MOLECULAR AND CELLULAR BIOLOGY, June 1997, p. 3194–3201 Vol. 17, No. 6
0270-7306/97/$04.00+0
Copyright © 1997, American Society for Microbiology

The Neuronal RNA Binding Protein Nova-1 Recognizes Specific RNA Targets In Vitro and In Vivo

RONALD J. BUCKANOVICH AND ROBERT B. DARNELL*

Laboratory of Molecular Neuro-Oncology, The Rockefeller University, New York, New York 10021

Received 7 January 1997/Returned for modification 24 February 1997/Accepted 11 March 1997

Nova-1, an autoantigen in paraneoplastic opsoclonus myoclonus ataxia (POMA), a disorder associated with breast cancer and motor dysfunction, is a neuron-specific nuclear RNA binding protein. We have identified in vivo Nova-1 RNA ligands by combining affinity-elution-based RNA selection with protein-RNA immunoprecipitation. Starting with a pool of ~10^{15} random 52-mer RNAs, we identified long stem-loop RNA ligands that bind to Nova-1 with high affinity (K_d of ~2 nM). The loop region of these RNAs harbors a ~15-bp pyrimidine-rich element $[UCAU(N)_{0-2}]_3$ which is essential for Nova-1 binding. Mutagenesis studies defined the third KH domain of Nova-1 and the $[UCAU(N)_{0-2}]_3$ element as necessary for in vitro binding. Consensus $[UCAU(N)_{0-2}]_3$ elements were identified in two neuronal pre-mRNAs, one encoding the inhibitory glycine receptor α2 (GlyR α2) and a second encoding Nova-1 itself. Nova-1 protein binds these RNAs with high affinity and specificity in vitro, and this binding can be blocked by POMA antisera. Moreover, both Nova-1 and GlyR α2 pre-mRNAs specifically coimmunoprecipitated with Nova-1 protein from brain extracts. Thus, Nova-1 functions as a sequence-specific nuclear RNA binding protein in vivo; disruption of the specific interaction between Nova-1 and GlyR α2 pre-mRNA may underlie the motor dysfunction seen in POMA.

RNA-protein interactions are important in the posttranscriptional regulation of RNA metabolism and expression. Nascent RNA transcripts associate with large multiprotein complexes that include hnRNP proteins and snRNP particles (19). 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 melanogaster*, the identification of sequence-specific targets for the sex-lethal (sxl) (5, 30) and transformer-2 (28) 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 (48, 69). Identification of specific RNA ligands for the human immunodeficiency virus Rev and Tat proteins has clarified their role in regulating viral RNA transcription, processing, and transport (14, 27, 34, 40, 70). There remain, however, many RBPs for which specific RNA targets have not been identified.

RNA selection has been used as an in vitro approach to identify RNA ligands for RBPs (61, 65). This approach was originally used to confirm the specificity of rRNA binding sites recognized by T4 DNA polymerase (65) and has subsequently been used to confirm and extend known binding sites for the U1-snRNP-A protein (64), sxl (55), and the viral Rev (3, 31) and Tat (66) proteins. 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 (12), SR proteins (29, 62), and the neuronal RBP (n-RBP) Hel-N1 (36), although in general these studies have yielded short in vitro consensus RNA ligands whose in vivo significance is currently being explored.

Nova-1 is a nuclear n-RBP identified as a target antigen in paraneoplastic opsoclonus myoclonus ataxia (POMA) (9, 10, 16). POMA is a neurologic disorder that develops when systemic tumors (typically breast tumors) ectopically express Nova, triggering the production of an autoimmune response characterized by high-titer anti-Nova antibodies in the cerebrospinal fluid (9, 38). The neurologic symptoms of POMA are thought to result, at least in part, from the failure of inhibition of brainstem and/or spinal motor systems (16). Cloning of the Nova-1 gene led to the demonstration that it was an n-RBP composed of three KH-type RNA binding domains (9, 10). Nova-1, together with the Hu family of n-RBPs, are distinct from most mammalian RBPs in that their expression is extremely tissue specific. For example, most hnRNP proteins are ubiquitously expressed, although some show different levels of expression among different tissues (13, 33). Nova-1 is expressed only in neurons of the subcortical central nervous system (9, 10).

