AD_____

AWARD NUMBER DAMD17-97-1-7097

TITLE: Functional Analysis of Nova Proteins: Gene Regulation and Breast Tumor Immunity

PRINCIPAL INVESTIGATOR: Kirk B. Jensen, Ph.D.

CONTRACTING ORGANIZATION: The Rockefeller University
New York, New York 10021

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE

Functional Analysis of Nova Proteins: Gene Regulation and Breast Tumor Immunity

5. FUNDING NUMBERS

DAMD17-97-1-7097

6. AUTHOR(S)

Jensen, Kirk B., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Rockefeller University
New York, New York 10021

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research And Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The investigation and molecular characterization of the paraneoplastic neurological disorders (PNDs) has provided the means to explore the elements of effective tumor immunity. The PNDs are autoimmune diseases which appear to develop when tumor express proteins that are normally expressed only in neurons.

One of the best studied PNDs, paraneoplastic opsoclonus-myoclonus-ataxia (POMA), is a motor disorder, and is most frequently associated with occult breast tumors. Antisera from POMA patients identifies a 55 kDa RNA-binding protein in both tumor and normal brain specimens, and has been used to expression clone the gene responsible for this antigen, Nova.

The effective anti-tumor immunity seen in breast cancer patients with tumors that express the Nova warrants further investigation. It is likely that Nova plays a role in RNA processing, both in neurons and in tumors. The specificity of the ectopic expression of Nova in breast tumors suggests that the study of Nova's *in vivo* function is directly relevant to a further understanding of breast cancer biology.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer , Nova, paraneoplastic neurological disorders, RNA binding protein, anti-tumor immunity, gene regulation	22
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

KS In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

KS In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

KS In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

KS In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Kirk B. Jones

PI - Signature Date

Table of Contents

Introduction

The detailed understanding of how the immune system participates, or fails to participate, in the recognition and effective destruction of tumors has long been a subject of intense research. The investigation and molecular characterization of a rare group of neurological diseases, the paraneoplastic neurological disorders (PNDs), has provided the means to explore the elements of effective tumor immunity[1]. The PNDs are autoimmune diseases which appear to develop when tumor express proteins that are normally expressed only in neurons. The immune response to these "onconeural antigens" is robust, leading to high titers of antibodies reactive with apparently identical antigens in both tumor and normal brain samples. Furthermore, the immune response, which is believed to be responsible for the severe neuronal degeneration seen in the PNDs, is highly correlated with effective tumor suppression; patients with PND are typically unaware of underlying malignancies, and some PND patients have shown documented tumor elimination.

One of the best studied PNDs, paraneoplastic opsoclonus-myoclonus-ataxia (POMA), is a motor disorder affecting a subset of neurons in the brainstem, spinal cord and cerebellum, and is most frequently associated with occult breast tumors. Antisera from POMA patients identifies a 55 kDa protein in both tumor and normal brain specimens, and high titer antibody from a POMA patient with breast cancer has been used to expression clone the gene responsible for this antigen[2]. The gene, Nova-1, and a second recently cloned gene, Nova-2, both encode proteins which are highly homologous to a class of RNA-binding proteins which include FMR-1 (the fragile-X gene), PSI from *Drosophila*, and MER-1 from yeast; both PSI and MER-1 have been implicated in the regulation of alternative splicing. The Nova-1 gene can be alternatively spliced itself, and suggests that Nova's behavior is complex. Strikingly, all antisera samples from POMA patients tested recognize the same region of the Nova-1 protein, the third RNA binding domain, and affinity purified antibody completely disrupts binding of Nova-1 to RNA.

The effective anti-tumor immunity seen in breast cancer patients with tumors that express the Nova protein warrants further investigation. It is likely that the Nova proteins play a role in RNA processing, both in neurons and in tumors. The specificity of the ectopic expression of Nova in breast tumors suggests that the study of Nova's *in vivo* function is directly relevant to a further understanding of breast cancer biology. Moreover, the tumor suppression seen in patients with high antibody titers to Nova, and the demonstration that these antibodies block Nova binding to RNA suggest that tumor suppression and neuronal degradation may be directly linked to abrogation of Nova function.

This proposal aims to answer several fundamental questions about the function of Nova proteins in breast tumors. The Technical Objectives for this study are:

1. Identification of the *in vivo* form of Nova expressed in breast tumors
2. Identification of Nova RNA ligands by *in vitro* RNA selection
3. Identification of Nova RNA sequences *in vivo*
4. Characterization of Nova RNA ligands identified by selection
5. Functional analysis of candidate RNA ligands

The first year of this three-year award has seen substantial progress towards all five of the Objectives.

Body

I. Identification of breast tumor variants of Nova-1

Characterization of the Nova-1 protein has led to the identification of several alternatively spliced forms of the Nova-1 transcript. In one striking example, a small cell lung cancer form of the Nova mRNA contains a 72 base exon, termed exon H, which codes for a serine- and proline-rich sequence, and suggests the exon may contain important phosphorylation sites; this and other possible regulatory elements will likely have a profound effect on Nova function. We had proposed screening a collection of anonymous breast tumor specimens from POMA patients (previously established in this laboratory) using Nova-1 and Nova-2 primers and RT-PCR to look for alternatively spliced forms of Nova. Recently, our laboratory completed the genomic mapping of the Nova-1 gene (Zhong and Darnell, unpublised data). The exon/intron structure of the gene has been determined, giving us precise details on all possible forms of the Nova-1 gene product. The Nova-1 gene does indeed contain a single alternatively spliced exon, exon H, which codes for a 24 amino acid region immediately upstream of the second RNA binding domain. We believe that all other exons are constitutively spliced. All of the POMA patient antisera tested in our laboratory is reactive with an epitope in the third RNA binding domain of Nova-1, which lies in the terminal exon of the gene. Thus, we believe that full length forms of Nova-1 are expressed in breast tumors of POMA patients.

