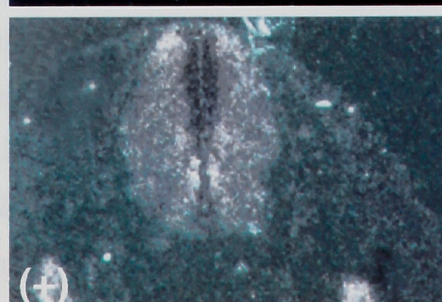
D



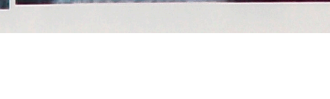
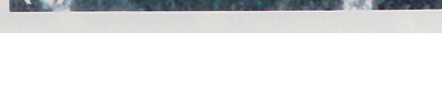
P.1



Adult



(+)





the Nova-1 domain of expression included islet positive cells and the islet negative interneurons (data not shown). At E.14, E.18, and P.0 Nova-1 encompassed the ventral  $\sim 1/2$ ,  $2/3$ , and  $4/5$  of the spinal cord, respectively (Fig. 14). In the adult Nova-1 protein and mRNA was expressed in all but the most dorsal neurons of the spinal cord; in the dorsal most neurons, lamina 1 and 2, Nova-1 is either absent or reduced. This expression pattern in the spinal cord is similar to the pattern of synaptogenesis observed of spinal cord neurons; the largest and ventral most neurons undergo synaptogenesis first starting as early as E.10 at the junction of the ventral horn and the white matter, while the smallest, dorsal most neurons undergo synaptogenesis last. The onset of Nova expression just prior to the onset of synaptogenesis suggest that Nova may play some role in this process.

#### Expression of truncated Nova-1 transcripts.

As previously mentioned, several truncated Nova-1 transcripts were identified. The Nova-1 transcripts share similar sequences proximal of KH2 but differ in their 3' sequences. The D1, D2 and D3 transcripts would not be identified by either of the previously utilized probes (3' coding region or 3' UTR probes). To assess the expression of the variant transcripts, we utilized a probe from the 5' UTR of Nova-1. This probe should detect Exon I, D1, D2 and D3 splice variants. The expression detected with the 5' UTR probe was more wide-spread within the CNS than was expression observed with the 3' coding region or 3' UTR probes. Expression of the 5' UTR included not only regions previously seen to express the Nova-1 3' UTR, but also the thalamus, the neocortex, and also ventricular zone cells in the neocortex (Fig 15). Within the spinal cord expression was strongest in the ventral half, but was also detected in the dorsal half. By E.14 the pattern of expression was similar to that seen with the other Nova-1 probes (data not shown); this may be due to a down regulation of expression of Nova-1 in the telencephalon and thalamus, however these seem unlikely considering the D3 variant was cloned from an adult forebrain cDNA library. Alternatively there may be an

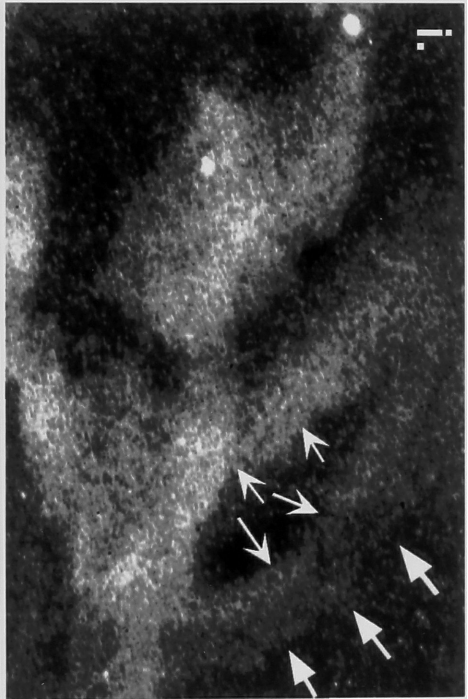
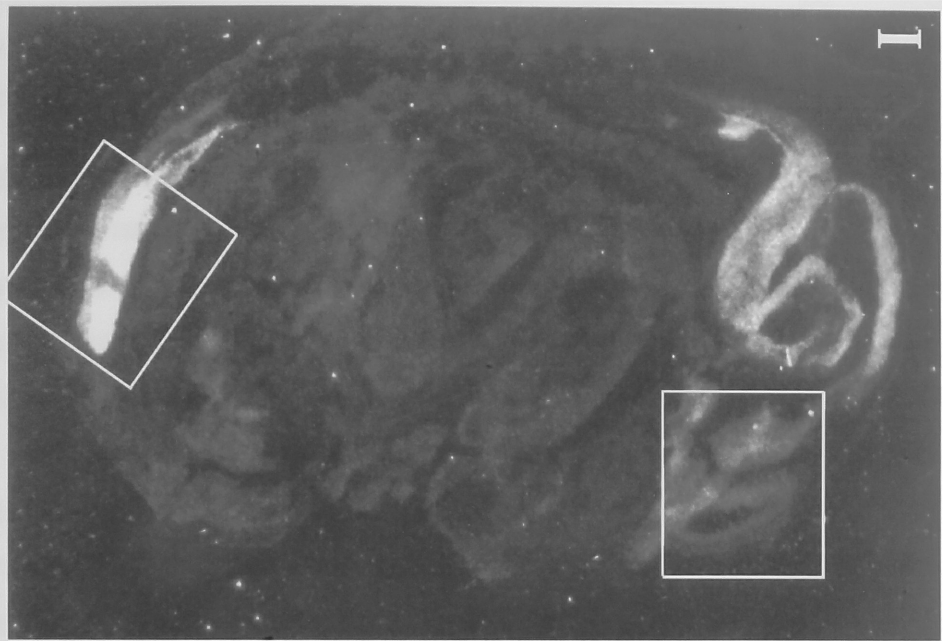
Figure 15. In-situ hybridization with Nova-1 5'UTR.

(A) E.11 mouse sagittal section revealing Nova-1 5' UTR expression restricted to the CNS.

(i) Higher magnification of the forebrain region boxed in A. Expression is detected in the dorsal thalamus and in ventricular zone neurons of the telencephalon. Thin arrows indicate the proliferating ventricular zones, thick arrows indicate the meningeal borders of the cortex.

(ii). Higher magnification of the spinal cord boxed in A. Strong expression is detected in the ventral spinal cord, and weaker expression is detected in the dorsal spinal cord. Probe consisted of 90 bp of the Nova-1 5' UTR.







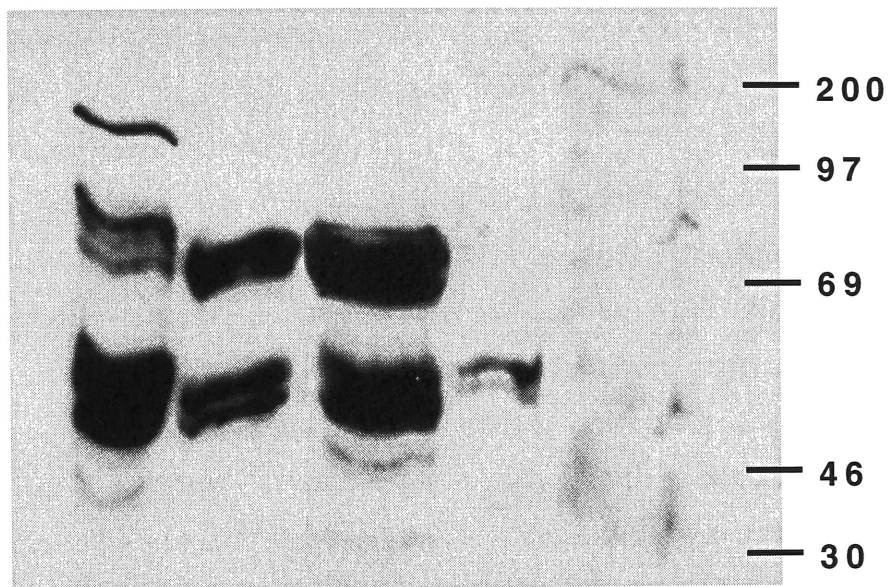
increased abundance or stability of Nova-1 Exon I RNAs in subcortical tissues as compared to that for D1, D2 and D3. These observations suggest that the restricted expression pattern of Nova-1 is regulated at the level of alternative splicing of the Nova-1 hnRNA.

Nova Expression: from *melanogaster* to Man. Proteins with important developmental function are often evolutionarily conserved. To assess whether proteins antigenically similar to Nova-1 are expressed cross species, we performed western blot analysis with human brain, mouse brain, xenopus larvae, Drosophila larvae and adult heads, using affinity purified antibodies generated against the Nova-1 fusion protein. Reactive antigens of approximately 53-55 and 74-78 Kd were seen in human neurons and identical reactivity was seen in mouse brain and Xenopus embryo extract (Fig 16). Two reactive antigens which exactly co-migrated with the ~55kd antigens were present in extract made from Drosophila embryos (Fig 16) and adult Drosophila heads (data not shown). However, the larger antigens were absent from Drosophila extract, suggesting that this protein family expanded between invertebrates and vertebrates. Immunohistochemical analysis of Nova expression in Drosophila embryos showed specific reactivity within all neurons of the developing CNS. These results demonstrate that the Nova proteins are conserved through evolution from invertebrates to mammals in the developing and adult CNS, and suggests that the Nova proteins play an important role in CNS development.

Figure 16. Zoo Blot analysis with anti-Nova antibodies.

Western blot analysis of human purkinje cells, mouse brain, xenopus larvae, drosophila embryo, and yeast extracts using affinity purified rabbit anti-Nova-1 antibodies. Molecular weight markers are indicated on the right.

Human  
Mouse  
Xenopus  
Drosophila  
Yeast





## Discussion

### The Nova Gene Encodes an Onconeural Antigen

Criteria for the clinical significance of paraneoplastic antibodies have previously been set (Posner and Furneaux, 1990). Among these criteria, antibodies need to be present in more than one patient, reactive with the same protein species, and target antigens need to be expressed in the clinically symptomatic regions of the nervous system. The Nova-1 cDNA encodes an antigen recognized specifically by the sera of 7/7 patients with POMA, providing an assay for the diagnosis and classification of this disorder. Furthermore, POMA antibodies specifically recognize the third KH domain of Nova-1, and mutation of a single amino acid in KH3 eliminates antibody recognition. Both full length NFP and KH3 fusion proteins are capable of affinity purifying POMA antibodies which reproduce the native serum's pattern of antigen recognition on both western blot and immunohistochemistry. Similarly, polyclonal sera generated against a full length Nova-1 fusion protein recognizes the same antigens on western blot and immunohistochemistry. *In vitro* transcription and translation of the Nova-1 cDNA yields a product recognized by POMA antisera which comigrates with the 55Kd antigen in brain extracts (data not shown). Finally, the Nova-1 *in-situ* pattern yields the same pattern as stringent immunohistochemistry with affinity purified antiserum, and both reveal that Nova-1 is expressed in a pattern which correlates with the clinically affected regions of brain. These data provide compelling evidence that the Nova-1 cDNA encodes an autoimmune target antigen in POMA. We recognize that there are likely to be a family of related proteins (as suggested by Western blot analysis), and a family of genes encoding them (as we have





found evidence for). The role of other family members in the disorder remains to be explored.

### Expression of Nova-1.

The expression pattern of Nova-1 is consistent with the predictions of the model of PND pathogenesis. Nova-1 protein and RNA are restricted to the immunologically privileged CNS throughout development and adulthood. In addition Nova-1 is expressed in the clinically affected areas of the brain including the midbrain and brainstem (potential sources of opsoclonus), spinal cord (potential source of myoclonus) and cerebellum (potential source of ataxia) (Anderson, et al., 1988a; Darnell, 1994). The restricted expression of Nova-1 may help to explain the apparent discrepancy between the focal neurologic deficits in POMA and the pan-CNS reactivity of POMA antibodies.

The perfect correlation between Nova-1 RNA expression and the restricted pattern of antigen expression detected using affinity purified antibody and stringent fixation suggests that the protein detected under these conditions is Nova-1. The fact that Nova-1 is not expressed in neocortex or medial thalamus, but POMA antiserum detects reactive antigens in both these regions of brain, strongly indicates the existence of other Nova family members. Screening an expression library with POMA antibody has identified a highly homologous protein Nova-2 (Yang and Darnell, unpublished data). The primary structures of Nova-1 and Nova-2 are 75% identical, and 100% identical in the core of KH3.

### Nova-1 encodes KH RNA Binding Domains.

The Nova-1 gene encodes three putative RBDs highly homologous to those of the hnRNP K protein. The RNA binding activity of hnRNP K has been attributed to the KH motifs (Siomi, et al., 1994). The presence of KH domains in Nova-1, suggests that Nova-1 functions as an RBP in neurons. RBPs are important post-transcriptional regulators of gene expression (Dreyfuss, et al.,



1993; Morris D.R., 1993). Nova-1 is the first nRBP to be expressed in a restricted pattern within the developing CNS. This suggests that similar to developmentally expressed compartmental transcription factors which have been proposed to regulate determination and differentiation, Nova-1 may play a role in neuronal determination or differentiation. The maintenance of Nova-1 expression in the adult suggests that Nova-1 also plays a role in the function of mature neurons.

Like many other RBPs Nova-1 has numerous alternative splice forms. Members of the Hu family of nRBPs have numerous alternative splices in the 5' UTR, 3' UTR, and in a spacer region between the second and third RRM (Okano and Darnell, 1996). Splicing of this spacer region may be functionally important as this region appears to mediate protein:protein interactions between the Hu proteins (Okano and Darnell, personal communication). The differential expression pattern of Nova-1 5' UTR containing mRNA vs. 3' coding region and 3' UTR containing mRNA suggests that a critical splice choice is made in the region of exon H. It appears that when exon H is spliced into the Nova-1 mRNA there are three potential outcomes: (1) exon H can be spliced to the KH2-KH3 containing giant exon (Exon I variant) leading to full length Nova-1 mRNA, or (2) exon H can be spliced to the stop codon containing D1 or D2 exons, leading to a truncated mRNA, or (3) the splice donor of exon H is not utilized and the D3 sequence is retained, also leading to a truncated mRNA (see Figure 3D). The proteins which result from the D1, D2 or D3 splice variants would contain KH1 and then terminate shortly thereafter. These proteins would likely be non-functional or have different functions than the full length Exon I variant. It is also possible that these truncated proteins may act as competitive inhibitors, competing for RNA binding sites with the full length protein. Similar alternative splicing occurs in the formation of mRNAs encoding the *Drosophila* alternative splicing factors *sxl* and *tra* (Baker, 1989). Both the *sxl* and *tra* RNAs can be spliced in a manner resulting in full length proteins, or



prematurely terminated proteins. Only the full length forms of these proteins are functional. Interestingly, both *sxl* and *tra* regulate sex specific alternative splicing of their own RNAs, stimulating the formation of full length, functional protein. Analogous to *sxl* and *tra*, Nova-1 may potentially regulate splicing of Nova-1 RNA encouraging the utilization of the Exon I splice and the formation of full length functional Nova-1 protein.



## Chapter IV

### Nova-1 is an RNA binding protein

Nova-1 encodes three potential RNA binding domains homologous to the KH domains of the previously discussed RNA binding proteins hnRNP-K, FMR-1, the *Drosophila* alternative splicing factor PSI, and the *Drosophila* Bicaudal-C proteins, the yeast alternative splicing factor MER-1, and others (Figure 3B; Burd and Dreyfuss, 1994a ). This domain appears to be a functional protein motif *in vivo*: a severely affected fragile-X syndrome patient harbors a point mutation (I367N substitution) within the core of the conserved KH element (DeBoulle, et al., 1993 ; see Figure 3B). Similarly, mutations of the core of the third KH domain of Bicaudal-C lead to loss of function *in vivo* (Mahone, et al., 1995). Mutations in the KH domains of hnRNP-K and FMR-1 decrease RNA binding activity *in vitro* (Siomi, et al., 1994). While these experiments suggests that KH domains are necessary for RNA binding they have not determined if a KH domain is sufficient for RNA binding.

In the absence of known RNA ligands or RNA binding function it is important to assess the RNA binding capabilities of potential RBPs. We wished to establish an assay to determine whether bacterially expressed Nova-1 had RNA binding capacity. This is important to determine before attempting more difficult, though more significant assays such as RNA selection. Many RNA binding proteins have rare codon usage or modified amino acids (Liu and Dreyfuss, 1995) and bacterially expressed proteins are often non-functional or truncated(Siomi et al., 1993b; Siomi, et al., 1994). In this case eukaryotically expressed fusion proteins or *in vitro* transcribed and translated products are necessary.

Putative RBP's have been functionally characterized and classified based on their ability to bind ribohomopolymers *in vitro* (Swanson and Dreyfuss, 1988; Dreyfuss, et al., 1993). Poly-pyrimidine tract binding protein (PTB) and





hnRNPK have been classified as poly C binding proteins whereas the FMR-1 protein binds preferentially to poly G and poly U. Typical interactions of known RBPs with homopolymers are ~200 nM Kd. The goal of this work was to determine if Nova-1 had the capacity to bind RNA, to determine the affinity of Nova-1's interaction with RNA, and to determine which if any of the Nova-1 KH domains mediated RNA binding.

#### Nova-1 binds to rG and rU *in vitro*.

To determine whether Nova-1 binds to RNA *in vitro*, we analyzed the ability of a T7 epitope-tagged Nova-1 fusion protein (NFP) to bind ribohomopolymers (see Fig. 17). Binding was detected by Western blot analysis using a monoclonal antibody directed against the T7 epitope. At low stringency (0.25M NaCl), NFP bound all ribohomopolymers except adenine, while control proteins showed no detectable binding. At intermediate stringency (0.5M NaCl) NFP bound preferentially to guanine (rG) and uracil (rU) ribohomopolymers, and at high stringency (0.75M NaCl) NFP bound only to rG (Fig. 18 A). Thus Nova-1 binds RNA *in vitro*, and binds with the same ribonucleotide preference as FMR-1 (rG>rU>rC>>>rA), in contrast to the binding preference (rC) of hnRNP-K and several other KH type RBP's.

To quantitate NFP binding to rG, we mixed increasing concentrations of protein with constant concentrations of RNA, separated bound from unbound protein, and quantitated the results using Western blot analysis. Binding plots revealed that NFP bound to rG with high affinity in a saturable fashion. Using Scatchard analysis, we estimate that NFP bound to rG with a dissociation constant (Kd) of 250nM in 0.25M NaCl (Fig. 18 B and C). The affinity of Nova-1 for rG is similar to that seen for other RBP's bound to ribohomopolymers (e.g. ~200nM for the polypyrimidine tract binding protein; McCarthy and Kollmus, 1995).

Figure 17. Diagrammatic representation of the fusion protein used.

Dark gray boxes indicate KH domains and the light gray box represents exon H. Numbers indicate the amino terminal and carboxy terminal amino acid positions relative to full length Nova-1 protein. The names of each fusion protein are indicated.