KH-type RBPs are an expanding family of proteins for which some functional data are available, although whether they act as sequence-specific RBPs is uncertain. KH-type proteins include FMR-1 (encoded by the fragile-X gene), which harbors two KH domains; loss of function mutations of the fragile-X gene is associated with mental retardation (11, 58). Nova-1 is also related to KH-type RBPs involved in regulating RNA splicing, including MER-1 in yeast (21), PSI in *Drosophila* (54), and the recently described mammalian proteins KSR and SF-1 (2, 41). Nova-1 is most closely related to hnRNP K, a protein whose function or targets as an RBP are unknown. In this paper, we report the identification of a specific stem-loop RNA which Nova-1 binds in vitro and in vivo in two neuronal pre-mRNAs. We discuss these findings in relation to the biology of Nova as an RBP and as a disease antigen.

MATERIALS AND METHODS

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 [22]) was synthesized on an ABI DNA synthesizer to yield 2 mg of DNA with an estimated complexity of ~3 × 10^{16} sequences; the oligonucleotide was characterized and PCR amplified by using forward and reverse oligonucleotide primers as described previously (20, 22). Following PCR amplification, the sequences of 24 random clones from this

* Corresponding author. Mailing address: Laboratory of Molecular Neuro-Oncology, The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-7460. Fax: (212) 327-7147. E-mail: darnelr@rockvax.rockefeller.edu.

FIG. 1. 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 (open squares), with each point defined as a percentage of protein eluted in the peak fraction. RNA elutions from selection round 5 (*RNA-Rd5*) or round 7 (*RNA-Rd7*) are shown. Multiple selection cycles yielded RNA pools in which ~87% of input RNA counts coeluted with Nova-1 protein (RNA selection round 7; see also Table 1). (B) Sequences of Nova-1 selected RNAs. Sequences of 12 of 14 PCR clones encoding RNA ligands cloned after seven rounds of RNA selection are shown; two additional clones in which no consensus was identified are not shown. UCAU repeats in each ligand are shown in boldface. Regions of the RNA which form the inverted repeat stem are underlined. Stem sequences which are derived from the nonrandom regions of the RNA ligand (primer binding sites) are in lowercase. A consensus sequence is shown below.

pool were determined; each clone was unique, and the overall base composition showed a slight excess of purines (24% A and 33% G) over pyrimidines (21% C and 20% U) and a random distribution of dinucleotide frequencies (data not shown). Two pool equivalents of purified library DNA (110 μg, $\sim 2.1 \times 10^{14}$ molecules) were transcribed in vitro with T7 RNA polymerase and [α-^{32}P]UTP as described previously (20). RNA was purified from denaturing acrylamide gels, heated to 70°C for 5 min, and applied to a precolumn containing an irrelevant histidine-tagged fusion protein to adsorb nonspecifically bound RNAs. The flowthrough was then applied to a histidine-tagged Nova-1 nickel affinity column in high-salt buffer (HSB; 0.5 M LiCl, 20 mM Tris-HCl [pH 7.6], 1 mM $MgCl_2$, 60 mM imidazole [20, 25]). Following washing in 10 column volumes of HSB, protein and RNA were coeluted by the addition of 1.0 M imidazole in HSB. Protein-bound RNA was extracted in phenol–0.5% sodium dodecyl sulfate at 50°C and ethanol precipitated with LiCl and glycogen.

Filter binding assays. Filter binding using purified histidine-tagged Nova fusion proteins and methods previously described (10). Briefly, the indicated concentrations of fusion proteins were incubated with 50 to 200 fmol of ^{32}P-labeled RNA as indicated; RNA was transcribed directly from PCR products encoding RNA selection clones or from oligonucleotides harboring T7 RNA polymerase promoters. Binding was performed in 100 μl of binding buffer (0.5 M LiCl, 20 mM Tris-HCl [pH 7.4], 1 mM $MgCl_2$, 10 μg of yeast tRNA/ml) for 30 min, samples were filtered through nitrocellulose and washed with 1 volume of binding buffer, and retained counts were 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 μl of protein buffer (50 μg of bovine serum albumin/ml, 0.5 M LiCl, 20 mM Tris-HCl [pH 7.6], 1 mM $MgCl_2$); 50 fmol of ^{32}P-labeled RNA was heated to 80°C for 5 min, cooled to room temperature, and then added to RNA buffer (0.5 M LiCl, 20 mM Tris-HCl [pH 7.6], 1 mM $MgCl_2$, 40 ng of yeast tRNA/ml) in a final volume of 10 μl. Protein and RNA samples were mixed and allowed to equilibrate for 30 min at room temperature. For supershift experiments, antibody was then added and incubated an additional 30 min at room temperature. Two percent of each sample was resolved with a 50 mM Tris-glycine–4% polyacrylamide gel, dried, and exposed to XAR-5 autoradiography film.