Accordingly, we have constructed Nova-1 fusion protein vectors that express full-length Nova-1 including exon H (see Figure 1), and we are currently cloning Nova-1 fusion protein lacking exon H. The work described below summarized the biochemical data collected using the exon H form of Nova-1. Additionally, we have begun to investigate the behavior of the individual RNA binding domains of Nova-1, starting with the third domain (KH3). This RNA binding domain is the antigenic epitope in all studied POMA patients, and discovery of its *in vivo* RNA targets should be highly informative for both breast cancer pathogenesis and for understanding Nova's role in neurons[3].

II. Construction of an *in vitro* selection library from *in vivo* RNA sequences

There exist two methods for construction *in vitro* RNA selection libraries. One may use synthetic, random libraries, or one may construct pools derived from *in vivo* RNA sequences. Our version of *in vivo* library production is know as transcribed sequence SELEX (TS-SELEX). Those pools constructed from RNA derived from *in vivo* sources allows one both to select for sequences with high affinity and identify those genes which contain this high affinity target sequence. We have pursued both methods with Nova, and outlined below are our recent experiments with *in vivo* derived selection libraries.

Figure 2-A. Tissue selection and RNA fractionation for *in vivo*-derived SELEX pools. Because Nova-1 expression is limited to discrete regions of the CNS, we have limited the tissue used for TS-SELEX to the "hindbrain" (midbrain, cerebellum, pons and medulla) of E17 mouse embryos. Hindbrain was gently treated with trypsin to generate a single cell suspension. Half of this material was subject to guanadinium extraction to give a "total" RNA preparation. Half of the total prep was purified for polyA+ sequences using oligo dT beads. The other half of the single cell suspension was lysed by hypotonic solution, and the nucleus and other large membranes sedimented to create a "nuclear" RNA preparation. Half of this material was subject to a mild detergent extraction, and Nova plus bound RNA was immunoprecipitated withanti-Nova sera affinity purified for Nova-1.

Figure 2-B. 1st and 2nd strand cDNA synthesis from the four RNA preparations. A 25-base oligonucleotide with an 8-base degenerate 3' end was used to randomly prime cDNA synthesis on the four RNA preparations. Next, the RNA was destroyed by alkaline hydrolysis and >95% of the 1st strand synthesis primer was removed by spin column chromatography. 2nd strand synthesis was initiated using Klenow and a 37-base oligonucleotide with an 8-base degenerate end. The first and second strand primers contain appropriate fixed sequences for subsequent PCR; the second strand primer also contains a T7 promoter for *in vitro* transcription.

Figure 2-C. Size selection of the cDNA. After 2nd strand synthesis, the cDNA is electrophoresed on a denaturing polyacrylamide gel. cDNA with inserts of 200±10 bases are purified and amplified by PCR with the same primer set *lacking* the 8-base degenerate ends. The *in vivo*-derived SELEX pool is now ready for use.

Figure 2-D. Mapping of the high-affinity protein binding domain with TS-SELEX. Theoretically, one should be able, by random priming cDNA synthesis, to create an entire set of overlapping clones which cover a protein binding domain of interest. If this is achieved, a TS-SELEX experiment should return this set of overlapping clones upon selection, and allow one to map the protein binding domain without further experiment.

III. *In vitro* selection of wild-type Nova using the *in vivo*-derived RNA libraries

Selection with the four *in vivo*-derived RNA pools. Selections were performed using standard nitrocellulose filter partitioning techniques. Binding was done in 200 mM KOAc, 50 mM Tris-OAc pH 7.7, 5 mM $MgCl_2$ and 10 mM DTT. RNA:Nova-1 ratios were kept between 10:1 and 100:1 for the selections. All four RNA pools have undergone 4 rounds of selection. The nuclear and Nova-1 IP pools have also passed though a fifth round using column partitioning. Additionally, after round 4, the four pools were combined and taken through another two rounds of selection.

Pool affinities for Nova-1. Selections progress was monitored by filter binding assays. Shown are binding curves from all fours pools from after round 1 to after round 4. Pool affinity increased ~400-fold in all four pools during these rounds.

IV. Results of the *in vitro* selection

Sequence analysis. To date, we have cloned from all round 4 pools, round 5 nuclear and Nova-1 IP pools, and the round 6 combined pool. We have sequenced 76 clones as described below:

Total	round 4	5
PolyA+	round 4	19
Nuclear	round 4	10
Nova-1 IP	round 4	10
Nuclear	round 5	8
Nova-1 IP	round 5	8
Combined	round 6	16

Based upon the restriction fragment analysis, we estimate that we'll have to sequence much more deeply within the current pools to get close to an accurate sampling. However, the sequences we've looked at so far are quite interesting.

Figure 3. The $(UCAU)_n$ motif. The majority of clones analyzed so far contain the tetranucleotide UCAU. This TS-SELEX result is in agreement with our laboratory's previous *in vitro* selection of Nova-1 (Buckanovich and Darnell, *Mol. Cell Biol.*, **17** 3194, 1997). Most of our $(UCAU)_n$ clones contain discrete runs of the motif, varying between 5 and 15 UCAUs. Two $(UCAU)_n$ clones have matched published sequences; one is in the 3' UTR of the anion exchanger, the other is in the 3' UTR of the *quaking* gene. One other $(UCAU)_n$ sequence matches a mouse EST, and the rest are not found within the databases.