**NFP**



**N1-2**



**N1**



**N3**



**N3L21N**



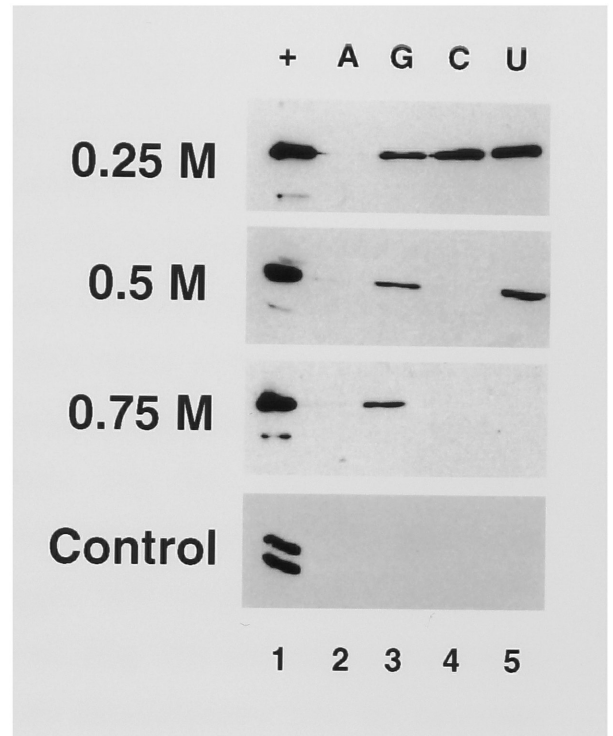
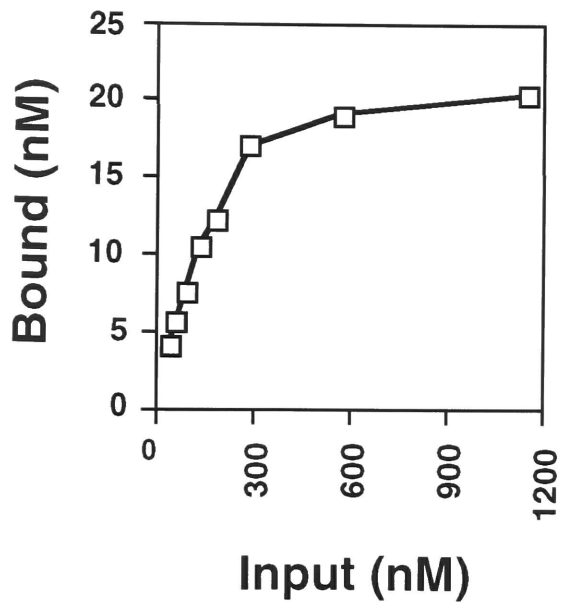
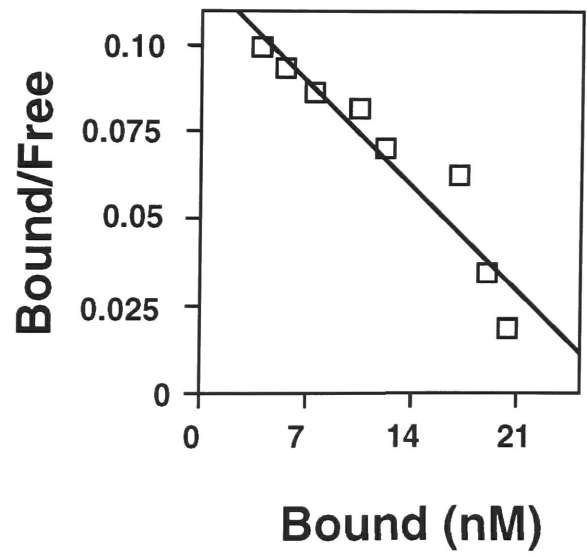


Figure 18. Nova-1 Binds RNA *in vitro*.

(A) T7 tagged Nova-1 fusion protein (NFP) was bound to the indicated ribonucleotide homopolymers at varying salt concentrations; after washing, bound protein was detected by Western blot analysis using a T7 monoclonal antibody (A, G, C and U binding assays are shown in lanes 2-5 respectively). NFP bound to G, C and U ribohomopolymers in 0.25M NaCl, to rG and rU in 0.5M NaCl, and only to rG in 0.75M NaCl. NFP bound to all 4 ribohomopolymers in 0.1M NaCl (data not shown). An irrelevant T7 tagged protein (Control) showed no detectable binding to A, G, C or U ribohomopolymers in 0.25M NaCl. To control for the amount of protein present in each assay, twenty percent of the protein from each reaction was reserved prior to ribohomopolymer binding and loaded directly in lane 1 (+).

(B) Binding plot of NFP to rG. The amount of NFP added to each reaction is indicated on the X axis and amount of NFP bound to RNA is indicated on the Y axis.

(C) Scatchard analysis of NFP binding to rG. The X axis shows the concentration of NFP protein bound to rG and the Y axis gives the ratio of protein bound to rG relative to unbound protein. Slope of the line ( $-1/K_d$ ) yields a  $K_d$  of approximately 250 nM.---

**A****B****C**





To define the domain of Nova-1 that binds to RNA *in vitro*, we constructed several truncated Nova-1 fusion proteins (see Fig. 17). One protein (N1) encompasses the amino terminus and first KH motif of Nova-1, a second (N1<sup>I14T</sup>) is identical to N1 but harbors an isoleucine to threonine mutation at position 14 of the first KH motif, a third (N3) encompasses the third KH domain and carboxy terminus of Nova-1, and a fourth (N3<sup>L21N</sup>) is identical to the N3 construct but harbors a leucine to asparagine mutation in position 21 of the third KH motif. The N3<sup>L21N</sup> mutation is precisely analogous to the FMR-1 I367N *in vivo* KH domain mutation (Fig 3B). Ribohomopolymer binding assays demonstrated that both N1 and N3 maintained the same sequence preference (rG>rU) as the full length NFP while N1<sup>I14T</sup> and N3<sup>L21N</sup> showed markedly reduced RNA binding to rG (Fig. 19A and data not shown). Quantitation of N3 and N3<sup>L21N</sup> binding to rG demonstrated that N3 has over 25 fold greater affinity for rG than N3<sup>L21N</sup> (N3 K<sub>d</sub>~750 nM, N3<sup>L21N</sup> K<sub>d</sub> ~ 20 mM; Fig. 19 B and C).

As an independent measure of Nova-1 RNA binding, Northwestern assays were performed. NFP, N3, N3<sup>L21N</sup> and a control fusion protein were immobilized on nitrocellulose and probed with a poly-rG riboprobe. NFP and N3 bound to the rG riboprobe, while N3<sup>L21N</sup> and the control fusion protein did not (Fig. 20). The blot was stripped and reprobed with T7 antibody, revealing fusion protein present in each lane (Fig. 20). These experiments demonstrate that the Nova-1 KH domain is sufficient for sequence-specific binding to RNA *in vitro*, and that disruption of this domain markedly interferes with that binding.

To determine if Nova-1 is capable of binding to *in vivo* brain RNA's, we incubated NFP with total mouse brain RNA. After immunoprecipitating NFP, RNA binding was detected by amplifying specific RNA's using reverse

Figure 19. RNA Binding and Mutational Analysis of the First (N1) and Third (N3) Nova-1 KH Domains.

(A) The first (N1) and third (N3) KH domains of Nova-1 bind preferentially to rG ribohomopolymers in 0.25M NaCl (A, G, C and U, lanes 2-5). N1<sup>I14T</sup> and N3<sup>L21N</sup> show no detectable RNA binding. Twenty percent of the amount of protein used in each binding assay was loaded in lane 1 (+). The N1 construct encodes amino acids 1-87 and the N3 construct amino acids 427-511 of Nova-1.

(B) Binding plot of N3 and N3<sup>L21N</sup> to rG. Input protein is indicated on the X axis and amount of protein bound to RNA is indicated on the Y axis.

(C) Scatchard plot analysis of N3 and N3<sup>L21N</sup> rG binding. The X axis shows the concentration of protein bound to RNA and the Y axis gives the ratio of protein bound to RNA and unbound protein. Slope of the line ( $-1/K_d$ ) yields a  $K_d$  of approximately 750 nM and 20 mM for N3 and N3<sup>L21N</sup> respectively.

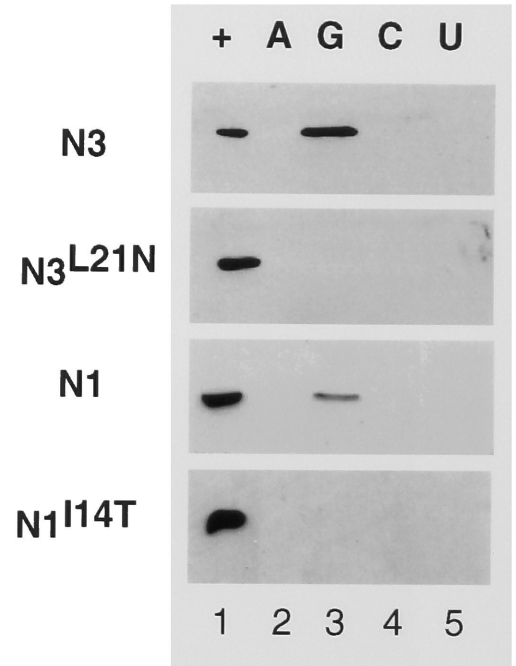
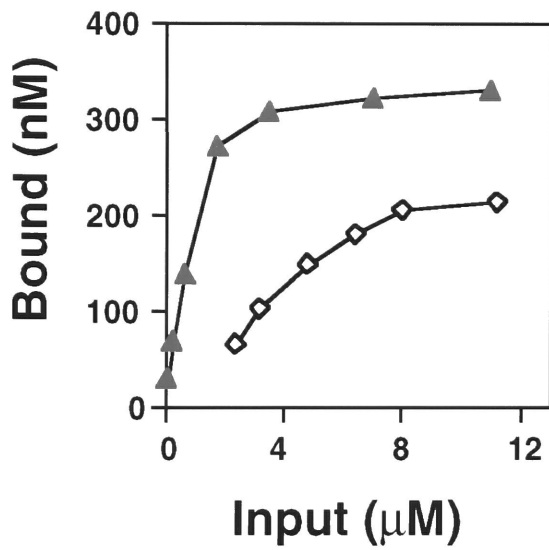
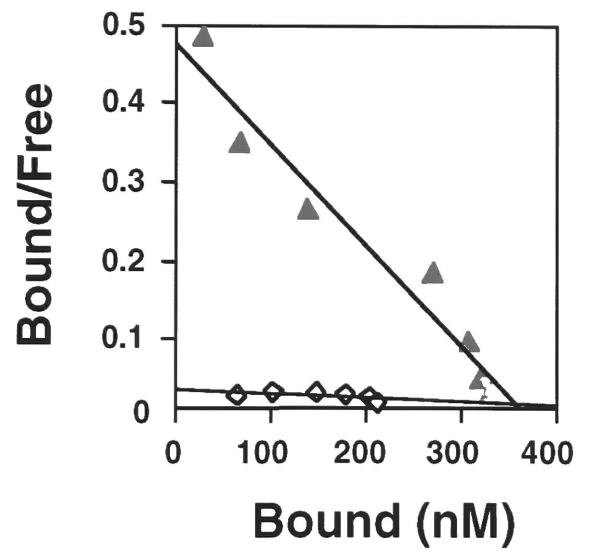
**A****B****C**

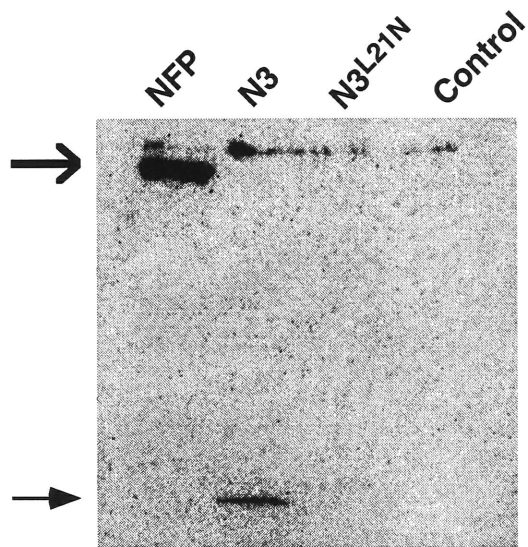




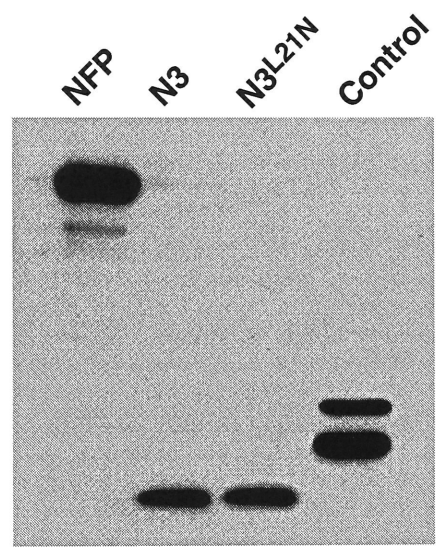
Figure 20. Northwestern Blot Analysis of Nova-1 RNA Binding.

NFP (large arrow) and N3 (small arrow), N3<sup>L21N</sup> or a control protein were transferred to nitrocellulose and bound to a poly-rG riboprobe (Northwestern). RNA bound only to NFP (large arrow) and N3 (small arrow). Some non-specific binding to bacterial proteins is present in the wells of all lanes. To confirm that approximately equal amounts of fusion protein were loaded in each lane, the blot was stripped and reprobed with a T7 monoclonal antibody (Western).\_\_

NorthWestern



Western







transcription PCR. By this analysis, NFP selectively bound some (Nova-1 and actin) but not all (Neuronal Adaptin-like Protein;  $\beta$ -NAP) abundant neuronal RNA's examined (Fig. 21). No bound RNA was detectable following Immunoprecipitation of an irrelevant protein, or following mock immunoprecipitation of NFP with normal human serum. These results demonstrate that Nova-1 is able to bind *in vivo* brain RNA's.

Since several RBP's bind to single-stranded DNA (ssDNA) as well as ribohomopolymers *in vitro* (Dreyfuss, et al., 1993), we examined whether Nova-1 was able to bind to ssDNA. While control proteins showed no detectable binding, NFP stably interacted with ssDNA even under stringent conditions (4M NaCl; Fig. 22A). Similarly strong interactions have been observed between ssDNA and hnRNP-K or FMR-1 (Dreyfuss, et al., 1993; Siomi, et al., 1994). Unlike NFP, neither the N1 nor N3 isolated KH domain proteins bound ssDNA. (Fig. 22B). These results suggest that the Nova-1 KH domains function preferentially as RNA binding domains.

#### Autoimmune Antisera Bind the Functional Nova-1 RNA Binding Motif

It has been suggested that autoimmune epitopes map to functional domains of target proteins (Tan, 1991). Since we have defined the Nova-1 KH domain as a functional RNA binding domain *in vitro*, and previously mapped the target epitope of POMA autoimmune sera to the third KH domain of Nova-1 (see Fig. 3E) we examined whether there might be a relationship between the function of Nova-1 and the binding of POMA antisera to the protein. When increasing amounts of N3 or N3<sup>L21N</sup> protein were blotted with autoimmune POMA antiserum, we found that N3 was recognized markedly better than the N3<sup>L21N</sup> point mutant (Fig. 23A). To confirm that equal amounts of N3 and N3<sup>L21N</sup> protein were present in the assay, the blot was stripped and reprobed with a T7 monoclonal antibody (Fig. 23A). To extend this result, obtained with a single POMA antiserum, we repeated this analysis with 5 additional POMA antisera. In each case, reactivity was robust with N3 fusion protein,

Figure 21. Nova Binds *in vivo* RNA.

NFP or a T7-tagged control fusion protein (Yo) were incubated with total mouse brain RNA. NFP was immunoprecipitated with either POMA sera (NFP; lanes 5-6) or normal human sera (Mock; lanes 3-4); control protein was immunoprecipitated with a T7 tag monoclonal antibody (Yo; lanes 1-2). Bound RNA's were extracted, reverse transcribed and PCR amplified using gene-specific primers to either Nova-1, actin, or  $\beta$ -NAP (an abundant neuron-specific mRNA; Newman et al., 1995). To ensure RNA integrity, total brain RNA was also amplified by RT-PCR (+ Control; lane 7). As a control for DNA contamination, each RNA sample was analyzed in duplicate with (+) or without (-) reverse transcriptase (RT). NFP bound to Nova-1 and actin, but not to  $\beta$ -NAP RNA.

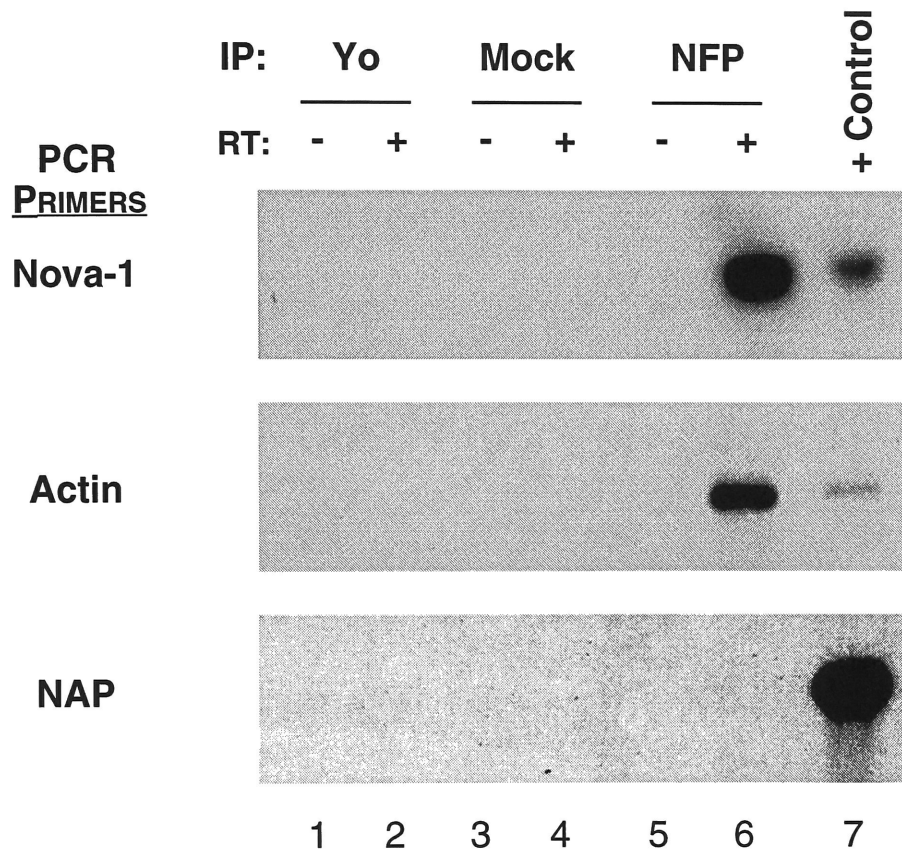




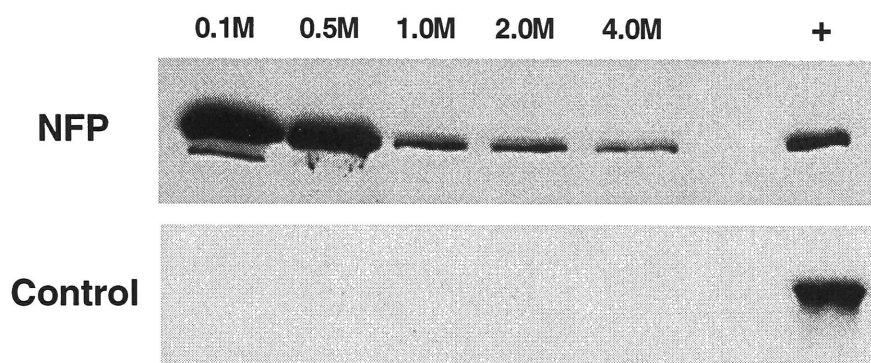


Figure 22. NFP, but not N1 or N3, binds ssDNA.

(A) NFP was bound to ssDNA in the presence of increasing salt (0.1M to 4.0M [NaCl]); bound protein was assayed as described in Figure 3. Twenty percent of the amount of protein used in each binding assay was loaded as a control (+).

(B) The binding of equimolar amounts of NFP, N1 and N3 to ssDNA was compared under relatively non-stringent conditions (0.25M NaCl). Under these conditions, NFP bound ssDNA while N1 and N3 did not. Twenty percent of the amount of protein used in each binding assay was loaded as a control (+).