Immunoprecipitation. Adult mouse brains were homogenized with a Dounce homogenizer 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 of NET-Triton, sonicated, and spun in a microcentrifuge, and the supernatant was precleared with protein A-Sepharose for 30 min at 4°C, followed by incubation with the indicated antibodies and protein A-Sepharose. After five washes with cold RIPA buffer (150 mM NaCl, 50 mM Tris [pH 7.4]. 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% deoxycholate), samples were resuspended in DNase buffer (50 mM Tris-HCl [pH 7.4], 6 mM $MgCl_2$, 3 mM $CaCl_2$) with 20 U of DNase and 20 U of RNase inhibitor at 37°C for 1 h, extracted with phenol-chloroform at 55°C, extracted with chloroform, and precipitated with ethanol and sodium acetate. Reverse transcription (RT)-PCR was performed as previously described (9). All PCRs except the PCR to detect clathrin were performed by using 30 cycles (1 min at 94°C, 30 s at 58°C, and 45 s at 72°C). PCR to detect clathrin DNA was performed by using 32 cycles of 1 min at 94°C, 30 s at 54°C, and 45 s at 72°C. Primers used were as follows: glycine receptor α2 (GlyR α2), AAAATACTAGTGGGAAGTTATCATGCA and CAT GGTGGTTTCTGTGACTGATC; Nova-1, GCGAATTCTCCAGATCGCATC AAACAA and ACTGAAGGCTCCAAAAGTCTTC; clathrin, TTAACCCTGT GCCTGCCTGTCTTG and GCTGCTGGTAGAACGCTTTGTCAG; HuD, GGACATGAATTACTTGCCAT and CATAGGCCATATTAAGCA; HelN1, ATTGCTGTAACCAATCTA and ATTCCATCGATCGTCATT; and brain-specific Na^+ channel, GGAATTCTGGAACTGGCTGGATTTCAGTG and TGCGGAATTTATCATGGCACC.

RESULTS

We have examined the function of Nova-1 by using an affinity elution-based RNA selection (25, 61) to identify specific Nova-1 protein–RNA interactions. RNA was transcribed from two pool equivalents of a PCR-amplified oligonucleotide library comprised of 52-nucleotide random-mers. The transcribed RNA, which had an estimated complexity of 2.1×10^{14} molecules, was applied to a nickel column containing an irrelevant histidine-tagged fusion protein, and the flowthrough was then applied to a nickel column containing an 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 monitor the coordinate elution of protein and RNA (Fig. 1A) 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 1).

Sequencing of individual PCR clones obtained after the seventh round of RNA selection identified a clear RNA consensus sequence (Fig. 1B). There were two components to the consensus, a structural element (an inverted 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 sequence motif that was nearly identical in each of the 11 clones and consisted of a pyrimidine-rich consensus sequence of a core of three UCAU repeats. Six of eleven clones matching the consensus sequence contain three UCAUs, and four of the five remaining clones have two UCAUs and a third NCAU. The

TABLE 1. Multiple selection cycles yield Nova-1 RNA ligand[a]

Selection round	Nova-1-bound RNA (% of input)
1	≤0.05
2	ND
3	0.26
4	3.4
5	10.2
6	51.0
7	87

[a] Percentages of total RNA input which specifically eluted with Nova-1 protein for each round of RNA selection are 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. ND, not determined.

UCAU repeats were separated by zero to two nucleotides; while these sequences were preferentially (~75%) pyrimidines, this frequency was not statistically significant, and evidence presented below suggests that pyrimidines between the UCAU repeats are not essential for Nova-1 binding. Therefore we have designated the Nova-1 consensus RNA ligand $[UCAU(N)_{0-2}]_3$.