The affinity of the $(UCAU)_n$ clones for Nova-1 is very high; the K_ds of the two clones shown are both sub-nanomolar.

Figure 4. The 28S rRNA sequences. Another set of clones are matches to 28S ribosomal RNA. Remarkably, these clones form an *overlapping* set which cover an 80-base region of the 28S– just the type of set one would predict could be generated during the selection of an *in vivo*-derived library! In addition, we have isolated a clone from the round 4 nuclear pool which is not a mouse sequence but E. coli 23S rRNA, *and overlaps the 28S clones in the same region.* Out of all 76 clones analyzed, we have found only one non-overlapping sequence from 28S, and none from 18S or 5.8S. We have found no other E. coli sequences in the evolved pools.

The region of overlapping clones in the 28S cover a very highly conserved region in domain IV. This region lies on the 60S subunit at the interface of the 60S and 40S, and has been shown to be close in space to the peptidyl transferase activity of the ribosome.

The ribosomal clones tested for binding with Nova-1 (p404 and r408) show Kds of 14 and 7 nM, about 10-fold less than the $(UCAU)_n$ motif sequences.

Other sequences. Several other clones are A-rich, although no clear specific sequence motif seems to be present. Many other clones sequenced have no clear "relatives." Some of our sequence show matches to the minus strand of known

messages. Finally, a substantial subset of clones (~15%) match mouse mitochondrial sequence; the clones we've tested show very high binding to nitrocellulose in the absence of protein– the switch to column partitioning is an effort to eliminate these sequences.

V. Nova protein construction for further *in vitro* selections; *in vitro* selection of the KH3 domain of Nova

***In vitro* selection of a single KH domain from Nova-1.** Nova-1 is composed of three KH-type RNA binding domains. It is unknown how these three domains interact with RNA– cooperating to bind a single RNA target, or as individual modules, each with a unique RNA binding site. While the UCAU sequence motif seems to bind solely to the KH3 domain of Nova-1[4], we were interested in performing *in vitro* selection on a protein construct containing only the isolated KH3 domain. A protein corresponding to amino acids 423-510 of Nova-1 was expressed as a cleavable-tag fusion protein in *E. coli* and purified for SELEX. The isolated KH3 domain binds RNA very poorly– appreciable binding by nitrocellulose filter binding assay was seen only at 10 μM. *In vitro* selection was performed using a 25-base random sequence pool for 11 rounds..

VI. Results of the Nova KH3 *in vitro* selection

Sequencing of individual clones from rounds 9, 10 and 11 revealed a conserved sequence motif consisting of GGACCnnnAUCACCCC (Figure 5). This motif was most often part of a potential stem-loop structure, which included the first two and last two bases of the conserved motif as the first two base pairs of the stem loop. Clone 1-11-01, one of the best fits to the "winner" consensus motif, binds Nova-1 KH3 with a Kd of approximately 500 nM. This same sequence binds full length Nova-1 with a Kd of approximately 5 nM. Unselected RNAs bind Nova KH3 with Kds of at least 400-fold less affinity.

VII. Biochemical characterization of the Nova RNA ligands

We have also shortened the 1-11-01 clone down to 20 bases encompassing the conserved stem-loop structure. This molecule displays identical affinity to both the Nova KH3 domain and full-length Nova. We have begun an extensive mutagenesis study of this molecule. Our data indicates that any change to the conserved loop nucleotides or to the GC basepairs at the top of the stem results in complete loss of binding to Nova. Also, the wild-type molecule shows no affinity to the isolated *KH2* domain of Nova.

VIII. Genetic database searching of the Nova RNA ligands; functional analysis of the Nova RNA ligands

The information generated from the mutagenesis study will allow us to search the existing sequence databases for similar elements in pre-mRNAs and mRNAs. This phase of the project is currently underway. Positive database matches would allow us to identify those pre-mRNAs or mRNAs which potentially bind to Nova *in vivo*. Also, the location of the potential Nova binding sites within the RNA (intronic, 5' UTR, 3' UTR, etc.) should illuminate the possible regulatory functions of Nova for the particular RNA. We envision that biochemical characterization of the Nova ligands, database searching for *in vivo* targets, and function characterization of the interactions will occupy most of the second year of the award.

IX. Direct identification of target RNAs bound to Nova

Using photocrosslinking to identify *in vivo* RNA targets of Nova-1. In a direct approach to identifying RNA ligands of Nova-1, we are using UV crosslinking as a means of isolating and purifying those RNAs that bind to Nova-1 *in vivo* (See Figure 6). Tissue from hindbrain and spinal cord of mice is isolated and protease treated to develop a single cell suspension of Nova-1 expressing cells. This cell suspension is irradiated with short wavelength UV light to crosslink nucleic acid to protein. The cells are then gently broken open and the lysate treated with RNAse T1 to release individual RNA:protein complexes from large hnRNPs and mRNPs. The lysate is then subjected to high-speed spin to pellet high molecular weight material, and the supernatant used in an immunoprecipitation using anti-Nova antiserum conjugated to protein A sepharose. The antibody-Nova beads are washed well and incubated with gamma-^{32}P ATP and polynucleotide kinase. Since the RNAse T1 digest releases RNA with a 5' OH group, the remaining RNA is efficiently labeled. Next, the beads are heated in SDS loading buffer and the bound protein:RNA complexes are separated by molecular weight on SDS-PAGE. The relevant immunoprecipitated complex can be visualized by autoradiography, removed from the gel matrix and free RNA isolated by protease digestion of Nova-1. The sequences in this pool can be made into cDNA by random priming of the RNA with an appropriate primer set. Purification of a discrete insert size is performed using denaturing gel electrophoresis, and the cDNA pool amplified using primers corresponding to the fixed sequence of the priming oligonucleotides.