**A**



**B**

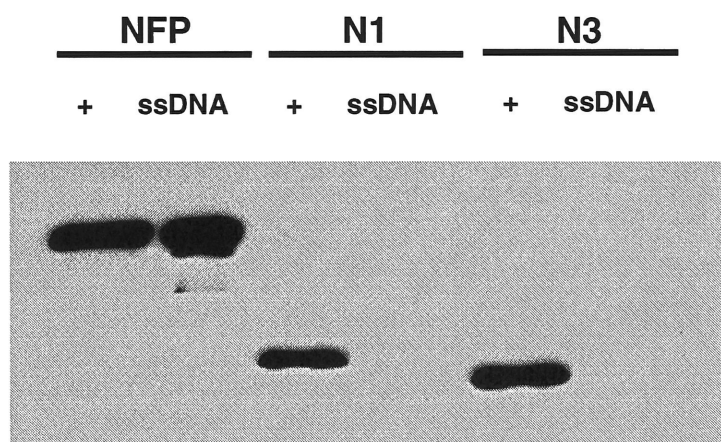






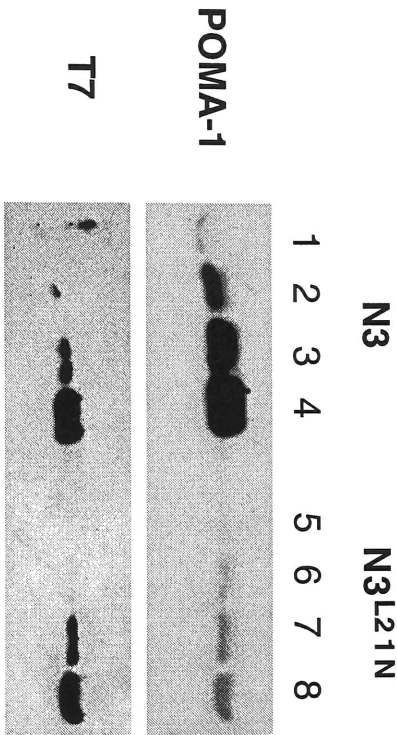


Figure 23. POMA Antisera Recognize Wild Type N3 but not the N3<sup>L21N</sup> Point Mutant.

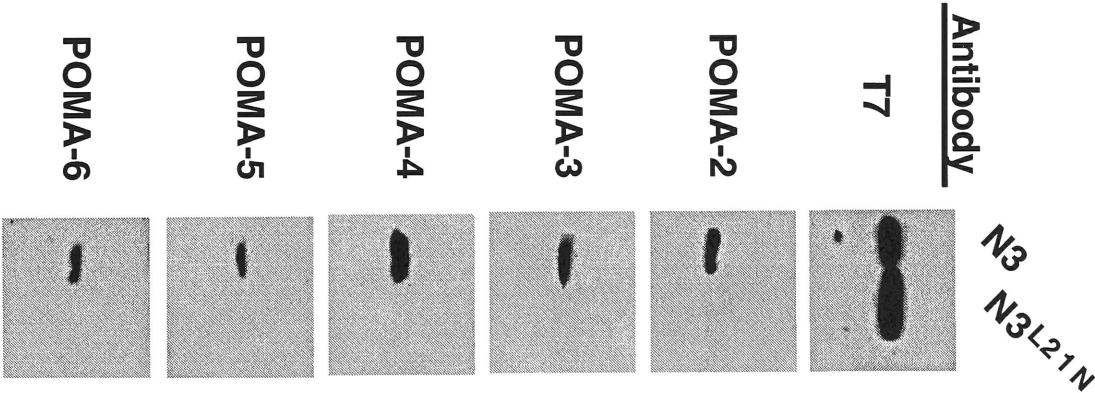
(A) Western blot analysis of 12.5, 25, 50, and 100ng of N3 (lanes 1-4) and N3<sup>L21N</sup> (lanes 5-8) using a POMA disease antiserum (POMA-1) or T7 monoclonal antibody.

(B) Western blot analysis with T7 monoclonal antibody and five additional POMA disease antisera (POMA 2-6) with 50ng of N3 and N3<sup>L21N</sup> proteins.

**A.**



**B.**



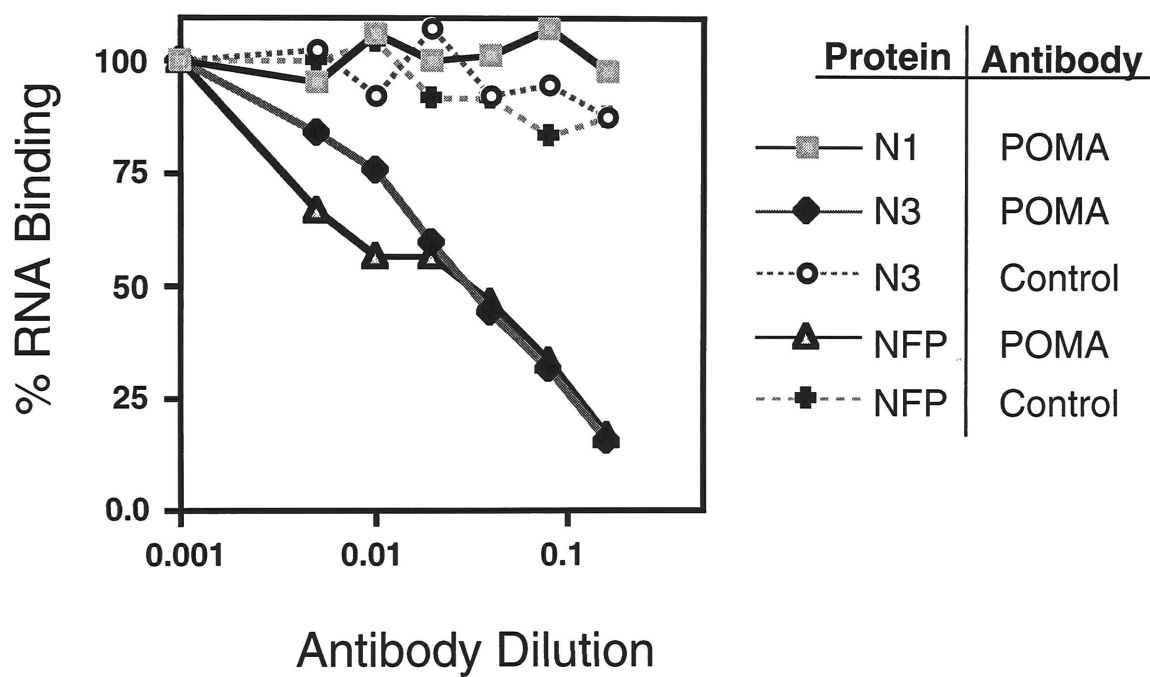


but nearly undetectable the N3<sup>L21N</sup> protein (Fig. 23B). These results indicate that the same amino acid which is essential for the RNA binding activity of N3 is also essential for recognition of the protein by autoimmune antibodies.

The dependence of both RNA binding and antibody recognition on the same amino acid suggest that the autoimmune POMA antibody may inhibit the function of the Nova-1 protein. To test this hypothesis *in vitro*, we assessed the ability of Nova-1 protein to bind soluble ribohomopolymers in the presence of antibody, using a filter binding assay. Binding of N3 to rG was inhibited by affinity purified POMA antibodies in a dose dependent fashion (Fig. 24). However, N3 binding was unaffected by T7 monoclonal or irrelevant autoimmune antibodies. In contrast, N1, which is not recognized by POMA antibodies, bound rG equally well in the presence of POMA or control antibodies. Surprisingly, POMA antibody inhibited full length NFP (which contains both N1 and N3 sequences) RNA binding as effectively as it inhibited N3 (Fig. 24). Taken together, these results indicate that the binding of POMA antibody to N3 effectively prevents the full length Nova-1 protein from binding to RNA.

Figure 24. POMA Antisera Inhibit RNA Binding of Nova-1 *in-vitro*.

RNA binding of NFP, and N3 proteins preincubated with affinity purified POMA or irrelevant autoimmune (Control) antibodies. Neither NFP or N3 RNA binding are affected by control antibodies, however both are inhibited in a dose dependent fashion by POMA antibodies. POMA antibodies do not recognize N1, and have no affect on N1 RNA binding. Dilution of antibody used is indicated on the X axis; percent RNA binding is indicated on the Y axis (binding with no antibody present [ $\sim 20,000$  CPM] was defined as 100%, and background counts when no protein was present [ $\sim 1,250$  CPM] was defined as 0%).







## Discussion

These observations define Nova-1 as an RBP. RBPs have been defined based upon their ability to bind ribohomopolymers, and classified based upon their ribohomopolymer preference (Dreyfuss, et al., 1993). Nova-1 binds to rG with high affinity and sequence specificity relative to other homopolymers. This binding is attributable to the KH domains of Nova-1; isolated Nova-1 KH domains (N1 and N3) bind RNA with similar affinity and specificity, and mutations within these KH domains disrupt RNA binding. Interestingly, unlike Nova-1, most other KH type RBPs analyzed including hnRNP K, hnRNP E (Dreyfuss, et al., 1993), PCB-1 and PCB-2 (Leffers et al., 1995), show a preference for rC. One notable exception is FMR1. FMR1 shows binding preferences similar to Nova-1, binding preferentially to rG>rU>rC>> >rA.

A compelling role for the isolated Nova-1 KH motifs as functional RNA binding domains reflects earlier studies of the hnRNP-K and FMR-1 proteins, but differs in several respects. Although KH domains are necessary for RNA binding in the intact hnRNP-K and FMR-1 proteins (Dreyfuss, et al., 1993; Siomi, et al., 1993b; Siomi, et al., 1994), our data provide the first evidence that they are sufficient for direct interaction with RNA *in vitro*. Quantitative analysis of the RNA binding of full length versus isolated N1 and N3 KH domains of Nova-1 reveal that they are competent to bind RNA with nearly equal affinity ( $K_d$  ~250-750nM in 0.25M NaCl; Figs 18, 19 and data not shown). These results provide a plausible mechanism for interaction of RNA with proteins (such as MER-1) that contain single KH domains.

The observation that POMA antibody recognizes the third KH domain of Nova-1, but not an inactive point mutant, is consistent with the general observation that autoantibodies are unusual in recognizing functionally important domains (Tan, 1991), POMA antibody, which binds only to the third KH domain (see Figure 3E), abolishes the Nova-1:RNA interaction, even in the presence of two upstream RNA binding domains (Fig. 24). This may be explained if the Nova-1 KH domains are in close physical proximity,



such that the presence of antibody bound to the third Nova-1 KH domain is able to sterically inhibit the first and second Nova-1 KH domains from interacting with RNA. Alternatively, in the full length Nova-1 protein, binding of RNA to the third Nova-1 KH domain may be a necessary prerequisite for binding of RNA to the first and second Nova-1 KH domains; such an effect could be mediated by structural or cooperative changes in Nova-1.

It is unclear why so many autoimmune target antigens, like Nova-1, are nucleic acid binding proteins. The major target antigens in Systemic Lupus Erythematosus (SLE), Sjogrens syndrome, scleroderma, and mixed connective tissue disorders are nucleic acid binding proteins. Target antigens include the snRNPs, hnRNPs, RNA polymerase III, RNA polymerase I, DNA polymerase, histones and others (Tan, 1991). Also, the Hu family of nRBPs are target antigens in the PND PEM/SN (Szabo, et al., 1991). It has been proposed that nucleic acid binding proteins may be particularly antigenic because they exist and function in large multi-protein complexes (Hassfeld et al., 1995), or because their association with nucleic acid makes them particularly immunogenic.



## Chapter V

### Identification of *in vitro* and *in vivo* RNA Ligands of Nova-1.

RNA-protein interactions are important in the posttranscriptional regulation of RNA metabolism and expression. Nascent RNA transcripts associate with large multi-protein complexes that include hnRNP proteins and snRNP particles Dreyfuss, et al., 1993. RNA protein complexes subsequently participate in polyadenylation, RNA splicing, RNA transport and translational control. Defining target RNAs with which RNA binding proteins (RBPs) interact has been critical in defining their function. In *Drosophila*, the identification of sequence-specific targets for the sex-lethal (Bell et al., 1988; Inoue et al., 1990) and transformer-2 (Hedley and Maniatis, 1991) RBPs has led to a precise understanding of their role in regulating RNA splicing. In mammals, the ability of U2AF(65) to bind to polypyrimidine tracts is believed to recruit U2 snRNP to the branch site (Ruskin, et al., 1988; Zamore et al., 1992). Identification of specific RNA ligands for the HIV Rev and Tat proteins has clarified their role in regulating viral RNA transcription, processing and transport (Malim et al., 1989; Zapp and Green, 1989; Heaphy, et al., 1990; Kjems, et al., 1991; Cullen, 1994). There remain, however, many RBPs for which specific RNA targets have not been identified.

Identification of RNA ligands is one means to try to associate an RBP with a biologic pathway and potentially identify its function. Biochemical purification and direct sequencing is one technique which has been used to identify RNA ligands of RBPs such as the snRNPs. However, this technique requires an abundance of identical protein:RNA complexes. This technique will likely not work for proteins such as alternative splicing or transport RBPs which are likely to have transient interactions with multiple RNA targets. RNA selection, or SELEX, has been used as an *in vitro* approach to identify RNA ligands for RBPs (Tuerk and Gold, 1990; Szostak and Ellington, 1993). This approach was originally used to confirm the specificity of ribosomal RNA binding sites recognized by T4 DNA polymerase (Tuerk and Gold, 1990), and has



subsequently been used to confirm and extend known binding sites for the U1-snRNP-A protein (Tsai et al., 1991), sxl (Singh et al., 1995), and the viral Rev (Bartel et al., 1991; Jensen, et al., 1994) and Tat proteins (Tuerk and MacDougall-Waugh, 1993). RNA selection experiments have also been used in efforts to identify RNA ligands for RBPs that do not have known sequence-specific binding sites, including hnRNP proteins (Burd and Dreyfuss, 1994c), SR proteins (Heinrichs and Baker, 1995; Tacke and Manley, 1995), and the neuronal RNA binding protein Hel-N1 (Levine, 1993). Careful analysis of selected ligands then allows for a directed search of the known databases for candidate *in vivo* ligands. Candidate ligands must demonstrate the proper timing and pattern of expression and make biologic sense.

#### Identification of in vitro ligands of Nova-1

We examined the function of Nova-1 by using an affinity-elution based RNA selection (Green et al., 1991; Szostak and Ellington, 1993) to identify specific Nova-1 protein:RNA interactions. From a library comprised of 52 nucleotide random-mers with an estimated complexity of  $\sim 10^{15}$  sequences, transcribed RNA was applied to a nickel column containing Nova-1-histidine tagged fusion protein. After washing, Nova-1 fusion protein, together with bound RNA, was affinity eluted in 1.0 M imidazole. In this way, we were able to follow the coordinate elution of protein and RNA (Fig. 25A), and found that the fraction of RNA bound to Nova-1 rose from essentially undetectable in the initial pool (<0.05%) to ~87% after seven cycles of selection (Table 2). Sequencing of individual PCR clones obtained after the seventh round of RNA selection identified a clear RNA consensus sequence (Fig. 25B). There were two components to the consensus, a structural element (an inverted repeat stem sequence) which bounded a sequence specific element (a loop sequence), which together accounted for nearly the full 52 randomized nucleotides present in each clone of the library RNA. The stem consisted of an average of 13 bp in each arm; there was no apparent sequence similarity between the stem of any two clones. The loop element contained a conserved

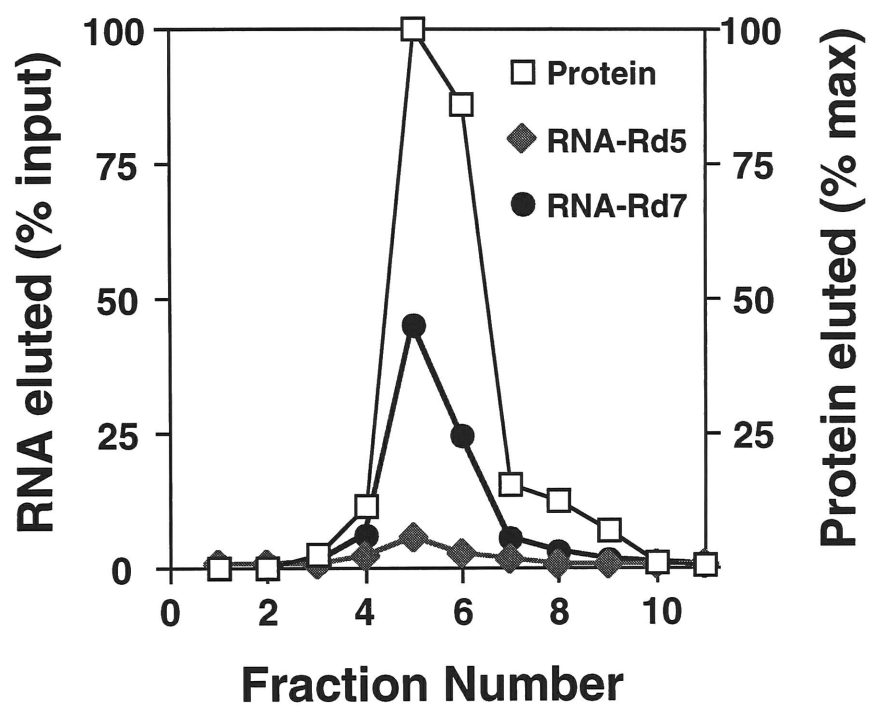
Figure 25. Selection of Nova-1 RNA ligands from a random RNA library. -

(A). Bound RNAs were enriched in each round of selection by specifically eluting histidine-tagged Nova-1 fusion protein in 1.0 M imidazole. A typical protein elution curve is indicated (squares) with each point defined as a percent of protein eluted in the peak fraction. RNA elutions from selection round 5 (diamonds) or round 7 (circles) are shown. Multiple selection cycles yielded RNA pools in which ~87% of input RNA counts co-eluted with Nova-1 protein (RNA selection round 7; see also Table 1).

(B). Sequences of Nova-1 selected RNAs. Sequence of 12 RNA ligands cloned after seven rounds of RNA selection are shown. UCAU repeats in each ligand are shown in bold capital letters. Regions of the RNA which form the inverted repeat stem are underlined. Stem sequences which are derived from the non-random regions of the RNA ligand (primer binding sites) are given as lower case. A consensus sequence is shown below.



**A**





B

clone

SB2	CCU <u>UAUC A UGCUGAC UCA</u> CG <b>UCAU</b> UU <b>UCAU</b> CU <b>CAU</b> CAA G <u>GGAGUCAGUG G GA</u> ua
SB4	<u>ccaqaaq CGUGAC</u> ACA CU <u>AU</u> <b>UCAU</b> <b>UCAU</b> <b>UCAU</b> <b>UCAU</b> GUUGAUUU <u>UGUCAUG GU CUUCUGGGCC</u>
SB7	AGCGUG <u>CAUGGGGC CAU UA CAUGUA</u> <b>UCAU</b> UU <b>UCAU</b> UU <b>CA</b> C UCGU <u>GCAUGGCU auq ugcgu</u> cua <u>cauq</u>
SB9	<u>qCGUAU</u> ACUG <u>CCGC A</u> <b>UCAU</b> <b>CACA</b> <b>U</b> <b>UCAU</b> <b>AAGACA</b> <b>U</b> UCA <u>GCGG ACCG AU</u> AC <u>GCC</u>
SB10	GUGGGAU <u>AUCC UGA</u> GGACCGGU <u>CGCCA</u> <b>UCAU</b> <b>UCAU</b> CGU <b>CAU</b> UUUAUC <u>GCG C</u> ua <u>u guqcguc</u> u <u>aca</u> u <u>gqau</u>
SB12	<u>GAU</u> GCAC GUU <u>ACGAG UU GCGCA</u> CU <b>UCAU</b> CG <b>CAU</b> UU <b>CAU</b> AA <u>UGCGC UC CU</u> CGU <u>u auquqc</u>
SB14	<u>UGCGGA</u> AA <u>CU</u> GAGG <u>ACG AGC AC</u> <b>UCAU</b> AA <u>GU</u> <b>CAU</b> AA <b>ACA</b> <b>U</b> C <u>GCU AAG CC</u> UCA <u>G</u> u <u>auqug cqucu</u>
SB20	<u>AGCAU</u> GGGCGUGC <u>UGGGG GACAC</u> AU <b>UCAU</b> <b>UCAU</b> <b>UCAU</b> <b>UCAU</b> ACAC <u>GU</u> GU <u>C AAG C</u> ua <u>uquqcqucu</u> a c
SB28	<u>agGAGAGCGAG</u> ACC <u>UA</u> AUAGAC <u>CC</u> AGCGU <b>UCAU</b> UA <b>ACA</b> <b>U</b> <b>UCAU</b> CUUA <u>CA</u> GCTGU <u>Ucu</u> auqugc <u>qucu</u> a <u>caug</u> g <u>au</u> ccu
SB31	GCAU <u>CA</u> CGCAAGUC <u>UG</u> CCCG <b>UCAU</b> <b>CAU</b> <b>UCAU</b> <b>UCAU</b> <b>UCAU</b> AC <u>CG</u> GU <u>GA</u> UUUU <u>GCGUGC</u>
SB37	<u>UGCGCAU</u> UU <u>UGCCGACA</u> CCC <b>UCAU</b> UU <b>UCAU</b> CU <b>ACA</b> UA <b>UCAU</b> <b>UCAU</b> UACGGGU <u>UGGGGcu</u> auquqc
SB6	<u>CUA</u> GUUGG <u>GCA</u> ACC <u>GAG</u> UUA <u>GAG UCU</u> GGCCAUGCAU <u>CA</u> GUA <u>G</u> GUUGC <u>GAGG</u> Cua

CONSENSUS

STEM - **UCAU**Y**UCAU**Y**UCAU**Y - STEM





Table 2. Multiple selection cycles yield Nova-1 RNA ligands.