To characterize the Nova-1–RNA interaction, and to map the domains of Nova-1 which are responsible for recognition of the $[UCAU(N)_{0-2}]_3$ 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. In a filter binding assay, NFP demonstrated high-affinity saturable binding to SB2 (K_d of ~20 nM in 0.5 M LiCl and of K_d of ~2 nM in 0.1 M LiCl [Fig. 2A and data not shown]). The affinity of N3 for SB2 was reduced approximately 10-fold (K_d of ~180 nM in 0.5 M LiCl and K_d of ~10 nM in 0.1 M LiCl), while neither N1, N1-2, nor an N3 protein carrying a single (leucine-to-asparagine) point mutation (N3 L213N [10]) showed detectable binding to SB2 (Fig. 2A). 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 $[UCAU(N)_{0-2}]_3$ 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 (SB2A3U) in which the $(UCAU)_3$ element was mutated to $(UCUU)_3$ (Fig. 2B). In addition, NFP binding to SB2 was competed with an excess of cold SB2 RNA (50% inhibition with a ~12-fold molar excess of competitor) but could not be competed with an excess of SB2A3U RNA (see Fig. 4C). A structural mutant, in which base pairing in the stem sequence was eliminated while the $[UCAU(N)_{0-2}]_3$ sequences were unchanged, showed a threefold reduction in binding of NFP (Fig. 2B). These results were confirmed by gel shift assays. Figure 2C shows that the SB2 RNA was almost completely shifted after binding to NFP but unaffected following incubation with an equimolar amount of an irrelevant control protein (amino acids 270 to 413 of cdr2 [16, 16a]). In contrast, the mutant SB2A3U RNA showed no gel shift when bound to NFP (Fig. 2C). We also found that the SB2-NFP complexes could be supershifted by using Nova-1 antibodies (Fig. 2C). SB2-NFP complexes were not shifted when incubated with preimmune antiserum or an irrelevant affinity-purified antibody, and anti-Nova-1 antibodies alone did not shift SB2 RNA (Fig. 2C 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; the loop sequence is the major determinant of Nova-1 binding, while the stem is not essential for binding.

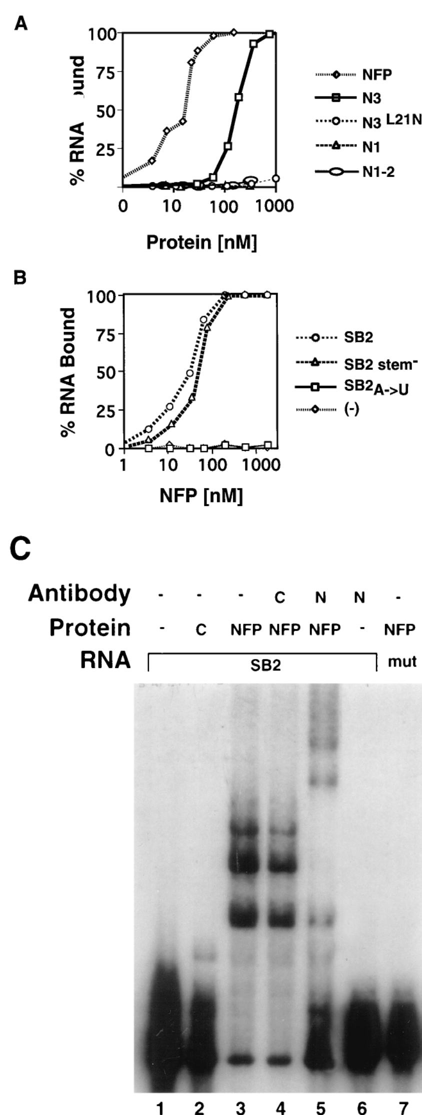

FIG. 2. Nova-1 is a sequence-specific RBP. (A) Nova-1 binding to an RNA selection ligand (SB2). RNA binding of full-length and truncated Nova-1 proteins was determined in 0.5 M LiCl by a filter binding assay (see Materials and Methods). Full-length NFP and the N3 domain proteins bound with K_ds of ~20 and 100 nM, respectively. Proteins N1, N1-2, and N3 L213N 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 SB2A3U RNA, the UCAU repeats have been mutated to UCUU, and in SB2 stem⁻ RNA, the base pairing of the stem has been disrupted by oligonucleotide-directed mutagenesis (the 3′ SB2 stem⁻ RNA sequence is **UCAU** CAAG GGUCUAACGCGCU, where the third UCAU of SB2 is in boldface and SB2 mismatches are underlined). There is no detectable binding to an irrelevant RNA transcribed from a random library clone (−). (C) Gel shift analysis of Nova-1 protein. SB2 RNA or SB2A3U mutant (mut) ^{32}P-labeled RNA was transcribed in vitro and incubated with 1 pmol of NFP or irrelevant protein (C), and complexes were run on nondenaturing polyacrylamide gels. These complexes were supershifted with affinity-purified rabbit anti-Nova-1 antibodies (N) but not with preimmune serum (C). No significant amounts of label were evident in the wells of any gel-shifted reaction (data not shown).