Figure 7-A. SDS-PAGE of a Nova:RNA covalent complex. Nova or control antiserum was used to IP from hindbrain lysate. The radioactive band at ~55 kDa is approximately the molecular weight of Nova protein, and is dependent on both the Nova antiserum and UV crosslinking. Also shown is the free RNA isolated from the Nova:RNA covalent complex after treatment with proteinase K. The RNA fragments range in size from approximately 15-50 nucleotides.

Figure 7-B. RNA isolated from the Nova covalent complex was used as a template from random primed cDNA synthesis. 1st strand synthesis was primed with an oligonucleotide consisting of a 9-base degenerate 3' end and a 5' fixed sequence. 2nd strand cDNA was initiated with a 10-fold excess of a similar oligonucleotide. The cDNA was separated using denaturing gel electrophoresis and bands corresponding to

"inserts" of 30-40 and 40-50 nucleotides were isolated and the cDNA extracted. This material was amplified by PCR using fixed sequence oligonucleotides corresponding to the fixed sequences of the cDNA primers. (The 5' PCR primer adds 21nt to the length of the cDNA.) Shown are autoradiographs of ^{32}P labeled PCR product. After the first PCR, faint bands are visible at the correct size for 30-40 and 40-50 nt inserts. This material was gel purified and used for a second PCR reaction. The autoradiograph of material from this reaction shows abundant product which can be used for the cloning of the cDNA pool. We are also modifying our RNAse T1 conditions to increase the size of the RNA fragments isolated by the crosslinking procedure.

Conclusions

We are actively pursuing both the *in vitro* selection and crosslinking methods described here. We are currently analyzing our first set of clones isolated from the crosslinking technique, and are extending this method to capture longer RNAs crosslinked to Nova. The *in vitro* selection data on Nova-1 suggests to us that Nova's KH domains might function to bind several RNA targets, possibly simultaneously. The domain IV sequence of 28S rRNA, which was isolated by TS-SELEX, exhibits high affinity binding to Nova-1, and retains much of this affinity to a Nova-1 construct which contains only KH1 and KH2. However, a Nova-1 construct containing only KH3 shows no measurable binding to the domain IV RNA. Our recent *in vitro* selection of the isolated KH3 domain indicates that this domain is capable of high affinity binding to a well-defined sequence with a potential stem loop structure. We are in the process of testing the KH3 ligands with Nova-1 constructs containing only KH1, KH2, or KH1-2. A compelling hypothesis is that Nova functions to condense two (or more) RNAs (Figure 8). Given that one of the possible Nova targets is rRNA, it is possible that Nova's function in RNA metabolism might lie in the transport and/or localization of mRNA, and our *in vitro* selection data indicates that Nova might even mediate an interaction between mRNA and the 60S ribosomal subunit. Thus, Nova might play a function in a new type of translational control, and this regulatory pathway might have important biological consequences in both neurons and in breast cancer.

References

1. Darnell, R.B., *Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity and the brain.* Proc Natl Acad Sci USA, 1996. **93**: p. 4529-4536.
2. Buckanovich, R., J.B. Posner, and R.B. Darnell, *Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system.* Neuron, 1993. **11**: p. 657-672.
3. Buckanovich, R.J., Y.Y. Yang, and R.B. Darnell, *The onconeural antigen Nova-1 is a neuron-specific RNA binding protein, the activity of which is inhibited by paraneoplastic antibodies.* J Neurosci, 1996. **16**: p. 1114-1122.
4. Buckanovich, R. and R.B. Darnell, *The neuronal RNA binding protein Nova-1 recognizes specific RNA targets in vitro and in vivo.* Mol. Cell Biol., 1997. **17**(6): p. 3194-3201.