Percent of total RNA input which specifically eluted with Nova-1 protein for each round of RNA selection is shown. The fraction of RNA bound to Nova-1 in each cycle of selection was calculated by dividing the number of counts eluted with Nova-1 protein by the number of counts loaded onto the Nova-1 column. In round 1, RNA was eluted in batch from the Nova column by phenol extraction.

**Table 2. Multiple Selection Cycles Yield Nova-1 RNA Ligand**

<i>Selection Round</i>	<i>Nova-1 bound RNA (% input)</i>
1	$\leq 0.05$
2	N.D.
3	0.26
4	3.4
5	10.2
6	51.0
7	87





sequence motif that was nearly identical in each of the eleven clones, and consisted of a pyrimidine-rich consensus sequence of three UCAU repeats separated by from 0-2 nucleotides which were preferentially (~75%) pyrimidines. Six of 11 clones matching the consensus sequence contain three UCAUs, and four of the five remaining clones have two UCAUs and a third sequence NCAU. We have designated the Nova-1 consensus RNA ligand [UCAUY]<sub>3</sub>.

Nova-1 is a sequence specific RNA binding protein.

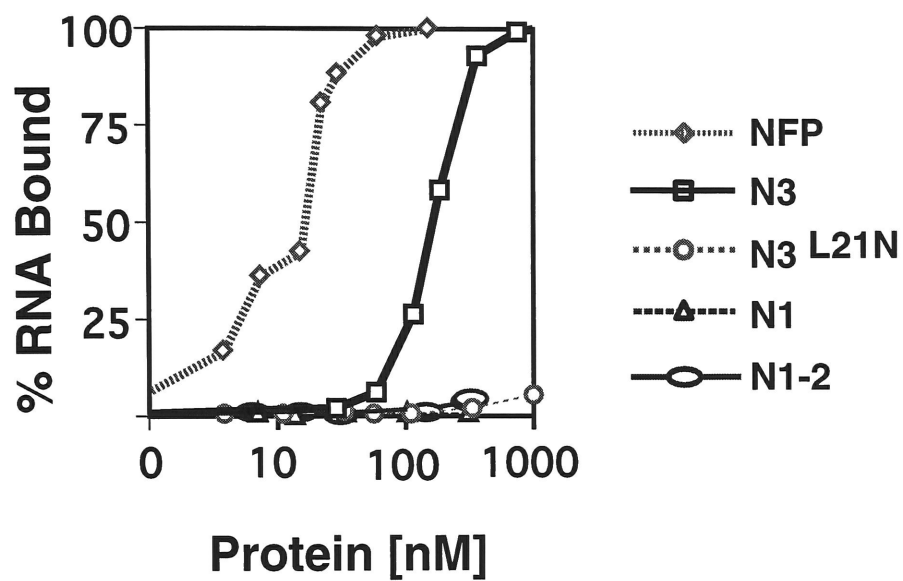
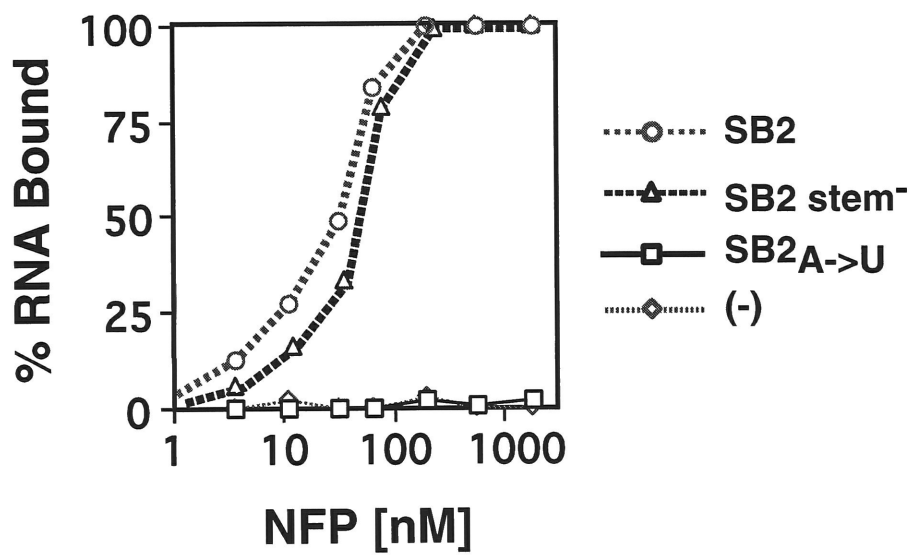
To characterize the Nova-1:RNA interaction, and to map the domains of Nova-1 which are responsible for recognition of the [UCAUY]<sub>3</sub> element, we quantitated binding of a selected RNA clone (SB2) to full length Nova-1 fusion protein (NFP), or to Nova-1 deletion constructs containing either the first (N1), first and second (N1-2), or third (N3) KH domains (see Fig. 17). In a filter binding assay NFP demonstrated high affinity saturable binding to SB2 (K<sub>d</sub> ~20nM in 0.5M LiCl, K<sub>d</sub> ~2nM in 0.1M LiCl; Fig. 26A and data not shown). The affinity of N3 for SB2 was reduced approximately 10-fold (K<sub>d</sub> ~180nM in 0.5M LiCl, K<sub>d</sub> ~10 nM in 0.1M LiCl), while neither N1, N1-2, nor an N3 protein carrying a single point mutation (N3 L21=>N) showed detectable binding to SB2 (Fig. 26A). Thus, Nova-1 binds to SB2 with high affinity, and this interaction is mediated at least in part by KH3.

We analyzed the sequence specificity and the structural requirements for Nova-1 binding to the [UCAUY]<sub>3</sub> RNA ligand by quantitating its interaction with a series of SB2 derived mutant RNA ligands. While NFP showed high affinity binding to SB2, there was no binding to an RNA (SB2<sub>A=>U</sub>) in which the [UCAUY]<sub>3</sub> element was mutated to [UCUUY]<sub>3</sub> (Fig. 26B). In addition NFP binding to SB2 was competed with an excess of cold SB2 RNA (50% inhibition with a 10-15 fold molar excess of competitor), but could not be competed with an excess of SB2<sub>A=>U</sub> RNA (see Fig. 29C). A structural mutant, in which base

Figure 26. Nova-1 is a sequence specific RNA binding protein.

(A). Nova-1 binding to an RNA selection ligand (SB2). RNA binding of full length and truncated Nova-1 proteins was determined in 0.5 M LiCl by a filter binding assay. NFP and N3 proteins bound with a  $K_d$  of ~20 and 100 nM respectively. N1 and N1-2 proteins, as well as N3 L21=>N show no binding.

(B.). Sequence-specific binding of Nova-1 to the RNA selection ligand. Filter binding assays were used to compare the binding of NFP to the wild type RNA selection ligand SB2, or SB2 derived mutant RNAs. In SB2A=>U RNA the UCAUY repeats have been mutated to UCUUY, and in SB2 stem<sup>-</sup> RNA the base pairing of the stem has been disrupted by oligonucleotide directed mutagenesis. There is no detectable binding to an irrelevant RNA transcribed from a random library clone (-).

**A****B**



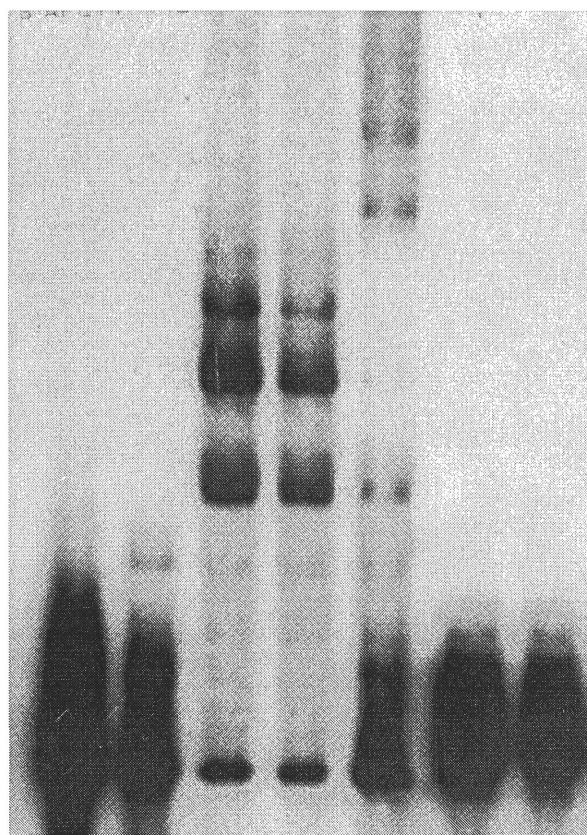
pairing in the stem sequence was eliminated while the [UCAUY]<sub>3</sub> sequences were unchanged, showed a three fold reduction in binding of NFP (Fig. 2B). These results were confirmed by gel shift assays. Figure 27 shows that the SB2 RNA was almost completely shifted after binding to NFP, but unaffected following incubation with an equimolar amount of control protein. In contrast, the mutant SB2<sub>A=>U</sub> RNA showed no gel shift when bound to NFP (Fig. 27). We also found that the SB2-NFP complexes could be supershifted using Nova-1 antibodies (Fig. 27). SB2-NFP complexes were not shifted when incubated with pre-immune antisera or an irrelevant affinity purified antibody, and anti-Nova-1 antibodies alone did not shift SB2 RNA (Fig. 27 and data not shown). We conclude that Nova-1 is a sequence specific RBP; the preferred Nova-1 RNA ligand is a stem-loop RNA, and the loop sequence is the major determinant of Nova-1 binding.

In order to further define the sequence requirements for high affinity Nova-1 RNA binding we generated small RNAs consisting of the [UCAUY]<sub>3</sub> loop sequence of SB2 or a series of mutant loop RNAs, and assayed their binding to Nova-1 by gel shift and filter binding assays. Gel-shift assays with the [UCAUY]<sub>3</sub> loop RNA revealed a shifted RNA species present at the lowest amount NFP tested (0.5 pmol) that increased with increasing NFP concentration. Gel-shift with an RNA in which a single UCAU repeat was mutated to UCUU only yielded a shifted species with a 10 fold higher concentration of NFP (Fig 28). These data also demonstrate that migration of the NFP-loop sequence RNA (a 21-mer) migrated as a single species even at the highest protein concentrations, in contrast to migration of NFP with the full length SB2 RNA (a 96-mer), which migrated as multiple shifted complexes. This suggests that the loop RNA retains the ability to bind NFP with high affinity, but is unable to support protein multimerization. Taken together, the results support the conclusion that NFP binds specifically [UCAUY]<sub>3</sub> and suggest that Nova-1 multimerization is not essential for high affinity binding.

Figure 27. Gel-shift analysis of Nova-1 and SB2

Gel shift analysis of Nova-1 protein. SB2 RNA or SB2A=>U mutant (mut) <sup>32</sup>P-labeled RNA was transcribed *in vitro* and incubated with NFP or irrelevant protein (C) and complexes run on non-denaturing PAGE. forms protein:RNA complexes with SB2 RNA, but not the mutant SB2A=>U RNA (mut). These complexes can be super-shifted with affinity purified rabbit anti-Nova-1 antibodies (N), but not with preimmune sera (PI) or an irrelevant affinity purified antibody (anti  $\beta$ -NAP; data not shown).

<b>Antibody</b>	-	-	-	C	N	N	-
<b>Protein</b>	-	C	NFP	NFP	NFP	-	NFP
<b>RNA</b>	SB2					mut	



1 2 3 4 5 6 7





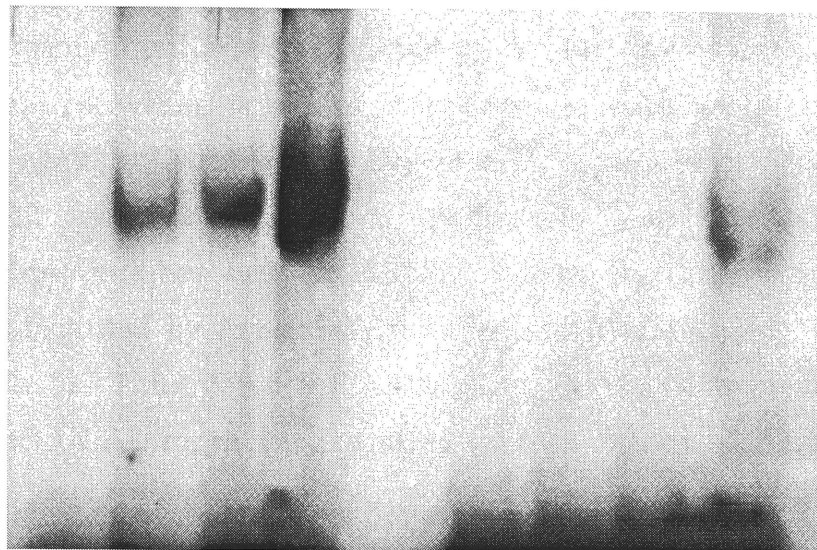


Figure 28. Gel shift analysis of [UCAUY]<sub>3</sub> or a mutant loop RNA bound to NFP.

Gel shift analysis of [UCAUY]<sub>3</sub> or a mutant loop RNA bound to NFP.

Increasing amounts of NFP (0, 0.5 pmol, 1.5 pmol, 5 pmol, respectively in lanes 1-4 and 5-8) were incubated with one fmol of <sup>32</sup>P-labeled RNA and analysed by non-denaturing PAGE. The RNA used in lanes 1-4 consist of the loop sequence in SB2 and is indicated below. The RNA used in lanes 5-8 consist of the loop sequence in SB2 with a single A=>U mutation is indicated below the lanes. UCAU repeats in the RNAs are in bold type. The mutated nucleotide is underlined.

**NFP:**



**RNA:**

1 2 3 4  
UCAUUUUCAUUUUCAU

5 6 7 8  
UCUUUUUCAUUUUCAU



We next assayed the binding of NFP and N3 to a series of [UCAUY]<sub>3</sub> loop RNAs harboring specific nucleotide substitutions (Table 3). Substitution of a single A to U residue within one [UCAUY]<sub>3</sub> repeat reduced binding approximately 9 fold, consistent with the gel-shift assay using this mutant (Fig. 27). Substitution of two of three As to Us completely abolished binding (Table 3). This result demonstrates that Nova-1 is not simply a polypyrimidine tract binding protein, but requires specific purine residues to be present within a pyrimidine-rich sequence. Substitution of any of the C, A or second U nucleotides within each of the three repeats completely eliminated binding, while substitution of the first U within each repeat reduced binding 7-12 fold (Table 3). Although the selected RNAs preferentially harbor pyrimidines between UCAU repeats (Fig. 25B), these sequences could be substituted by A residues with no significant change on binding affinity (Table 3). Thus Nova-1 RNA binding is highly sequence-specific and three intact UCAU repeats are required for high affinity binding.

#### Identification of candidate *in vivo* ligands of Nova-1.

Based on the consensus RNA selection sequence we identified two potential *in vivo* Nova-1 RNA binding sites, one within an intron of the glycine receptor  $\alpha 2A$  hnRNA (glyR $\alpha 2A$  ; Kuhse et al., 1991), and one within the Nova-1 hnRNA itself (Fig. 29A). The glyR $\alpha 2A$  intron has three distinct UCAU repeats, each separated by two nucleotides. A fourth UCAU repeat overlaps the first and second in a pattern similar to that seen in RNA selection clone SB31 (Fig. 25B). In the Nova-1 intron there are two UCAU repeats separated by four pyrimidines, followed by five nucleotides (3/5 pyrimidines) and a third CCAU repeat. In both genes, the UCAU sequences are present in potential stem loop structures, and are adjacent to alternatively spliced exons (80 bp upstream of glyR $\alpha 2A$  exon 3A (Kuhse, et al., 1991) and 8 bp downstream of Nova-1 exon H (Fig. 29A). In Nova-1, the UCAU element lies within a 35 nucleotide intronic sequence which is 95% identical between human and

Table 3. Nova-1 binding to mutant RNA ligands. The indicated RNA's were *in vitro* transcribed from DNA oligonucleotides containing T7 RNA polymerase promoter sequences. The K<sub>d</sub> values are indicated for NFP and N3 binding to RNAs in 0.5 M LiCl; stars indicated no significant binding (K<sub>d</sub>>>5μM). K<sub>d</sub>'s were determined by filter binding assays.

RNA	Kd (nM)	
	NFP	N3
UCAU UU UCAU UU UCAU UU	50	230
C - - - - C - - - - C - - - -	340	*
G - - - - G - - - - G - - - -	600	*
- U - - - - - U - - - - - U - - - -	*	*
- G - - - - - G - - - - - G - - - -	*	*
- - G - - - - - G - - - - - G - - - -	*	*
- - - C - - - - - C - - - - - C - - - -	*	*
- - - G - - - - - G - - - - - G - - - -	*	*
- - U - - - - - - - - - - - - - - - -	420	*
- - U - - - - - - - - - - - U - - - -	*	*
- - U - - - - - U - - - - - U - - - -	*	*
- - - - AA - - - - AA - - - - AA	50	280







Figure 29. Nova protein binds to glycine receptor  $\alpha 2A$  and Nova-1 RNAs.

A. Alignment of *in vivo* [UCAUY]<sub>3</sub> elements with SB2. UCAU elements present in the rat glycine receptor  $\alpha 2A$  (Kuhse, et al., 1991) and Nova-1 genomic DNA sequences are shown aligned with UCAU elements (bold) present in RNA selection clone SB2 (from Fig. 1B), with gaps indicated by ( ). Nova-1 genomic sequences were determined from human and mouse genomic libraries. The Nova-1 UCAU elements are present within an intron (IVS) 8 nucleotides downstream of an alternatively spliced exon (Exon H; Buckanovich, et al., 1993), and the glycine receptor  $\alpha 2A$  sequences are present ~80 nucleotides upstream of alternatively spliced exon 3A (Kuhse, et al., 1991).