Appendices

Figures 1-8 follow

Figure 1

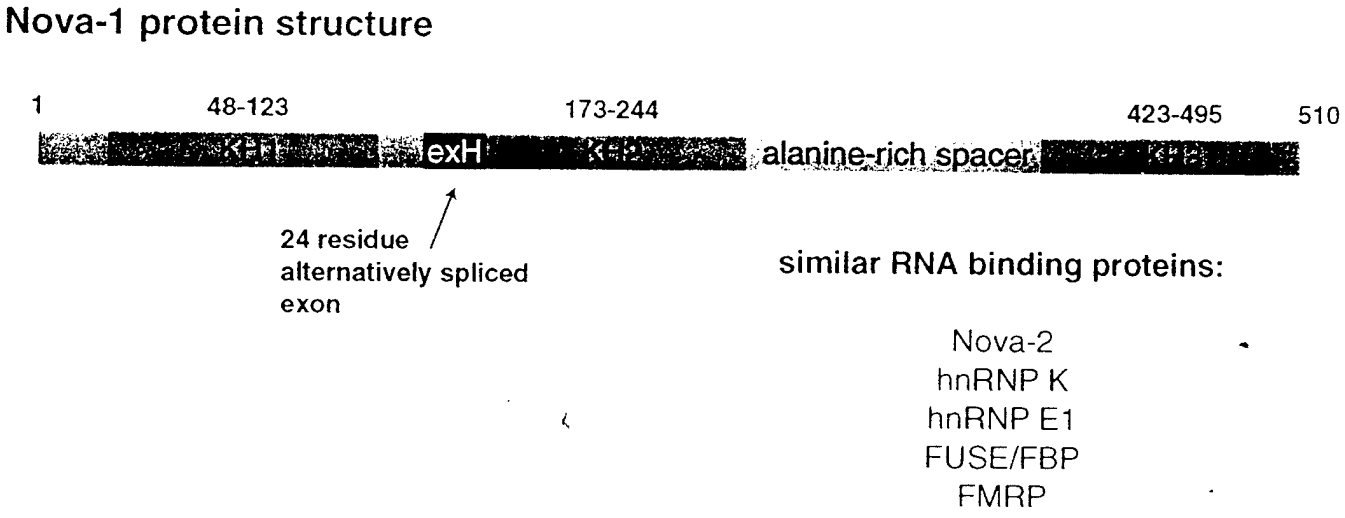

Nova-1 protein structure
1
48-123
173-244
423-495
510
KH1
exH
KH2
alanine-rich spacer
KH3
24 residue alternatively spliced exon
similar RNA binding proteins:
Nova-2
hnRNP K
hnRNP E1
FUSE/FBP
FMRP

Figure 2


A.
mouse E17 hindbrain
single cell suspension
nuclear and membrane-associated RNA
hypotonic lysis
RNA immunoprecipitated with polyclonal rabbit anti-Nova
protein A sepharose beads
oligo dT magnetic beads
B.
1st strand synthesis
alkaline hydrolysis; removal of primer
2nd strand synthesis
5'
3'
C.
PAGE
size select products; PCR
~200 base insert
D.
selected clones
protein binding domain

Figure 3

$(UCAU)_n$ motif sequences

~40% of clones analyzed

p402/r505- anion exchanger 3' UTR 3462-3667
CCCTGTACAGAAATACCCCTAATGACTTCCTCCCATTTTCTGTCCCAGCCCCACCCCAAGA
CTCCCTTCTCTGCATCCAGAGAAGCGTCCTGGTGAGAATAAAGGACTGGGGCTCAGACTCC
TANGTNCTTACTCTCTCCCACTAAGTAT**TCATTCATTTGCTCATTCATTCATTCATTCACT**
TATTCATTCATTCATTCATACACAGCCAGACAAATGTGG

p420- mouse EST match
TGTGTCTTCCTTGTCT**TCATCCTCATCATCATCTTCATCATCAT**CAGGATAATCTACCAGA
CCCACGAGTCCTCCCTTTGTGGTAATAGCTGCCGTCTGAGATGTACTCTTAGGGACGGATC
CAGGAGAGCCCGGAGAGCCCGGGGAGCCAGGTGACCCTGGAGAACCAGGCAGGCTTGTTGC
GGATGACTGGCTGGTGAGGTTAGTCTTTGTCCTACTTGAG

c604/c606 unknown
TCCCGGGTCTGATTTGCCTGTCTGTTAGTACCAAAAAGTCTCTGTGGGCATTTCTCTTTTG
GAAATGTACTTTCTGCCCATCTCCTCTCAAATAATGTAACACTTGCTGCTATTCATTTGCT
GTCTTTCTTT**TCATTCATTCATTCATTCATTCATTCATTCATTCATTCAT**GTGTGTGTGAT
GCACATGTGAGGGCAGGTGTCTAGAGGTC

c611 unknown
TATGCCTGCCTTTGCCTCTCAAGCTCTGGAATTCAAGGCATCTTGTACTATCTGGCTTTCC
ACTTGTGCGTTTGTTCGTTCGTTCGT**TCATTCATTCATTCATTCATTCATTCACTCATTCA**
TAGTGCCACGTGGTGCTCAGGCTGGCCTTGAATTTGTGCAGTAAAGCAGCTCTGCCTCAGG
GCCCAGAGCAGTTAGGCTACAGGGTAGTAATTCTACTCTGACCCAGGATT

p423 unknown
TGTGTTTGTGCATGCCTTAGTGCTGTGTGGAGGTCAGAGTCAGTTCTCTCAATGGTATGAG
CTCCAGGGATCAAACTCAGGCTTGGCTGAAATCACCTTGATGACCTCACCTATGTTAT**TCA**
TTCATTCATTCATTCATTCATTCATTCATTCATTCATTCATTCAAGACAAAGTCTCT
AGCCCTGCTGGCTCTCCTGGAACCCAA

r508 unknown
TGTTTTCTTTTTCTCTTTTCTGTTTTTGGTGTGTATATGAGTATGTCCATTTGTGTATGTG
CTACGGTGCAGGTGTGTACGTGCANGTACCTNAATATGGAGACTCGAAGAGAGATCAACTT
CTGGTGTCATCTCTCAGGAGCCTCCCACCTTA**TCATCATCATCATTATCATCNTCATTATC**
ATCATCATCATCATCATCATCGTTATCACCATCATT

Figure 4

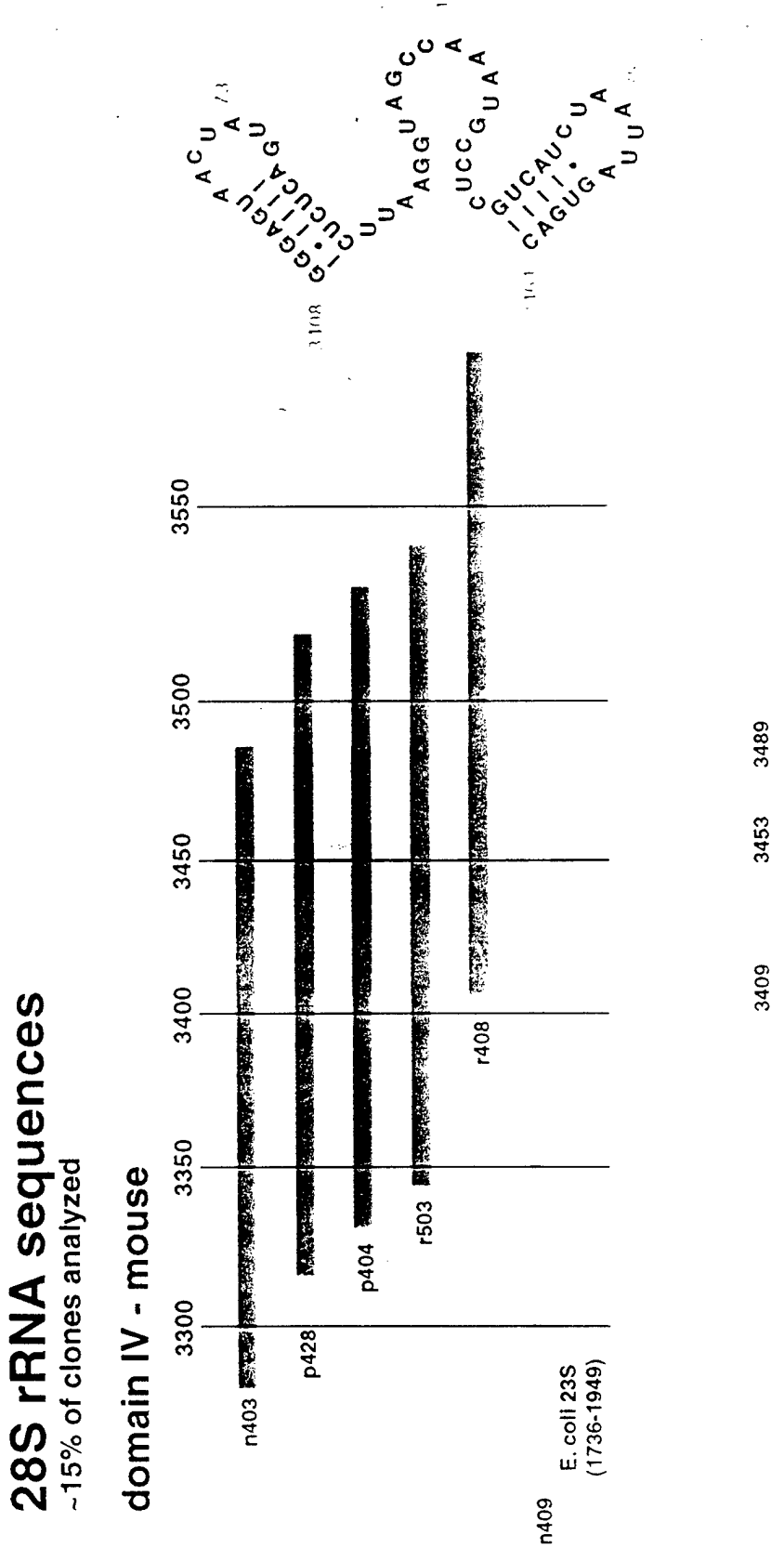

28S rRNA sequences
~15% of clones analyzed
domain IV - mouse
3300
3350
3400
3450
3500
3550
n403
p428
p404
r503
r408
n409
E. coli 23S
(1736-1949)
3409
3453
3489

Figure 5

Nova-1 KH3 domain SELEX sequences

lowercase- fixed sequence
uppercase- random region

```
11101    gggaggacgaugcggUACGGACCUAGAUCACCCCGUGCUGcagacgacgagcggga
11003    gggaggacgaugcggACAGGACCCAGAUCACCCCUGGCUGcagacgacgagcggga
10904    gggaggacgaugcggUCAGGACC-ACAUCACCCCUGUCCGcagacgacgagcggga
10903         gggaggacgaugcggACCUAAAUCACCCCGCAUUACCCGCcagacgacga...
20902   gggaggacgaugcggUCAAGGAUCACGAUCACCCCUUGGCcagacgacgagcggga
20903    gggaggacgaugcggAUUGCAUCACCAUCACCCCUCCCCCcagacgacgagcggga
10901 gggaggacgaugcggUAGCAAGGACCUAAUUCACCCCUGcagacgacgagcggga
```

potential consensus
secondary structure

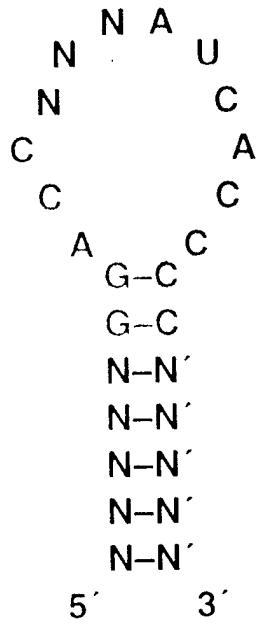
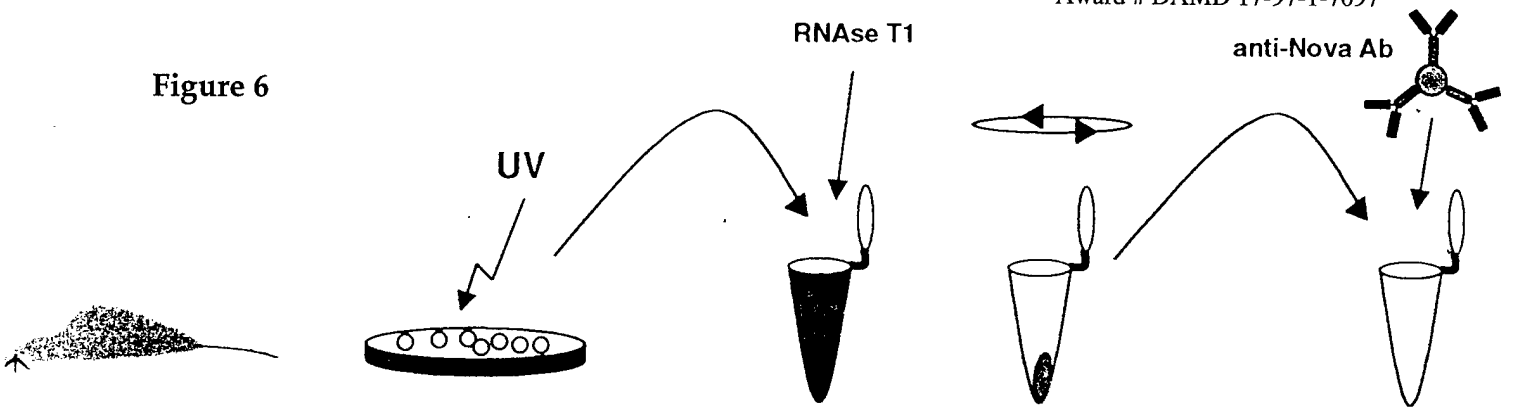