B. NFP binds to [UCAUY]<sub>3</sub> elements in GlyRa2A and Nova-1 RNAs *in vitro*. Fragments of the GlyRa2A and Nova-1 genes encoding putative Nova-1 binding elements were transcribed *in vitro* and used in filter binding assays with NFP. NFP bound to GlyRa2A (squares), Nova-1 (open circles), and SB2 RNAs (diamonds) was saturable with a K<sub>d</sub> ~ 20 nM (standard deviation ~ 2 nM). Mutation of the UCAU elements in either GlyRa2A (closed circles) or Nova-1 RNAs (triangles) eliminates binding.

C. NFP binding to the GlyRa2A can be competed with SB2 RNA. NFP was incubated with radiolabelled ligand (GlyRa2A or SB2) and increasing amounts of cold competitor RNA (SB2 or SB2 A $\Rightarrow$ U). Binding of NFP to either SB2 or the glyRa2A RNA could be competed with an 10-15 fold molar excess of cold SB2 (SB2 open squares, glyRa2A open triangles) RNA but not with an excess of cold SB2 A $\Rightarrow$ U (SB2 closed diamonds, glyRa2A inverted triangle).

**A**

SB2 UUAUCAUGCUGACUCACG **UCAUU**...**UCAUC**...**UCAU** CAAGGAGUCAGUGGG

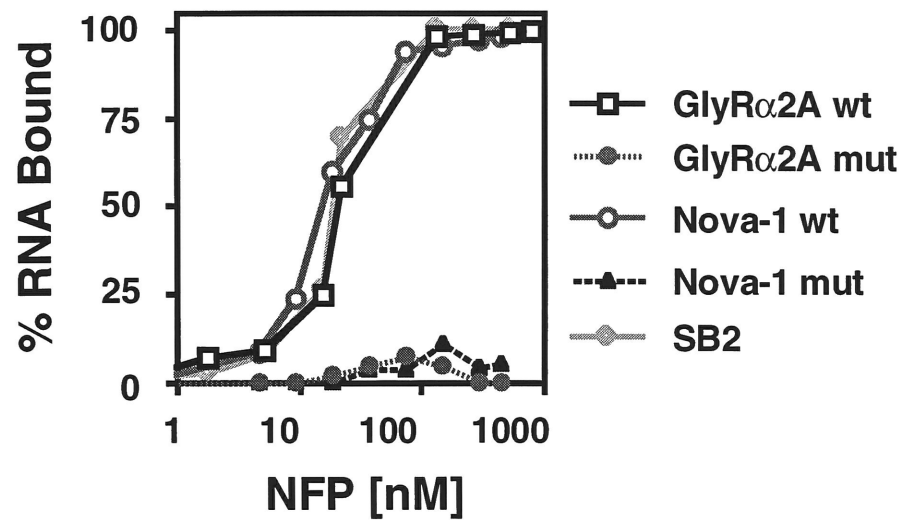
Gly Receptor UUUUUUUUCUUGCAGUC **UCAUCA..UCAUU..UCAU** UUUUUUACAUCGCUA

Nova-1	(mus)	GCCAAUCAG	GUACAGUA	<b>UCAUCCUCUACA<b>CCA</b></b> CAU	ACUUUCACAGCGGUG
Nova-1	(hum)	GCUAUCAG	GUACAGUA	<b>UCAUCCUCUACA<b>CCA</b></b> CAU	ACUUUCACGGGUGAU

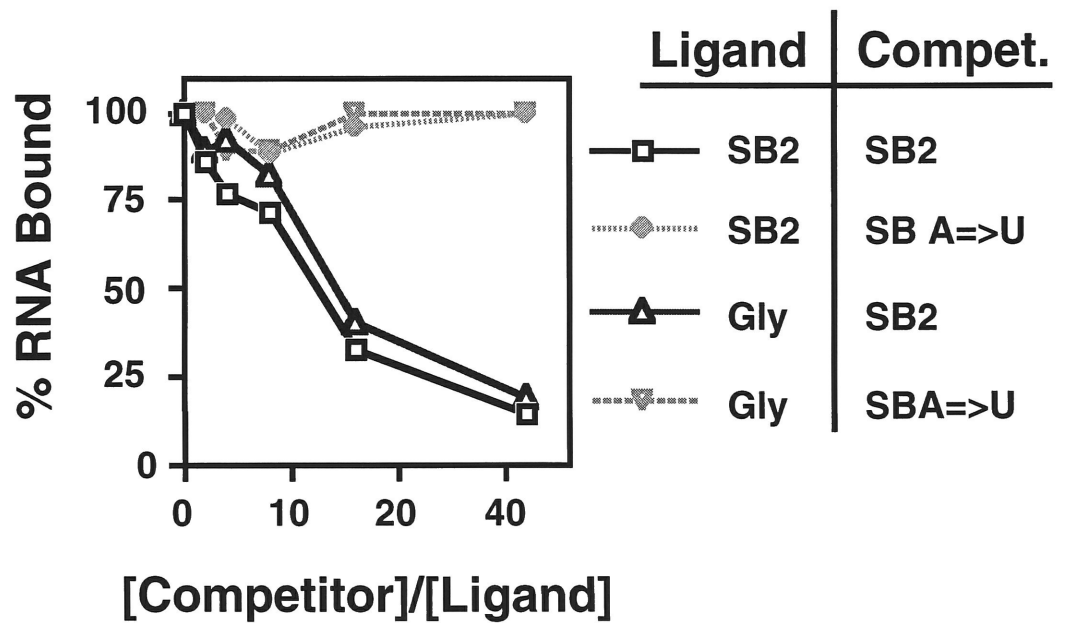
## Exon H IVS ...



**B**



**C**





mouse. In both the glyR $\alpha$ 2A and Nova-1 hnRNAs, the sequence CAGU is present one nucleotide upstream of the first UCAU, and a pyrimidine rich stretch (7/8 in glyR $\alpha$ 2A RNA, 6/8 in mouse Nova-1 RNA, and 7/9 in human Nova-1 RNA) is present downstream of the UCAU element. We cloned these regions of the glyR $\alpha$ 2A and Nova-1 genes and *in vitro* transcribed them for RNA binding studies. Deletion analysis of the glycine receptor RNA mapped the Nova-1 binding site to a 50 base pair region containing the UCAU motif (data not shown). Nova-1 bound SB2, glyR $\alpha$ 2A, and Nova-1 RNAs in an identical manner by filter binding assay (Kd's 20 nM, 20 nM and 15 nM respectively in 0.5 M LiCl; Fig. 29B). Mutation of the glyR $\alpha$ 2A RNA [UCAUY]<sub>3</sub> repeats to [UAAUY]<sub>3</sub> eliminated Nova-1 protein binding as did mutation of the Nova-1 RNA [UCAUY]<sub>3</sub> repeats to [UACUY]<sub>3</sub> (Fig. 29B). In addition, Nova-1 protein binding to glyR $\alpha$ 2A RNA could be competed by an excess of SB2 RNA but not by the mutant SB2 A $\Rightarrow$ U RNA (Fig 29C). These results demonstrate that Nova-1 binds to both the glyR $\alpha$ 2A and Nova-1 RNAs *in vitro* with high affinity via the [UCAUY]<sub>3</sub> motif.

We have shown that POMA antibodies target the third KH domain of Nova-1 and can disrupt the low affinity binding (300nM Kd) of Nova-1 to poly ribohomoguanine RNA *in vitro*. Having identified the third KH domain of Nova-1 as both necessary and sufficient for sequence specific binding, we tested whether POMA antibodies could disrupt the sequence specific interaction between Nova-1 and the high affinity glyR $\alpha$ 2A or Nova-1 RNA ligands. Pre-incubation of Nova-1 protein with affinity purified POMA antibodies strongly inhibited Nova-1 binding to both RNAs; a three fold molar excess of antibody reduced RNA binding 80% (Fig 30). Nova-1 binding to glyR $\alpha$ 2A was unaffected by the presence of irrelevant autoimmune antibodies and incubation of RNA with antibodies alone did not lead to RNA



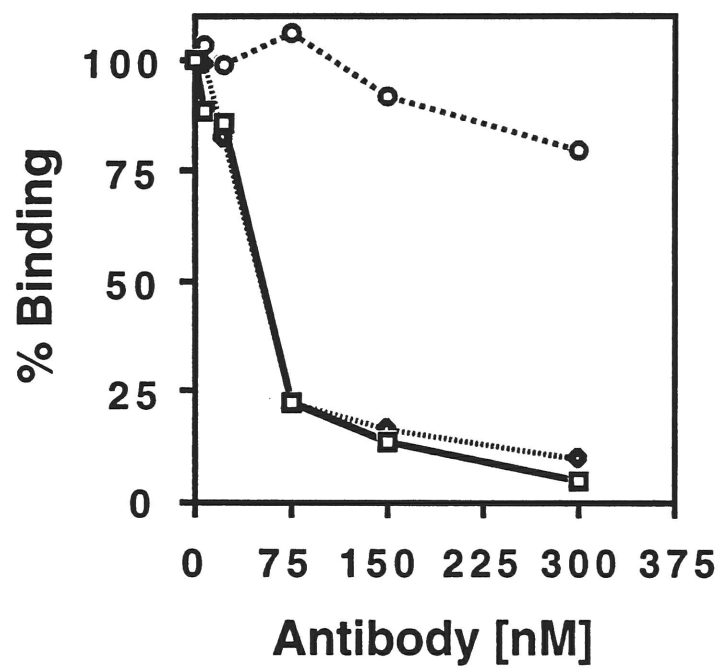


degradation (data not shown). Thus POMA antibodies are capable of disrupting the high affinity interaction of Nova-1 and its RNA ligands.

Immunoprecipitation in combination with nucleic acid sequence analysis permits the identification of specific *in vivo* ligands for nucleic acid binding proteins. In previous studies this method has been used to identify the RNA ligands of autoimmune target antigens and DNA elements bound by transcription factors (see discussion). To determine whether Nova-1 is bound to glyR $\alpha$ 2A and Nova-1 hnRNAs *in vivo*, we immunoprecipitated nuclear extracts of mouse brain with affinity purified rabbit anti-Nova-1 antibodies. Immunoprecipitates were phenol extracted, and RNA was reverse transcribed and PCR amplified with primers specific for glyR $\alpha$ 2A, Nova-1, or a series of abundantly expressed neuronal hnRNAs. Nova-1 immunoprecipitated RNA gave strong RT dependent PCR products using both glyR $\alpha$ 2A and Nova-1 primers, but not with control primers (Fig. 31). Control immunoprecipitation with antiserum that recognizes a different family of abundantly expressed n-RBPs (Hu) yielded no detectable bound glyR $\alpha$ 2A RNA or Nova-1 RNA. Similarly, immunoprecipitations with preimmune rabbit serum, normal human serum, paraneoplastic cerebellar degeneration anti-Yo antibodies, or an irrelevant affinity purified rabbit antibody to an abundant neuronal autoantigen (anti- $\beta$ -NAP; Newman, et al., 1995) failed to immunoprecipitate neuronal RNAs. These immunoprecipitations demonstrate that Nova-1 binds specifically to the glyR $\alpha$ 2A and Nova-1 hnRNAs *in vivo*, and offer an opportunity to identify additional *in vivo* ligands.

Figure 30. Nova-1 binding to glycine receptor  $\alpha$ 2A and Nova-1 RNAs can be inhibited by paraneoplastic antibodies.

NFP binding to Gly $\alpha$ 2A and Nova-1 hnRNAs can be inhibited by disease antibodies. Preincubation of NFP with increasing amounts of affinity purified disease antibodies (POMA) leads to complete inhibition of RNA binding. Preincubation with similar concentrations of affinity purified cerebellar degeneration antibodies (Control) had no affect on RNA binding.



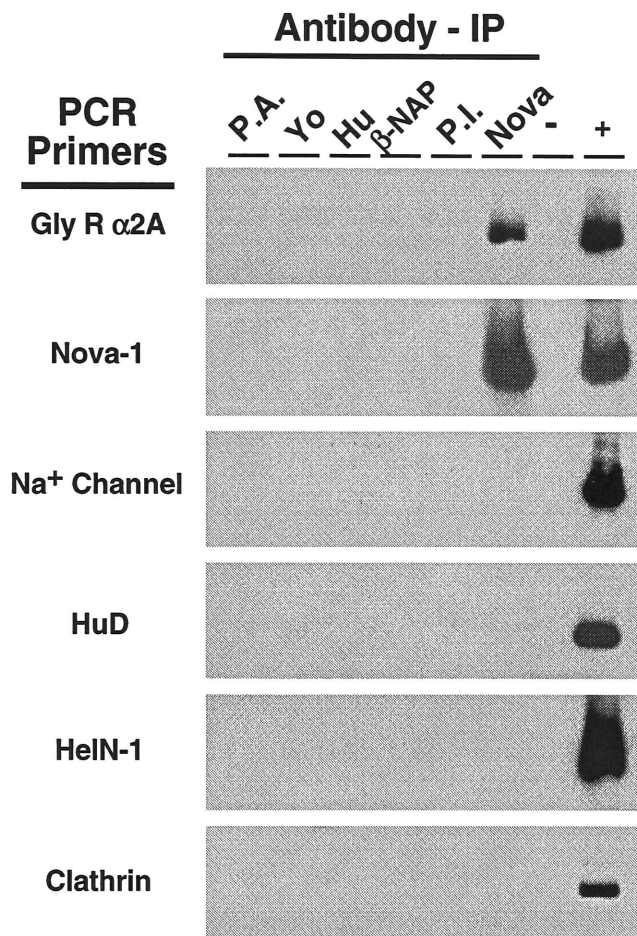
Ab	RNA
POMA	GlyR $\alpha$ 2A
POMA	Nova-1
Control	GlyR $\alpha$ 2A





Figure 31. Nova protein co-immunoprecipitates of with glycine receptor  $\alpha 2A$  and Nova-1 hnRNAs.

Nova protein co-immunoprecipitates of with glycine receptor  $\alpha 2A$  and Nova-1 hnRNAs. Immunoprecipitations of mouse brain were performed as described Steitz, 1989 with affinity purified rabbit anti-Nova-1 antibodies (Nova), or controls, including protein-A sepharose (P.A.), paraneoplastic cerebellar degeneration serum (Yo), paraneoplastic antiserum directed against the Hu family of n-RBP's (Hu), affinity purified rabbit anti- $\beta$ -NAP antibodies, or pre-immune serum (P.I.), as indicated. RNA present in the precipitates was phenol extracted as described Steitz, 1989 and subjected to RT-PCR amplification with the indicated primers. A minus reverse transcriptase control (-) was included to ensure the RNA dependence of RT-PCR product, and genomic DNA was amplified (+) to ensure primer fidelity. The fidelity of each immunoprecipitation was confirmed by Western blot analysis (data not shown).







### Nova-1 Enhances splicing of the glyR $\alpha$ 2A.

To test the functional implications of Nova-1 binding to the glyR $\alpha$ 2A hnRNA, we constructed a glyR $\alpha$ 2A minigene construct consisting of exon 2 and surrounding intronic sequences, together with exons 3A and 3B and their surrounding intronic sequences (including the Nova-1 binding site). We co-transfected this minigene construct into a mouse breast cancer cell line (RAC) with a Nova-1 expression construct, or a series of control plasmids. Protein expression was confirmed by western blot analysis (Fig. 32A). We then assayed for the presence of spliced products from the glyR $\alpha$ 2A minigene construct by quantitative RT-PCR. In the presence of a control vector we detect splicing to both exons 3A and 3B. With transfection of increasing amounts of Nova-1, we detect an increase (3-5 fold) in splicing from exon 2 to exon 3A and a minimal increase in splicing to exon 3B (0-2 fold) (Fig. 32 B and C). To assure that this is a specific effect, we co-transfected the glyR $\alpha$ 2A minigene construct with a Nova-1 deletion mutant in which the critical third KH domain and carboxy terminus have been deleted. The Nova-1 $\Delta$  mutant had no apparent effect on splicing of either exon 3A or 3B (Fig. 32C). In order to demonstrate that this effect is dependent on the Nova-1 binding site in the glyR $\alpha$ 2A we made a glyR $\alpha$ 2A minigene construct in which the Nova-1 binding site was mutated. Co-transfection of Nova-1 with this construct had no effect on splicing (Fig. 32E). Thus we conclude that Nova-1 can function as a sequence specific splice enhancer protein. Nova-1 primarily stimulates splicing of the nearer splice acceptor (exon 3A) and thus may act to increase exon 3A utilization in vivo.

Figure 32. Nova-1 enhances splicing of the glyR $\alpha$ 2A exon 3A.

(A). Western blot analysis of cells transfected with 3  $\mu$ g pcDNA vector (V), 1, 2, or 3  $\mu$ g pcDNA Nova-1, or 1  $\mu$ g pcDNA  $\Delta$ mut. Full length Nova reactive antigen is present only in Nova-1 transfected cells and the amount of reactive protein increases with increasing amounts of transfected DNA. A smaller reactive species of the appropriate size is abundantly expressed in cells which have been transfected with a Nova-1 mutant (mut) from which the third KH domain has been deleted.

**A.**

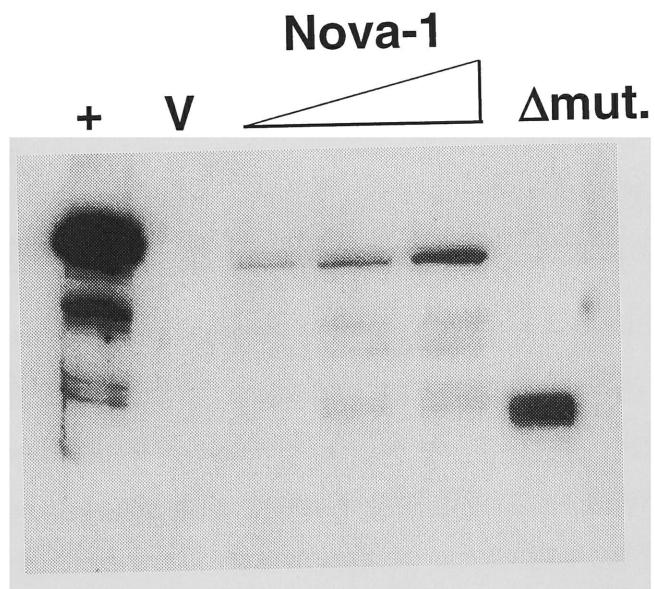




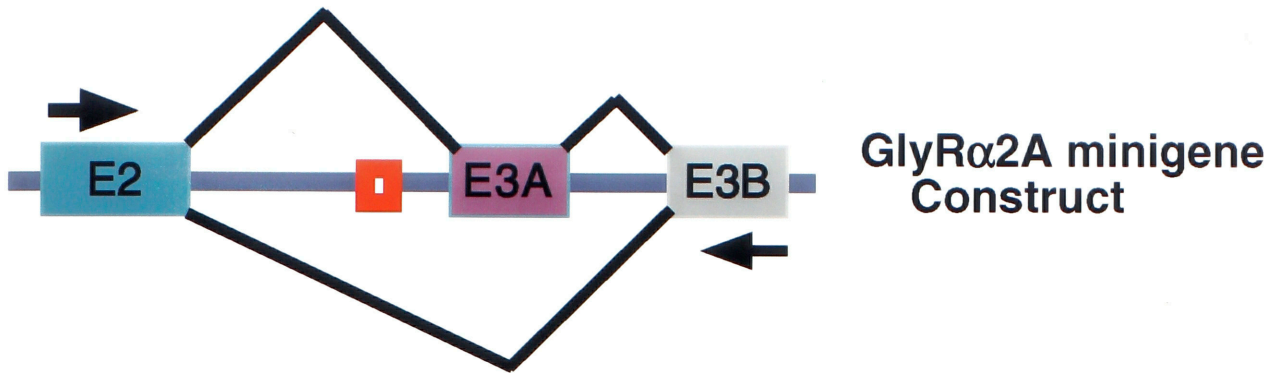


Figure 32 cont.

(B). Diagrammatic representation of the glyR $\alpha$ 2A minigene construct used in transfections. Arrows indicate the location of the PCR primers used for splice analysis. The Nova-1 binding site is indicated as a small box upstream of exon 3A.