Figure 6



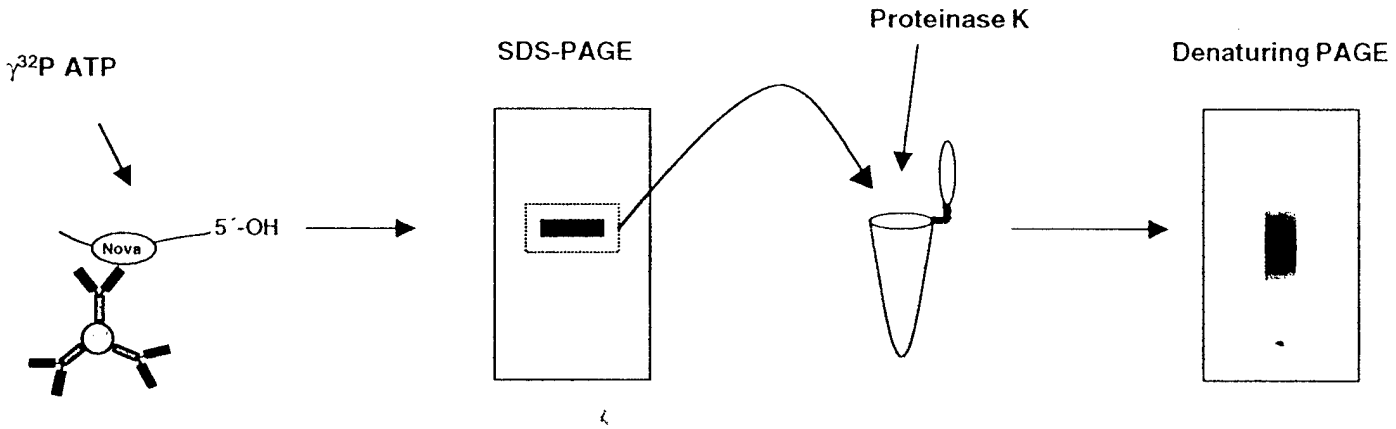
Isolate hindbrain

Make single-cell suspension and crosslink

Lyse cells and digest with RNAse T1

Spin down high molecular weight material

Incubate supernatant with antibody /protein sepharose

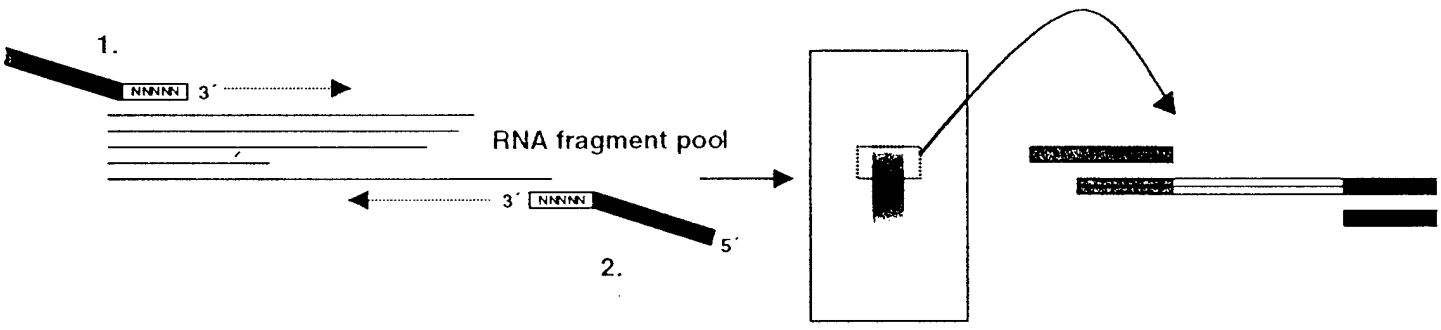


Wash PAS beads and label RNA with ^{32}P

Run on SDS-PAGE and cut out Nova-RNA covalent complex

Digest protein

Run free RNA on denaturing ge for analysis



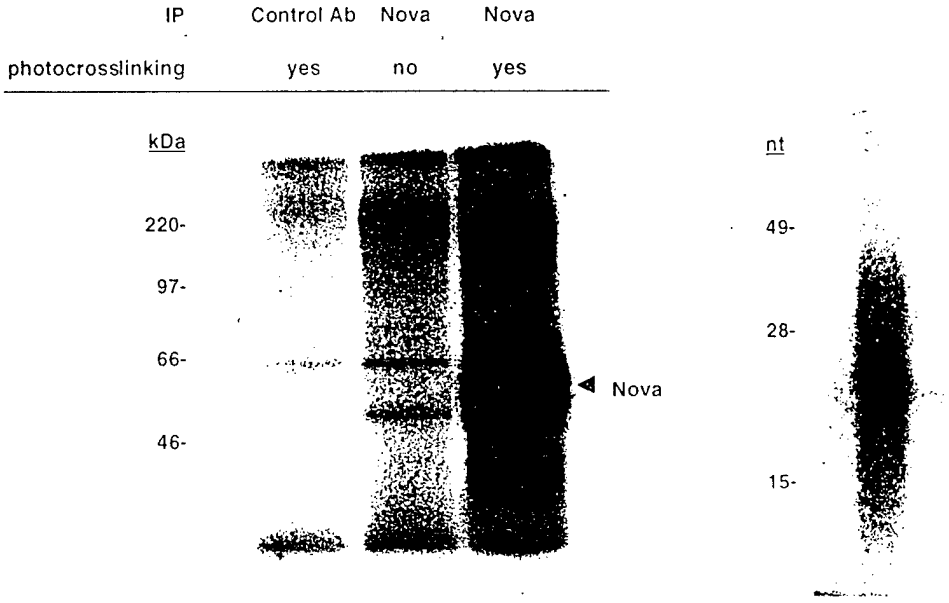
Make 1st strand cDNA with random primer/fixed sequence oligo; add 10-fold excess of 2nd primer and make 2nd strand cDNA

Run cDNA on denaturing gel and isolate band with desired length insert

PCR with fixed sequence primers; clone amplified DNA

Figure 7

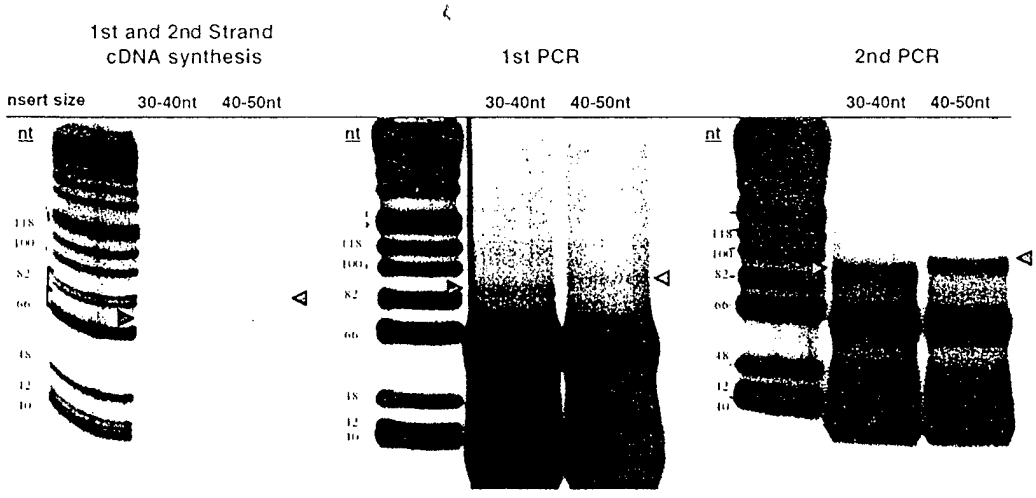
A. Isolation of RNA fragments covalently linked to Nova



SDS-PAGE of crosslinked RNA:protein complex

Purified RNA

B. Random priming of crosslinked RNA



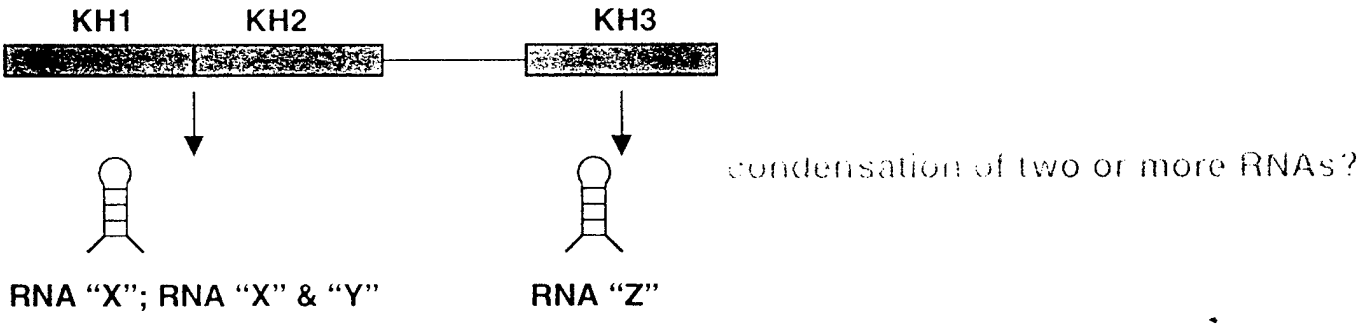
Blind gel purification of cDNA with inserts of 30-40 nt and 40-50 nt

Gel purify correct size PCR products (5' fixed primer adds 21 nt)

Gel purify correct products and clone using dU primers and UDG

Figure 8

A. Nova-RNA interactions: a model



B. possible Nova functions

- –nuclear and cyto-dendritic sub-cellular localization
- –Nova present in hnRNPs and mRNPs; shuttling protein?
- –functions:
  - I. Transport/localization of mRNA?
  - II. Role in translation- mediates interactions between mRNA and ribosome?