(C). RT-PCR analysis of splice products from glyR $\alpha$ 2Aminigene cotransfections with pCDNA vector (Vector 4  $\mu$ g-lane 1) or of pCND A Nova-1 (1, 2, 3, or 4  $\mu$ g, lanes 2, 3, 4, and 5). The slower migrating product is the result of an RNA spliced from exon 2 to exon 3A to exon 3B. The faster migrating product is the result of an RNA spliced directly from exon 2 to exon 3B. Nova-1 cotransfected cells demonstrate an increase in the amount of exon 3A included spliced products (lanes 3 and 4). At highest levels of Nova-1 transfection there is a toxic effect, resulting in inhibition of both spliced products (lanes 5 and 6). Spliced products detected are indicated in diagrammatic form (see A). The asterisk indicates a non RT dependent PCR artifact.

B



C

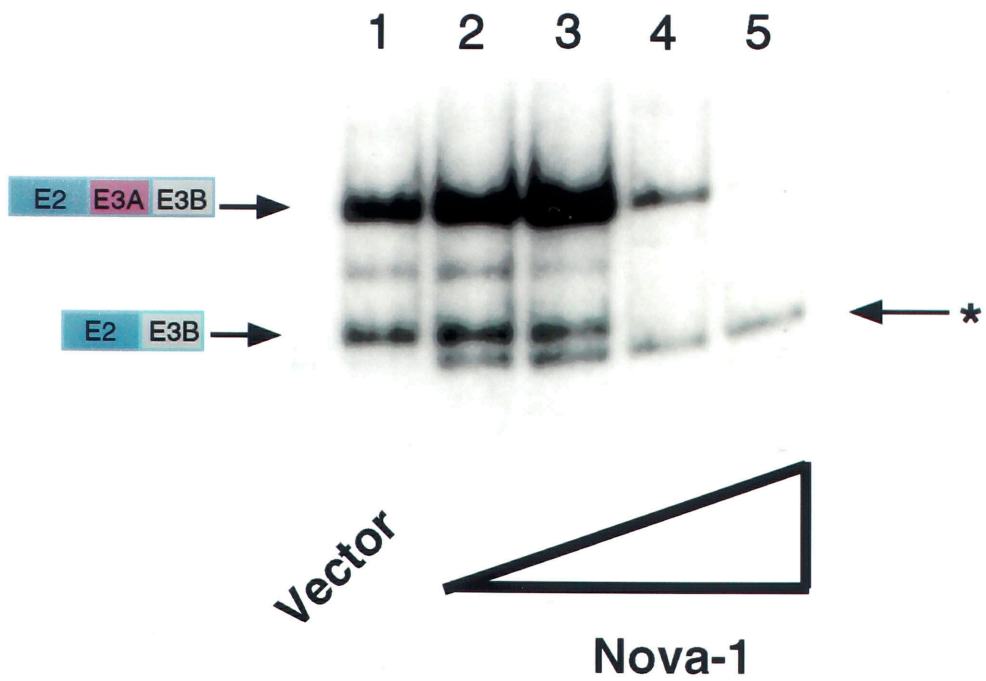




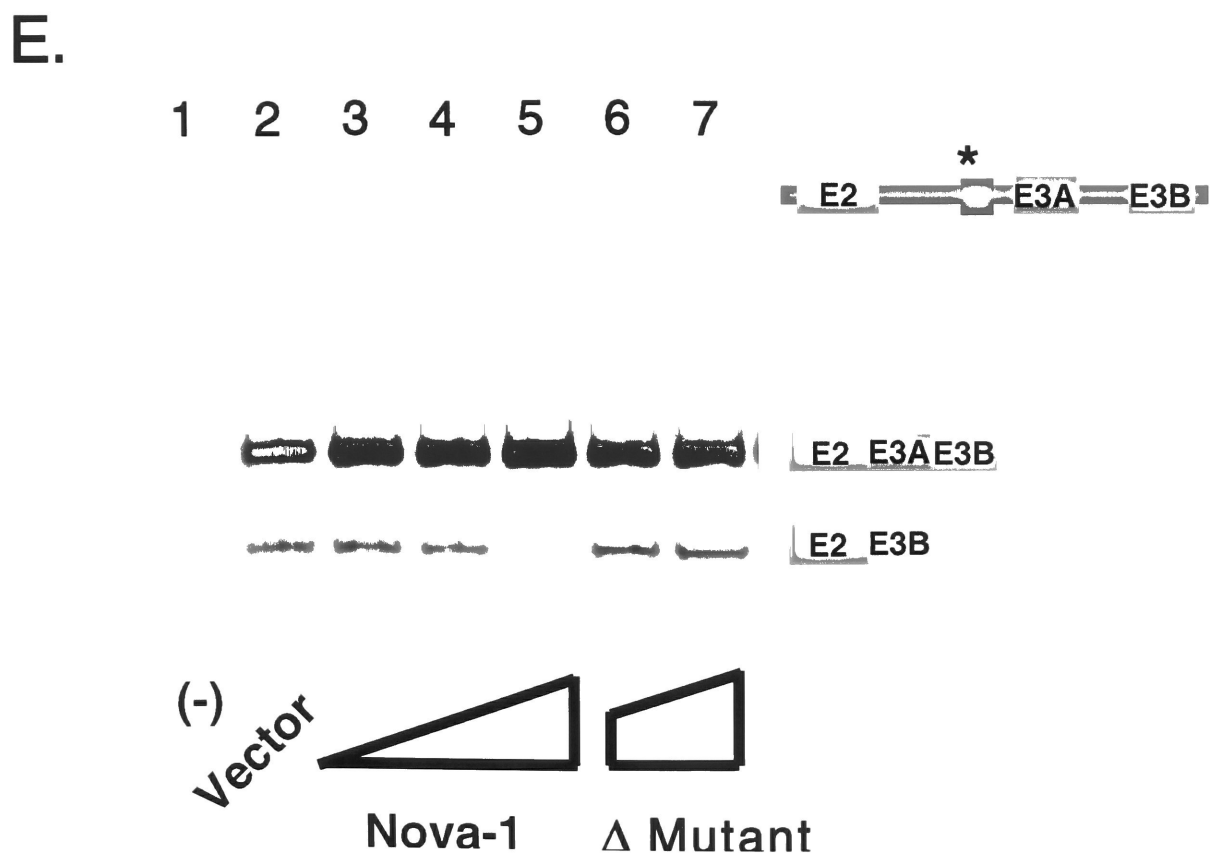
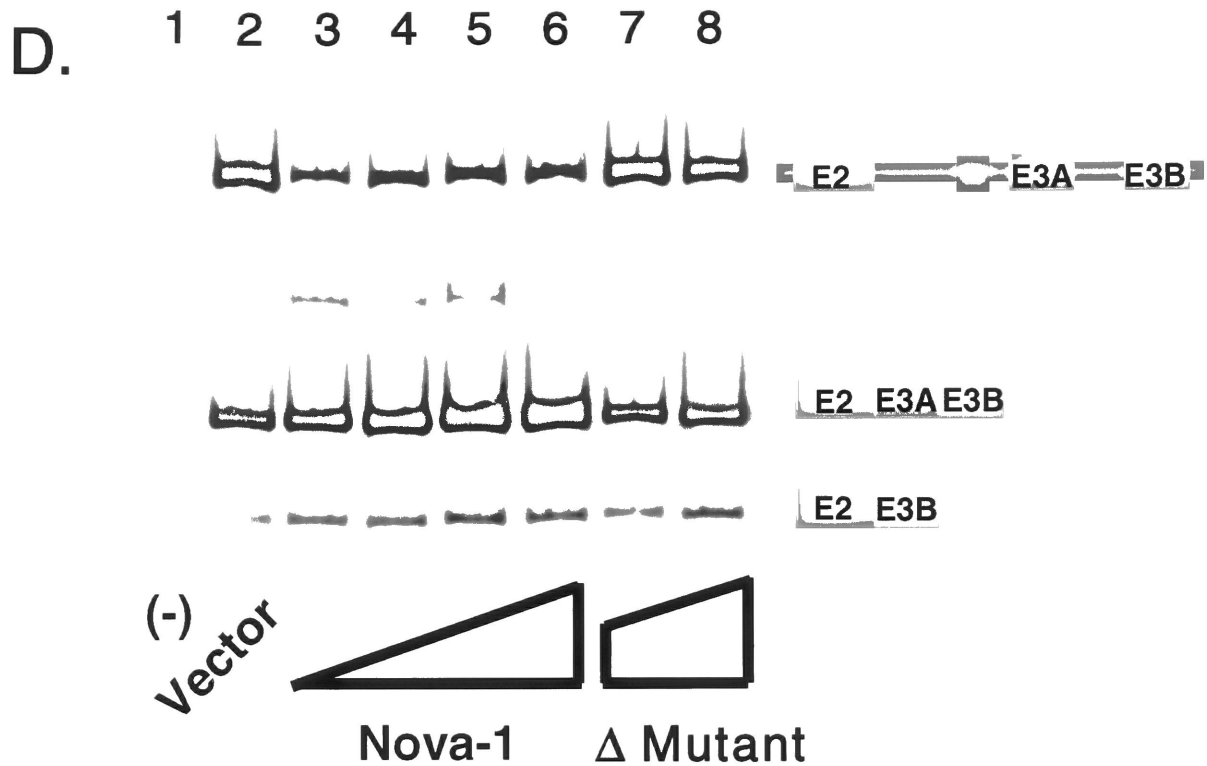




Figure 32 cont.

(D). RT-PCR analysis of splice products from glyR $\alpha$ 2A minigene cotransfections, comparing the effects of Nova-1 versus, vector alone, or a Nova-1 deletion mutant lacking KH3 ( $\Delta$  mut). Nova-1 cotransfected cells demonstrate a dose dependent increase of exon 3A included spliced products (1, 1.5, 2, and 3  $\mu$ g pcDNA Nova-1 lanes 3, 4, 5 and 6) as compared to both vector (lane 2) alone or Nova-1 deletion mutant ( $\Delta$  mut 2 and 3  $\mu$ g, lanes 7 and 8) cotransfected cells. Lane 1 is a (-) reverse transcriptase control. Spliced products detected are indicated in diagrammatic form (see A).

(E). RT-PCR analysis of splice products from a mutant glyR $\alpha$ 2A minigene construct in which the Nova-1 binding site has been mutated from [UCAU]<sub>3</sub> to [UAAU]<sub>3</sub>. Nova-1 has no effect on splicing of this mutant construct. Lane 1 is a (-) reverse transcriptase control. Cotransfection with pcDNA vector (lane 2), pcDNA Nova-1 (1, 2, or 3  $\mu$ g lanes 3, 4, and 5) or Nova-1 deletion mutant ( $\Delta$  mut 2 and 3  $\mu$ g, lanes 6 and 7). Lane 1 is a (-) reverse transcriptase control. Spliced products detected are indicated in diagrammatic form (see A).





## Discussion

Nova-1 is a Sequence-Specific KH Type RNA Binding Protein. We have shown that Nova-1 is a neuron-specific KH type RBP which binds to stem-loop RNAs in a sequence specific manner *in vitro* and *in vivo*. By using affinity elution and stringent binding conditions in our RNA selection protocol, we identified a relatively long, specific, pyrimidine rich sequences that binds Nova-1 with nanomolar affinity. At least 11 nucleotides in the consensus loop sequence are necessary and sufficient for high affinity binding, while the stem element confers approximately a three-fold increase in binding affinity but is not essential. Although the high divalent cation concentration present in our RNA selection binding buffer may have promoted selection of RNA ligands harboring stem elements, we note that a number of RBPs bind stem loop structures both *in vitro* and *in vivo*. These include the interactions between Rev and the RRE (Heaphy, et al., 1990; Jensen, et al., 1994), the U1-A protein and U1A RNA (Scherly et al., 1989; Scherly, et al., 1990; Tsai, et al., 1991), and the interactions between the L32 RNA (Vilardell and Warner, 1994) and Epstein Barr viral RNA 1 (Toczyski and Steitz, 1993; Dobbstein and Shenk, 1995) ligands and their ribosomal proteins (L32 and L2, respectively). In addition, the two *in vivo* Nova-1 RNA ligands we have identified are both surrounded by elements that preferentially fold into stem structures as assessed by the Zuker RNA folding algorithm ( $\Delta G \cong -15$ ; data not shown; Zuker, 1989).

The length of the loop sequence element identified for Nova-1 compares favorably with core consensus RNA ligands identified for other RBPs, which typically are on the order of 6-9 nucleotides (Levine, et al., 1993; Burd and Dreyfuss, 1994c; Heinrichs and Baker, 1995; Tacke and Manley, 1995). Based on our mutagenesis of the consensus *in vitro* Nova-1 RNA ligand and the sequences of the *in vivo* Nova-1 RNA ligands, at least 11 specific pyrimidine-rich



nucleotides present in three repeats appear to be strictly necessary for high affinity binding. These repeats are interspersed by sequences varying in length that are preferentially (~75%) pyrimidines, suggesting a consensus sequence (UCAU[Y]<sub>0-2</sub>UCAU[Y]<sub>0-4</sub>NCAU). The length of this sequence suggests a limited set of potential *in vivo* targets, on the order of  $1/4^{11}$  (with no interspersed pyrimidines). Considered in the context of the length of the mammalian genome ( $\sim 3 \times 10^9$  nt), the length of the Nova-1 consensus sequence suggests that there are on the order of tens to perhaps hundreds of possible high affinity binding sites within the genome. This limited target range increases the relevance of the *in vivo* Nova-1 RNA targets we have identified.

Identification of *in vivo* Nova-1 RNA Ligands. We have found evidence that the Nova-1 protein interacts with glyR $\alpha$ 2A and Nova-1 hnRNAs *in vivo* by co-immunoprecipitation and RT-PCR analysis. In previous studies protein:RNA co-immunoprecipitation has been used to purify and identify the RNA ligands of numerous autoimmune target antigens (e.g. snRNPs, Ro, La; see Steitz, 1989). This approach has also been used to identify DNA elements bound by transcription factors (e.g. ultrabithorax, thyroid hormone receptor and myc/max DNA binding elements; Gould et al., 1990; Bigler and Eisenman, 1994; Grandori et al., 1996). Presumably as a result of low levels of hnRNA ligands bound to Nova-1 we were unable to directly identify bound RNA ligands (Buckanovich and Darnell, unpublished data), but were able to analyze candidate RNA ligands by RT-PCR analysis.

The biology of the candidate Nova-1 RNA ligands (glycine receptor  $\alpha$ 2A and Nova-1 RNA) are consistent their binding Nova-1 protein in neurons. Both RNAs are expressed in Nova-1 expressing cells. Expression studies demonstrate that glyR $\alpha$ 2A mRNA is expressed in many brain regions which co-express Nova-1 including midbrain, brainstem, and spinal cord in both embryonic development and continuing into the adult. Single cell studies





reveal that Nova-1 protein and glyR $\alpha$ 2A mRNA are co-expressed within individual neurons of the rat spinal cord (Darnell et al., unpublished observations). An interaction between Nova-1 protein and its own hnRNA would suggest that Nova-1, like many other RBPs in *Drosophila* and mammals (Bell et al., 1991; Boelens et al., 1993; Rongo, et al., 1995), may regulate post-transcriptional processing of its own RNA.

### Function of Nova-1

Co-expression of Nova-1 with a glyR $\alpha$ 2A minigene construct leads to increased levels of an exon 2 to exon 3A spliced product (~3 fold) and exon 2 to exon 3B spliced product (~50%). A similar co-expression with a glyR $\alpha$ 2A minigene in which the Nova-1 binding site has been deleted shows no effect of Nova-1 on splicing. Finally, co-expression of a glyR $\alpha$ 2A minigene with a KH3 deleted Nova-1 protein had no effect on splicing. These observations suggests that Nova-1 acts as a sequence specific splice enhancer and that Nova-1 may act to regulate alternative splicing of the glyR $\alpha$ 2A in brain. However the exact role of Nova-1 remains elusive. Splicing of the glyR $\alpha$ 2A minigene in the tissue culture cells assayed to date does not lead to mutually exclusive use of exons 3A and 3B as is seen in brain. It is likely that other neuronal proteins may be necessary to work in conjunction with Nova-1 to lead to mutually exclusive exon use. The identification of potential Nova-2 binding sites adjacent to exon 3B and the demonstration that Nova-1 and Nova-2 may interact (Yang and Darnell unpublished data) suggests that a combination of Nova family members interacting with the glyR $\alpha$ 2A may be necessary for mutually exclusive splicing. Yeast two hybrid analysis indicates that Nova-2 interacts with numerous proteins believed to be involved in splice site regulation including SRp75 and PTB (Zahler et al., 1993; Lin and Patton,



1995). Thus numerous protein-protein contacts are likely to be necessary for regulating splicing of the glyR $\alpha$ 2A hnRNA.

Finally, while we have yet to test the functional role of Nova-1 binding to Nova-1 RNA, the Nova-1 binding site within the Nova-1 hnRNA appears to lie within a region of extensive alternative splicing (see chapter III, Figure 3D). The Nova-1 binding site is adjacent to the splice donor of the alternatively spliced exon H. The splice donor of exon H can be spliced to either of three different splice acceptors (Exon I, D1 or D2), or an alternative splice donor (D3-which contains the Nova-1 binding site) can be used. Thus Nova-1 binding to the Nova-1 hnRNA in this region may lead to Exon H inclusion or exclusion, or may enhance or inhibit utilization of Exon I, D1, D2 or D3. Nova-1 inhibition of the Exon I splice acceptor, or stimulation of either the D1, D2 or D3 variants would be consistent with potential feed back inhibition of gene expression demonstrated by some RBPs (Boelens et. al., 1993). Considering the pan neuronal expression of Nova-1 5' UTR and the restricted pattern of expression of full length Nova-1, we feel that this is unlikely. Alternatively Nova-1 may enhance utilization of the Exon I splice acceptor, or inhibit the D1, D2 and D3 variants. This would be reminiscent of the manner in which the alternative splicing factors *sxl* and *tra* regulate their own messages to produce full length functional proteins, and consistent with the restricted expression pattern of full length Nova-1 .



## General Discussion

### The expression pattern of Nova-1.

Morphologically the CNS develops from the spinal cord and five vesicles. Rostral to caudal they are the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. A basic principle of evolution is that the CNS generally evolves in a caudal to rostral manner (cephalization), with the rostral most neurons being evolutionarily the 'youngest' (Johnson 1906). Comparative neuroanatomy also suggests that more dorsal structures are evolutionarily younger than their ventral counterparts (e.g. dorsal telencephalic 'neo'cortex vs. the ventral 'arche'cortex). Similarly a general principle of CNS development is that the CNS develops caudal to rostral ventral to dorsal, and lateral to medial (Treux and Carpenter, 1969.).

Based upon these principles, Nova-1 appears to be a molecular marker for distinct evolutionary and developmental CNS compartments. Nova-1 is expressed in a phylogenetically older, caudal CNS compartment. At the rostral most borders of Nova-1 expression, Nova-1 is restricted to ventral and lateral regions of these compartments. In the telencephalon, Nova is expressed in the basal ganglia (amygdala and striatum, ventral structures), but is not expressed in the dorsal neocortex. In the diencephalon, Nova-1 is expressed in the hypothalamus (ventral), the lateral thalamus and lateral habenula, but is not expressed in the medial habenula or dorsal/medial thalamus. If Nova-1 is a marker of older phylogenetic age, these observations would suggest that the dorsal/medial thalamus is phylogenetically younger than the more rostral, ventral telencephalon. This is contrary to what would be predicted by the principle of cephalization, which would suggest that the more rostral structure are phylogenetically younger.



If the borders of Nova-1 expression represent boundaries of distinct evolutionary/developmental compartments there should other genes which respect these boundaries. Indeed, early in development, the oct-2 transcription factor has a pattern of expression very similar to Nova-1, predominantly in the diencephalon, mesencephalon, rhombencephalon and spinal cord (He et al., 1989). The Dlx-2 homeobox gene is expressed in a developmental compartment, similar to the rostral most domain of Nova-1 expression, composed of the ventral telencephalon and ventral diencephalon (Bulfone et al., 1995). In contrast, the T-br1 transcription factor has a dorsal telencephalic expression pattern complementary to Nova-1 (Bulfone, et al., 1995). Similarly the transcription factor BF-1 is restricted to the olfactory bulb and dorsal telencephalon, while BF-2, a relative of BF-1, is expressed in the ventral diencephalon (Tao and Lai, 1992; Hatini et al., 1994). Thus we conclude that Nova-1 is a marker for an evolutionary and developmental compartment within the CNS.

These observations are generally consistent with a developmental compartments model proposed by Rubenstein and others (Shimamura et al., 1995) who have used recently identified transcription factors to define distinct longitudinal and transverse boundaries of the brain. They have proposed that the CNS develops as a series of longitudinal columns with distinct transverse (neuromeric) borders. Based upon these models, the dorsal thalamus, one of few archecortical brain regions not to express Nova-1, is a distinct molecular compartment. The rostral and ventral borders of Nova-1 in the basal ganglia of the forebrain also perfectly correlate with both the prosomeric and longitudinal compartments postulated.

Nova-1 expressing cells may also be a part of a functional 'motor' compartment. The brain regions which most prominently express Nova-1, include the superior colliculi and pretectum, optic tectum, midbrain and pontine tegmentum, medial midbrain and brainstem reticular formation, cerebellar granule cells and deep cerebellar nuclei. The superior colliculi,





pretectum, and optic tectum serve primarily as reflex centers in control of ocular movements, integrating sensory signals and determining motor output. The midbrain and pontine tegmentum consist of multiple nuclei with varying or unknown functions. The most prominent nuclei of the midbrain tegmentum are the large Red nuclei. The Red nuclei express Nova-1 and are an important part of cerebrospinal and cerebellar-spinal motor circuits. Clinically, lesions of the Red nuclei and midbrain tegmentum result in contralateral motor disturbances part of the 'syndrome of Benedikt.' This syndrome consists of tremor, ataxia, choreiform movements and oculomotor palsy. Pontine nuclei which express Nova-1 include the motor nuclei of the cranial nerves. Nova-1 expression in the lateral sensory nuclei is greatly reduced. The midbrain and brainstem reticular formation consist of smaller scattered nuclei and are predominantly involved in regulation of motor reflexes. Stimulation of the more rostral reticular formation leads to bilateral inhibition of blink reflexes, patellar and foreleg reflexes, and a loss of extensor tone. Caudal regions of the reticular formation also regulate reflex responses, but can augment as well as inhibit these reflexes. Finally, the cerebellum is concerned primarily with mechanisms of coordination, motor activity, equilibrium and muscle tone. Nova-1 is also expressed, although at somewhat lower levels, in the striatum, a telencephalic region involved in regulating motor activity. Lesions of the striatum lead to choreiform movement disorders (Treux and Carpenter, 1969.).

Nova-1 is also expressed in a few regions which are not classically considered motor nervous system, but may have important interactions with and control over parts of the nervous system. Nova-1 is expressed in the lateral geniculate nuclei (LGN) of the thalamus. While the LGN are generally considered sensory structures, they do directly communicate with the superior colliculi and pretectal areas suggesting that they may be important in ocular motor reflexes (Treux and Carpenter, 1969.). Other thalamic nuclei which express Nova-1 include the zona incerta which is considered an



extrapyramidal motor nuclei (Snell), and the lateral habenula. The function of the habenula remains unclear, but based upon afferent and efferent projections, the medial habenula appear to be part of a sensory pathway integrally linked to the medial thalamic nuclei, while the lateral habenula may be motor nuclei, integrally linked to the red nuclei and tegmentum. Nova-1 is also expressed in the hypothalamus a critical regulator of the autonomic motor nervous system. Finally, the amygdala expresses Nova-1. The potential for motor association of the amygdala is not apparent. The amygdala receives multiple inputs from olfactory structures which do not express Nova-1, and is also an important regulator of emotional and sexual behavior.

Figure 33. shows a diagrammatic representation of the CNS regions which contribute to the motor system of lower vertebrates and mammals (taken from Sarnat and Netsky, *Evolution of the nervous system*; Treux and Carpenter, 1969.). Nova-1 is expressed in all of the regions which contribute to motor control of a lower vertebrate and all the mammalian motor/control regions except the dorsal thalamus and motor cortex. While some of these structures are considered primarily sensory structures, (e.g. the vestibular nuclei) they are critical regulators of the motor nervous system. The dorsal thalamus is a cortico-sensory relay area important for proprioception (as well as tactile sense, and pain). The highly evolved motor cortex is primarily concerned with fine dexterous movements, whereas the archecortical motor system controls the more crude axial skeletal and appendicular muscle movements. This is consistent with the motor neurologic symptoms in POMA which are primarily oculomotor, truncal, and appendicular, yet spares fine movements of the hands.

The islet/Lim family of transcription factors have been defined as motor neuron markers. While no individual family member marks all neurons of the motor system, motor neuron identity in the spinal cord can be predicted based upon combinatorial expression of these transcription factors (Tsuchida et

Figure 33. Regions of the CNS involved in motor control.

Diagrammatic representation of the regions of the CNS, and their interconnections, which are involved in regulation of motor function in lower vertebrates and mammals. Taken from Sarnat, H.B. and Netsky, M.G. Evolution of the nervous system. 1981. New York. Oxford University Press.

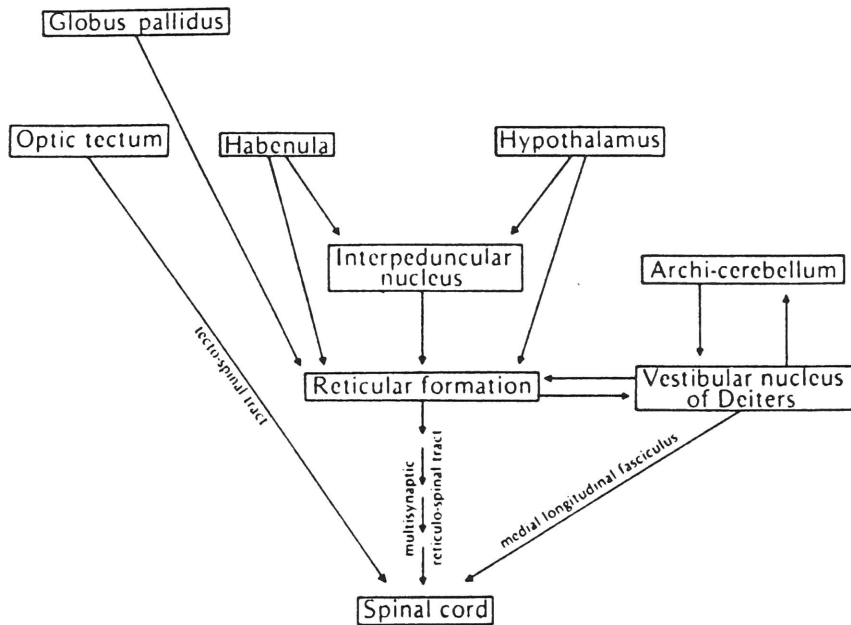


Figure 3-1 Diagram of the suprasegmental motor system of a lower vertebrate.

#### PLAN OF VERTEBRATE NERVOUS SYSTEM

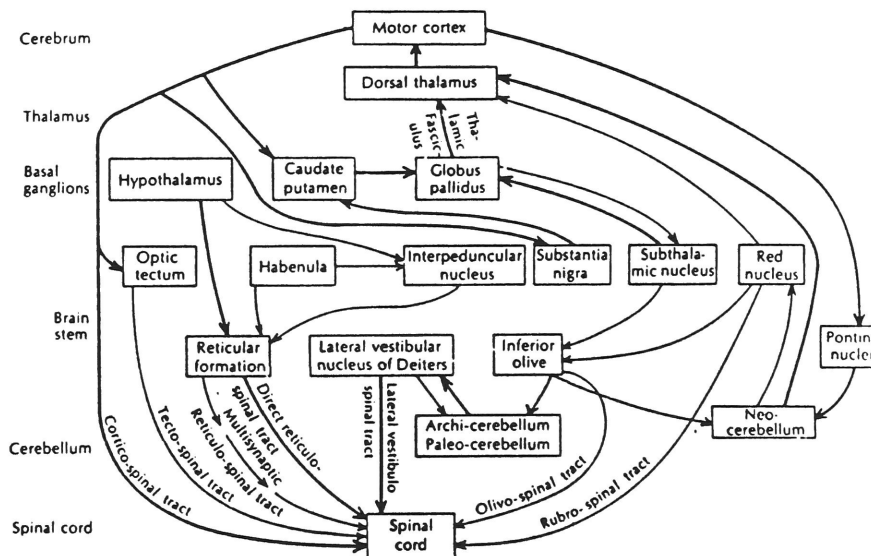


Figure 3-2 Diagram of the suprasegmental motor system of a mammal. The basic system of the lower vertebrates has been retained and supplemented, not replaced. The most important pathways are indicated by heavy lines.



al., 1994). The islet-1 and 2 proteins mark the ventral horn and intermediolateral motor neurons of the spinal cord, as well as various brainstem nuclei, the dorsal pons, hypothalamus, septal nuclei, and ventral telencephalon (Thor et al., 1991; Ericson et al., 1992). However, the islet proteins are not expressed in other neuroanatomical structures critical for motor function such as the cerebellum, or spinal interneurons. Lim1 primarily marks commissural neurons of the spinal cord, cerebellum, brainstem, and lateral diencephalon (Barnes et al., 1994). Similarly, lim3 and gsh-4 (a lim family member) mark specific regions involved in motor control (Li et al., 1994; Zhadanov et al., 1995). While no one of these proteins can account for the expression pattern of Nova-1, the sum of their respective expression domains is similar to that for Nova-1, suggesting that Nova-1 may be a marker for motor function.

Based upon the co-expression of Nova-1 and various lim proteins in different regions of the CNS, it is possible that Nova-1 transcription may be regulated by a lim responsive element. Alternatively, based upon the similar pattern of Nova-1 and oct-2 expression, at least early in development, it is possible that oct-2 may regulate Nova-1 gene expression. However, since it is possible that Nova-1 is transcribed in all neurons of the CNS, but restricted to the sub-cortical CNS by regulated alternative splicing (Exon I vs. D1, D2 and D3 splice variants), it seems more likely that Nova-1 transcriptional activity will be regulated by a pan-CNS transcriptional regulator. One potential pan-neuronal transcription factor is the AP-2 protein. Knockout experiments have demonstrated that AP-2 is essential for neurogenesis, with AP-2 null mice exhibiting severe neural tube defects (Zhang et al., 1996). AP-2 promoter elements have been identified in the light neurofilament and neuron specific enolase pan-neuronal genes (Sakimura et al., 1995).





### Function of Nova-1 in brain.

Clearly Nova-1 has the capacity to bind RNA *in vitro* and *in vivo*, suggesting that it functions as an RBP *in vivo*. RBP's play many important roles in posttranscriptional regulation of gene expression, ranging from pre-mRNA splicing and editing, to transport, translational control, localization, and stability (Pinol-Roma and Dreyfuss, 1992b; Dreyfuss et al., 1993; Burd and Dreyfuss, 1994; McCarthy and Kollmus, 1995). There are two potential categories of RBPs, those which co-exist with their cognate ligands such as the snRNP proteins and ribosomal proteins, and those which interact transiently with their RNA ligands. Because of the transient nature of the interaction with their RNA ligands, proteins in the second category of RBP can act upon numerous ligands. The second category of RBPs can be further sub-divided into two groups of RBPs. One group is composed primarily of the hnRNP proteins which are thought to bind in a non-specific manner to nearly all RNAs acting as RNA 'histones' or 'chaperones' (Herschlag et al., 1994). The second group is composed of proteins which act in a sequence specific manner performing discrete functions. Proteins belonging to this group of RBPs include *Drosophila* sex determination splicing factor *sxl* and the viral Rev protein which are involved in regulating alternative splicing and RNA export, the iron regulatory protein which regulates mRNA translation and stability, and others (Klausner et al., 1993; Valcarcel et al., 1993; Fischer et al., 1994; Gontarek and Derse, 1996). We have shown that Nova-1 is a sequence specific RNA binding protein. Moreover we have identified two specific *in vivo* Nova-1 ligands. Therefore Nova-1 falls into the latter grouping of RBPs of transiently interacting, site specific RBPs and indicates that Nova-1 will perform a discrete, site specific, function.

The restricted expression of full length Nova-1 not only to the CNS, but also to a subset of developing and adult neurons makes it unique among RBPs and n-RBPs. The localized expression of Nova-1 during development of the CNS suggests that n-RBP's perform specific roles in the development of



discrete groups of neurons. Similar to compartmentalized transcription factors (Shimamura, et al., 1995), developmentally compartmentalized RBPs may play a role in regulating gene expression essential for neuronal determination, differentiation, or synaptogenesis. In the adult, n-RBP regulation of gene expression may be essential for neuronal function and synaptic plasticity.

Based upon the location of the Nova-1 binding sites within introns neighboring alternatively spliced exons, and the preliminary evidence that Nova-1 can act as a splice enhancer, it seems likely that Nova-1 will act as an alternative splicing factor. This is consistent with the proposal that neuron specific factors may be necessary to regulate neuron specific splicing (Amara et al., 1982; Rosenfeld et al., 1983; Stamm et al., 1992). Numerous hnRNAs are differentially spliced, in neural vs. non-neural tissues, in different brain regions, at different developmental stages, and with varying neuronal activity. One class of RNAs, regulation of which will clearly be critical to neuronal development and function, encodes the neurotransmitter receptors. Electrical activity triggered by neurotransmission is not only essential for function of a mature neuron, but in development has been shown to be critical for neuronal maintenance, synaptogenesis and pattern formation. Alternative splicing of neurotransmitter receptors can drastically affect receptor characteristics including ligand affinity/preference (Schmieden et al., 1993; Garcia-Guzman et al., 1995), localization (Ehlers et al., 1995), desensitization (glurB), phosphorylation status (Wafford and Whiting, 1992; Tingley et al., 1993; Raymond et al., 1994), and G protein coupling (Montmayeur et al., 1993), all of which affect receptor signaling. In some cases alternative splicing of receptors can completely inactivate signaling (Sokoloff et al., 1992). The timing of Nova-1 expression, and the identification a neurotransmitter receptor RNA as a target for Nova-1, indicate that Nova-1 plays a role in regulating neurotransmitter receptor expression and therefore may regulate



neurotransmission and the important developmental and functional consequences of neural activity.

While it seems most likely that Nova-1 will be involved in regulating alternative splicing, it is possible Nova-1 may perform other functions in addition. Many RBPs perform dual functions. The iron response element binding protein (IRBP) is also the TCA cycle enzyme aconitase. Many viral coat proteins including R16 and HIV coat proteins also act as RBPs. The hnRNP A1 protein has been directly implicated in regulating alternative splicing; in tissue culture hnRNP A1 influences splice site choice promoting utilization of distal splice acceptors (Mayeda and Krainer, 1992; Cáceres et al., 1994), and is capable of functionally replacing the U1 snRNP in splicing extracts (Crispino et al., 1994; Crispino et al., 1996). However the hnRNP A1 protein has also been implicated in RNA transport and RNA stability. While predominantly a nuclear protein, hnRNP A1 is one of several RBPs known to shuttle between cytoplasm and nucleus (Pinol-Roma and Dreyfuss, 1992a; Michael et al., 1995 ). Electron microscopy demonstrates that an hnRNP A1 like protein of *C. tentans*, rapidly binds to newly synthesized Balbiani ring pre-mRNA, and remains in contact with the RNA as it is targeted to the nuclear pore, passes through the pore and remains on the RNA as it is translated by ribosomes (Visa et al., 1996). Studies of RNA stability suggest that the hnRNP A1 protein may specifically cross link to AUUUA elements in the 3'UTR of cytokine mRNAs and regulate mRNA stability (Hamilton et al., 1993; Henics et al., 1994).

Based upon the observations of hnRNP A1, Nova-1 may potentially perform multiple functions. Nova proteins are present in both the cell nucleus and cytoplasm. It remains uncertain whether this is due to protein shuttling or to the presence of antigenically related family members which reside in different cellular compartments. However, immunofluorescence analysis of Nova-1 transfected cells suggests that Nova-1 protein is predominantly nuclear, but



can also be detected in the cytoplasm. This is similar to that seen for shuttling hnRNP A1 protein, suggesting that Nova-1 protein may also cycle between the nucleus and cytoplasm. Chimeric cell fusion experiments and deletional analysis of the Nova-1 protein will be necessary to definitively determine whether or not the Nova-1 protein shuttles between the nucleus and the cytoplasm or is strictly nuclear.

A role for Nova protein in the cytoplasm is suggested by preliminary evidence that Nova:RNA co-immunoprecipitates may contain Nova-1 mRNA in addition to Nova-1 hnRNA. This observation taken together with the identification of potential Nova-1 binding sites in the Nova-1 3' UTR indicates that Nova-1 may regulate Nova-1 mRNA translation and/or stability. Interestingly, one potential Nova-1 binding site in the 3' UTR of Nova-1 mRNA lies adjacent to two potential RNA degradation signals, suggesting that Nova-1 binding to this site may prevent these degradation signals from being identified and lead to increased mRNA stability. Similarly, preliminary evidence suggests that Nova proteins may co-localize with the glyR $\alpha$ 2A mRNA in the cytoplasm and dendrites of neurons in the rat spinal cord (A. Triller personal communication). However, the only documented, or potential Nova-1 (or Nova-2) binding sites identified in the glyR $\alpha$ 2A thus far are within intronic sequences. It is possible that other Nova proteins bind to unidentified signals in the glyR $\alpha$ 2A mRNA 5' or 3' UTR to regulate RNA localization, translation and/or stability. This would be interesting in that it would suggest serial functions of Nova family members in regulating expression of the glyR $\alpha$ 2A. Alternatively, it is possible that the co-localization of Nova-1 and the glyR $\alpha$ 2A RNA in the cytoplasm may be Nova-1 binding to the defined intronic binding site retained in the glyR $\alpha$ 2A. One function for RNA export RBPs may be to allow intron containing RNAs to exit the nucleus. Several viral RBPs function to export intron containing viral RNAs to the cytoplasm (Fischer, et al., 1994; Qian et al., 1994). Thus it is





possible that Nova-1 might act to allow export of the unspliced glyR $\alpha$ 2A and localize this RNA to the dendrites. Localized splicing and translation of neurotransmitter RNA would be a rapid mechanism for regulating synaptic plasticity. Interestingly, yeast two-hybrid analysis with the Nova-2 protein identified the hnRNP L protein as a potential interacting protein. The hnRNP L protein has been functionally implicated in export of non-spliced RNAs (Liu and Mertz, 1995).

Finally, one last potential function for Nova-1 may be as a transcription factor. Of the KH proteins identified, Nova-1 is most similar to hnRNP K in not only the homology of the KH domains, but also in the modular structure of the proteins (see Fig. 3C). hnRNP K binds to ssDNA avidly *in vitro*, and has been implicated in binding to a ssDNA element in the c-myc promoter and acting as an enhancer of transcription (see chapter I). Nova-1 also binds avidly to ssDNA, even in extremely high salt (see figure 22). In addition the immunofluorescence pattern of Nova-1 in the nucleus, a fine speckled pattern (data not shown), is more reminiscent of a transcription factor than that seen for some alternative splicing proteins (e.g. SC35). Interestingly, numerous proteins have been identified which act as both transcription factors and RNA binding proteins including TFIIIA, bcd, and p53 (Theunissen, et al., 1992). These observations suggest that Nova-1, in addition to or in conjunction with its RNA binding activity, may function as a regulator of transcription.

#### The Role of Nova-1 in PND tumors.

While Nova protein is normally restricted to neurons, expression has also been detected in paraneoplastic tumors. The selected and maintained expression of PND antigens by tumors, in the presence of a strong anti-tumor immune response suggests that either PND antigens are either co-expressed with a transcription factor which is essential for the tumor, or that the PND



antigens themselves perform a necessary function for tumors. Nothing is known about the promoter elements of Nova-1. However, a particularly interesting candidate transcriptional regulator of Nova-1 in both neurons and breast cancer (the predominant tumor type associated with POMA) is AP-2. AP-2 is important for neurogenesis (Zhang, et al., 1996) and regulates the transcription of numerous neuronal genes (Beaudet et al., 1992). AP-2 activity is also associated with breast cancer, shown to activate the c-erb-2/HER2 protooncogene which is upregulated in breast carcinomas (Bosher et al., 1995). A second interesting candidate is oct-2, which is expressed in a similar pattern to Nova-1 in the developing CNS (He, et al., 1989). While oct-2 has not been specifically linked to breast cancers, it has been associated with Hodgkin's disease and glioblastomas (Schreiber et al., 1994; Bargou et al., 1996). Also, oct-2 normally regulates the expression of IgG, and MHC on B cells (Corcoran et al., 1993; Natkunam et al., 1994; Radomska et al., 1994), and thus if ectopically expressed in a PND tumor oct-2 may lead to expression of immune modulators, leading to the strong anti-tumor immune response.

Regulation of alternative splicing is one way that Nova-1 itself may be necessary for PND tumors. Alternative splicing has been documented to drastically alter function of numerous proteins. Splice variants of some proteins have been directly implicated in various stages of tumorigenesis. Transcription factors can be switched from enhancers to inhibitors with splice switches. For example STAT1 can be alternatively spliced to eliminate its activation domain, as a result STAT1 function switches from an activator to an inhibitor of transcription (Bandyopadhyay et al., 1995). Both STAT1 and STAT 3 family members are upregulated in some breast cancers (Watson and Miller, 1995). It is possible that Nova-1 may regulate the expression of various tumorigenic factors. One interesting possibility is that Nova-1 may inactivate tumor suppressor genes; it has been suggested that the Wilms tumor gene WT1, originally identified as a tumor suppressor transcription factor, is switched from a transcription factor to a splicing factor with the



exclusion/inclusion of a single exon (Larsson et al., 1995). Loss of function of the tumor suppressor genes BRCA-1, BRCA-2 and ATM has been correlated with development of breast cancer (Eccles and Phipps, 1993; Schutte et al., 1995; Ormiston, 1996), however the role of splicing of these RNAs remains unclear. Another possible target for Nova-1 activity in breast cancer cells may be the immune system co-stimulators. For example CD44 is expressed in both breast and small cell lung cancers and has 10 different splice variants. The CD44 v6 splice variant is correlated with a metastatic phenotype, while the CD44 v3 and v5 variants are associated with a more indolent course (similar to that seen for POMA patients) (Kaufmann et al., 1995). Nova-1 may also regulate the activity of various oncogenes. The RON gene encodes a tyrosine kinase receptor which functions to control cell dissociation, motility, and invasion of extracellular matrices. A splice variant of RON,  $\Delta$ RON, has an alternative 49 amino acid encoding exon excluded leading to constitutive receptor activation and the acquisition of an invasive/metastatic phenotype.

Finally, another class of genes which is regulated by alternative splicing is the programmed cell death (PCD) genes. Both PCD regulators and effector genes are regulated by alternative splicing. Different splice forms have opposite functions, pro-death versus anti-death. The PCD regulators Bclx, Bax, and Bad, each have long and short splice variants with opposing function (Boise et al., 1993; Tanaka et al., 1993; Bargou et al., 1995; Minn et al., 1996; Xerri et al., 1996). Similarly, the Fas/Apo1 receptor and Iceh protease, cell death effector proteins, have both long and short splice forms with opposing functions (Wang et al., 1994; Cifone et al., 1995; Fernandes-Alnemri et al., 1995; Papoff et al., 1996). Nova-1 splice regulation of cell death genes would be consistent with the maintenance of Nova expression in tumors in spite of the anti-tumor immune response; down-regulation of apoptosis has been shown to contribute to tumorigenesis *in vivo*. Unfortunately, very little is known about the factors or sequences involved in the regulation of splicing of these genes. Interestingly, many of the cell death genes are expressed in brain



exhibiting different splice forms. Nova-1 may regulate splicing of these PCD genes in neurons and then when expressed in tumors may switch splicing of the PCD genes to a neural, anti-death, form.

#### Nova-1's Role in disease.

The sub-cortical expression of Nova-1 in developing and adult brain correlates with the restricted motor symptoms of POMA patients. This is an indication that this epitope plays some role in the generation of the motor disorder, and gives a first insight into why the disease may be so specific in its symptomatology. POMA patients suffer from opsoclonus, a chaotic eye movement disorder attributable to a lack of inhibition of burst neurons in the brainstem, myoclonus, a disorder attributable to a failure of inhibitory control over spinal motor neurons, and truncal ataxia, referable to cerebellar dysfunction; there are no apparent cortical or sensory deficits (Anderson et al., 1988; Darnell, 1994). Nova-1 is expressed at very high levels in the superior colliculus, tegmentum, and nucleus of cranial nerve III (oculomotor), all areas involved in control of ocular movements. Similarly Nova-1 is also expressed at very high levels in deep cerebellar nuclei, pontine nuclei and the spinal cord, all areas involved in control of motor function and potentially targeted in disease. Other regions of the CNS, including the neocortex which is involved in higher integrated functions, the medial thalamus which is an important sensory relay region, and the inferior colliculus which is involved in audition, do not express Nova-1. However, Nova-1 expression is not completely restricted to motor tissues, for example the lateral geniculate nucleus of the lateral thalamus, an important relay area of visual information also expresses Nova-1.

The expression pattern of Nova-1 also correlates with the pathologic findings in some POMA patients. There are few pathologic studies of POMA, attributable to the small number of documented antibody positive POMA patients (23- Dropcho, 1994), and the indolent course of PND tumors. Unlike





the other PNDs there is usually minimal pathology in POMA brains, restricted to mild perivascular lymphocytic cuffing (primarily B cells; Posner and Furneaux, 1990). However, of the patients with reported pathology the regions predominantly affected in the respective patients included, the amygdala, brainstem and pontine nuclei, the spinal cord, the cerebellum, the olivary nucleus and pontine nuclei, and the mesencephalon and pontine tegmentum. (Anderson, et al., 1988; Luque et al., 1991; Hormigo et al., 1994) These are all regions which express Nova-1 at high levels. In one patient assessed there was particularly high levels of interstitial NK cells in the pons and mesencephalon (contrasted with the absence of NK cells in the visual cortex of the same patient and the absence of NK cells in Hu patients at autopsy) as well as high concentration of POMA antibodies in these areas (relative to serum). These observations lead the authors to suggest that antibody dependent cell cytotoxicity mediated by NK cells may play a role in POMA pathogenesis (Hormigo, et al., 1994).

While the restricted expression of Nova-1 correlates with POMA symptomatology, additional Nova antigens (Nova-2 and possibly others) are expressed more broadly than symptoms would predict. This suggests some greater level of complexity to the disorder. Affected neurons may be particularly sensitive to immune insult. For example, Purkinje and hippocampal neurons are the first to be affected by ischemic insult, while large motor neurons are primarily affected in amyotrophic lateral sclerosis (ALS) which is due to a defect in the ubiquitously expressed superoxide dismutase (Andersen et al., 1995; Orrell et al., 1995). Alternatively, all neurons may be equally targeted by the immune response, but loss of function of Nova proteins in some neurons may have no obvious phenotype.

As a disease related protein Nova-1 shares many similarities with FMR-1. Both are RBPs associated with neurologic disease. Both have KH type RBDs and mutation of the respective KH domains abrogates RNA binding function



*in vitro* (see Figs. 19, 20, 26 and Siomi et al., 1994). An *in vivo* point mutation of the second KH domain of FMR-1 is associated with severe mental retardation leading to the suggestion that interference of specific RNA binding may lead to the neurologic disease (Gibson et al., 1993; Siomi, et al., 1994). Since affinity purified POMA antibodies inhibit the high affinity *in vitro* RNA binding activity of Nova-1 (Fig. 30), we propose that interference with the RNA binding activity of Nova-1 may lead to neurologic disease, similar to the proposed pathogenesis of FMR-1, but by an immune-mediated rather than a genetic mechanism.

The identification of a glycine receptor RNA as an *in vivo* ligand is particularly relevant to Nova-1's role in neurologic disease. The neurologic symptoms of opsoclonus and myoclonus are thought to be due to a loss of inhibition of brainstem and spinal cord motor pathways. Glycine is the most abundant inhibitory neurotransmitter in the spinal cord and brainstem. Moreover mutations of the glycine receptor gene family lead to a myoclonic phenotype in both humans and mice. The human myoclonic disease hereditary hyperekplexia is due to point mutations in the glycine receptor alpha-1 gene (Shiang et al., 1993). Similarly the myoclonic *spasmodic* mouse mutant is due mutations in the glycine receptor alpha-1 (Ryan et al., 1994; Saul et al., 1994). Finally, a splicing deficit due to a transposable element insertion in an exon splice acceptor of the glycine receptor  $\beta$  gene, causes the myoclonic phenotype of the *spastic* mouse mutant; transgenic expression of a wild type glycine receptor B gene can rescue the myoclonic phenotype of the *spastic* mouse (Becker et al., 1992; Mulhardt et al., 1994; Hartenstein et al., 1996). Since altered expression of glycine receptor genes can lead to a myoclonic phenotype, the potential aberrant expression of the glyR $\alpha$ 2A gene due to disruption of Nova-1 regulation of alternative splicing of exons 3A and 3B of the glyR $\alpha$ 2A may cause the myoclonic phenotype in POMA. The glyR $\alpha$ 2A alternatively spliced exons 3A and 3B encode highly homologous 22 amino acid peptide sequences which are part of the extracellular domain of the



glycine receptor (Kuhse et al., 1991). This region may therefore play a role in ligand binding affinity. Single amino acid substitutions in the extracellular domain of the glycine receptor can drastically alter the strychnine binding pharmacology of the glyR $\alpha$ 2A (Schmieden, et al., 1993).

Assuming that antibody may be taken up by some neurons (see below), and that Nova-1 mediates the alternative splicing of the glyR $\alpha$ 2A, these observations suggests a model for the development of opsoclonus and/or myoclonus in POMA patients (Fig. 34). In this model antibody binding to the third KH domain of Nova-1 prevents Nova-1 from interacting with glyR $\alpha$ 2A hnRNA. The absence of Nova-1 binding to glyR $\alpha$ 2A results in an abnormal pattern of splicing of the glyR $\alpha$ 2A, or even RNA degradation in a particular subset of neurons. The resulting glyR $\alpha$ 2A protein translated from aberrantly spliced RNA would have altered functional capacity, may lead to loss of glycine mediated inhibitory pathways, and result in the phenotype observed in POMA. Abnormal regulation of the glyR $\alpha$ 2A would be further amplified if loss of Nova-1 function also leads to abnormal regulation of Nova-1 pre-mRNA splicing and the production of truncated inactive, or dominant negative Nova-1 protein.

Interestingly, treating patients with hereditary hyperekplexia with GABA receptor agonists leads to resolution of symptoms; loss of glycine mediated inhibition can be overcome by utilizing an alternative (GABA) inhibitory pathways (). Consistent with the idea that the myoclonic phenotype in POMA is due to a loss of inhibition due to improper glycine signaling, treatment of POMA patients with GABA agonists can also relieve symptoms in some patients. While it is possible that remission of symptoms could have been spontaneous, the reappearance of symptoms with the withdrawal and disappearance of symptoms with return of the drug suggests a direct cause and effect. (Dropcho et al., 1993; Casado et al., 1994).



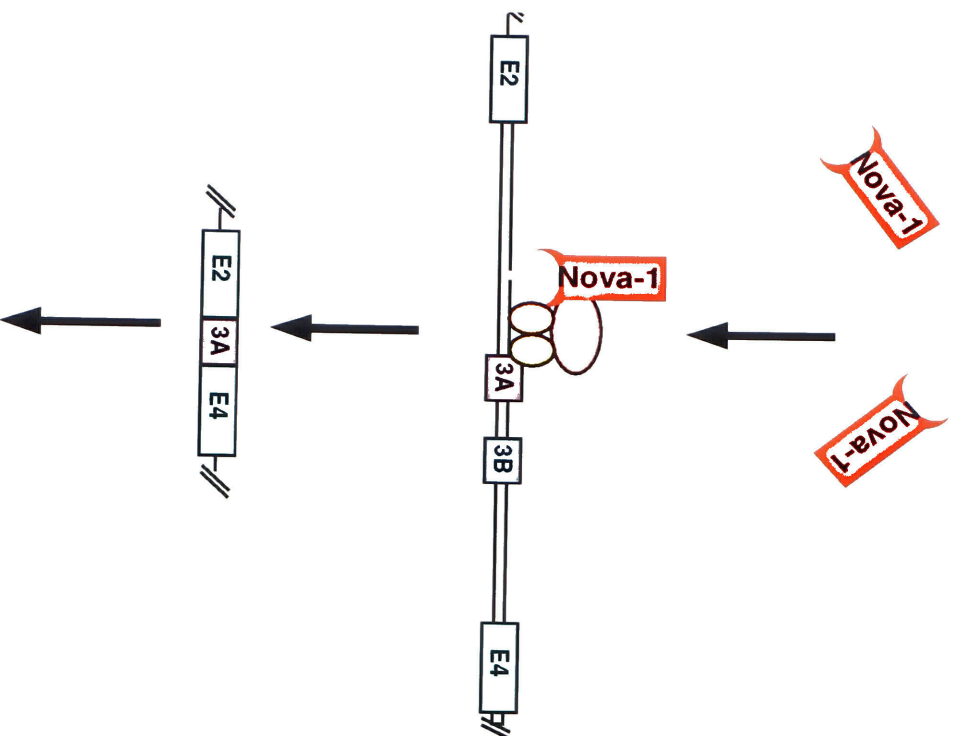
Antibody-mediated disruption of protein function would provide a mechanism compatible with the observation that neurologic symptoms are reversible in some POMA patients. In addition to spontaneous and GABA agonist induced remissions, some patients symptoms are helped by immunosuppressive steroid therapy. The disappearance of symptoms correlated with decreasing POMA antibody titers. When steroid therapy was withdrawn, antibody titers rose and symptoms returned, and when therapy was reinitiated symptoms and titers decreased (Casado et. al. 1994; Dropcho, et al., 1993). More evidence for the role of antibodies in disease pathogenesis comes from the documentation of a higher specific activity of antibodies in the CSF than in the serum of PND patients (Furieux et al., 1990), suggesting that B cells invade the nervous system and actively synthesize antibody. One difficulty with the suggestion that antibody mediates neurologic disease is the fact that Nova-1 is intracellular, being present in both the cytoplasm and nucleus. Antibody mediated inhibition of Nova-1 function would minimally require antibody penetration to the cell cytoplasm. There is some experimental evidence to suggest that paraneoplastic antibodies may accumulate within neurons following injection of IgG into the CSF *in vivo* (Graus et al., 1991) or following incubation of IgG with neurons *in vitro* (Greenlee et al., 1993). Also, Purkinje neurons *in vivo* have been shown to specifically take up large molecules such as IgG (Fabian and Petroff, 1987). In addition some extracellular neurotoxins such as tetanus (M.W. ~150 Kda) are capable of being translocated into the cell cytoplasm (Matteoli et. al., 1996). These observations suggest that at least some neurons may have the ability to internalize pathogenic antibodies, where they may act to inhibit the function of specific target proteins. The susceptibility of particular neurons to antibody penetration may contribute to the specificity of symptoms seen in the PNDs.

Figure 34. Model of the pathogenesis of POMA neurologic symptoms.

Normally, Nova-1 may function to enhance the utilization of the glyR $\alpha$ 2A alternative exons 3A and 3B, with predominant utilization of Exon 3A. In POMA patients, anti-Nova antibodies may prevent Nova-1 from binding to RNA. In the absence of Nova-1 binding to RNA, splicing of the glyR $\alpha$ 2A in these neurons would be altered such that either (i) exon 3B is utilized instead of exon 3A, (ii) in the absence of the enhancer function of Nova-1 neither exon 3A or 3B is utilized and exon 2 is spliced to exon 4, or (iii) intronic sequences are retained in the glyR $\alpha$ 2A RNA resulting in nuclear retention/degradation of the RNA. Therefore in the absence of Nova-1 function glyR $\alpha$ 2A protein, (i) may be expressed with abnormal ratio of 3A:3B splice containing forms, (ii) without a critical extracellular domain and therefore may have altered ligand binding affinity or even act in a dominant negative fashion, (iii) may be absent in these cells. Either of these possibilities could result in abnormal glycine receptor signaling and neurologic disease. A representation of a portion of the glyR $\alpha$ 2A hnRNA, exons are represented as large boxes while the intron is a thinner gray line. The Nova-1 binding site is represented as the grey stem-loop. Nova-1 is represented in red, POMA antibodies are in black, exons are color coded as indicated.

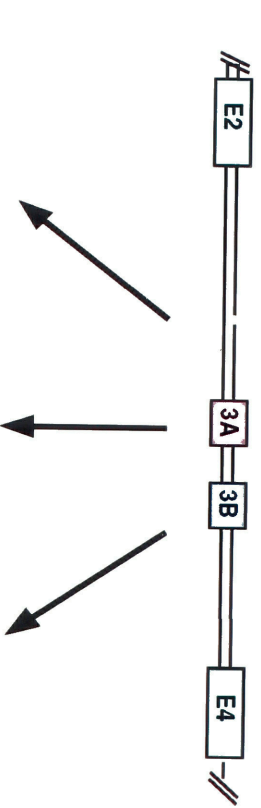


## Normal



## POMA Patients

α Nova Ab



Aberrant Receptor Expression

Opsoclonus / Myoclonus



In sum, we have identified a gene, Nova-1, which encodes a target antigen of the paraneoplastic neurologic disease paraneoplastic opsoclonus myoclonus ataxia. Nova-1 protein and RNA are restricted to the subcortical CNS throughout development and adulthood, in a manner which correlates with the neurologic symptoms of POMA. Nova-1 encodes a KH type RNA binding protein which is capable of binding RNA both *in vitro* and *in vivo*. Using RNA selection in conjunction with protein:RNA immunoprecipitation we have identified two sequence specific *in vivo* RNA ligands for Nova-1. This work establishes a methodology for the identification of RNA ligands for other Nova proteins and RBPs in general. Moreover, the ability to co-immunoprecipitate Nova-1 protein and RNA may lead to the identification of numerous RNA ligands for Nova-1. RNA co-immunoprecipitation followed by RNA selection of immunoprecipitated RNAs would be one way to identify specific Nova-1 RNA ligands from embryonic brain, adult brain, or tumors.

The identification of Nova-1 binding sites adjacent to alternatively spliced exons suggests a role for Nova-1 in the regulation of alternative splicing. Work presented here has helped to establish an assay for the functional analysis of Nova-1 in regulating splicing of the glyR $\alpha$ 2A and for future analysis of the role of Nova-1 in regulating splicing of Nova-1 pre-mRNA.

The identification of an inhibitory glycine receptor RNA as a target ligand for Nova-1, and the demonstration that Nova-1 can affect splicing of this RNA in tissue culture, is particularly important considering the neurologic symptoms of POMA are attributable to a loss of motor inhibition. Glycine is an abundant inhibitory neurotransmitter and mutations of members of the glycine receptor gene family, in both humans and mice, result in myoclonic symptoms similar to those seen in POMA. Moreover, the demonstration that



paraneoplastic antibodies can inhibit Nova-1 binding to the glyR $\alpha$ 2A *in vitro* suggests a model in which the disruption of Nova-1 regulation of the glyR $\alpha$ 2A pre-mRNA may lead to the neurologic phenotype of POMA.



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