To further define the sequence requirements for high-affinity Nova-1 RNA binding, we generated small RNAs consisting

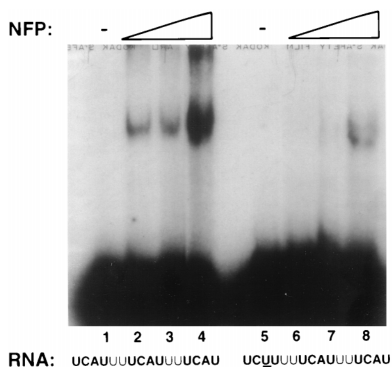

FIG. 3. Gel shift analysis of $[UCAU(N)_{0-2}]_3$ or a mutant loop RNAs bound to NFP. Increasing amounts of NFP (0, 0.5, 1.5, and 5 pmol, respectively, in lanes 1 to 4 and 5 to 8) were incubated with 200 fmol of ^{32}P-labeled RNA and analyzed by nondenaturing polyacrylamide gel electrophoresis. The RNA used in lanes 1 to 4 consists of the loop sequence indicated below the lanes. The RNA used in lanes 5 to 8 consists of the loop sequence with a single A→U mutation and is indicated below the lanes. UCAU repeats in the RNAs are in boldface. The mutated nucleotide is underlined. All loop RNAs contain a 5′ leader sequence (AGG) needed for efficient T7 transcription.

TABLE 2. Nova-1 binding to mutant RNA ligands[a]

RNA	K_d (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

[a] The indicated RNAs were in vitro transcribed from DNA oligonucleotides containing T7 RNA polymerase promoter sequences. All loop RNAs also contain a 5′ leader sequence (AGG) needed for efficient T7 transcription. The K_d values are indicated for NFP and N3 binding to RNAs in 0.5 M LiCl; dashes indicate no significant binding ($K_d \gg 5$ μM). K_ds were determined by filter binding assays.

of the $[UCAU(N)_{0-2}]_3$ loop sequence 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 $[UCAU(N)_{0-2}]_3$ 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 assays with an RNA in which a single UCAU repeat was mutated to UCUU yielded a shifted species only with a 10-fold-higher concentration of NFP (Fig. 3). These data also demonstrate that the NFP-loop sequence RNA (a 21-mer) complex migrated predominantly as a single species, in contrast to migration of NFP-SB2 RNA complex, which migrated as multiple shifted species (Fig. 2C). These data suggest that the isolated loop RNA is unable to support protein multimerization and consequently that Nova-1 multimerization is not essential for high-affinity binding; alternatively, the multiple bands in our gel shift (Fig. 2C) could reflect heterogeneity in RNA secondary structure. Taken together, these results confirm that NFP binds the $[UCAU(N)_{0-2}]_3$ RNA loop sequence specifically and with high affinity.

We next assayed the binding of NFP and N3 to a series of $[UCAU(N)_{0-2}]_3$ loop RNAs harboring specific nucleotide substitutions (Table 2). An A-to-U residue substitution within any one of the three UCAU repeats reduced binding approximately ninefold, consistent with results of the gel shift assay using this mutant (Fig. 3 and data not shown). Substitution of two of three A's with U's completely abolished binding (Table 2). 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- to 12-fold (Table 2). Although the selected RNAs preferentially harbor pyrimidines between UCAU repeats (Fig. 1B), these sequences could be substituted by A residues with no significant change in binding affinity (Table 2). Thus, Nova-1 RNA binding is sequence specific: three intact UCAU repeats are necessary and sufficient for high-affinity binding.

We used the consensus RNA selection sequence to search GenBank by using the BLAST server, to search a database of neuron-specific alternatively spliced exons (59), and to search our own Nova-1 genomic sequence data. These searches initially identified only two potential in vivo Nova-1 RNA binding sites, one within an intron of the GlyR α2 pre-mRNA (35) and one within the Nova-1 pre-mRNA itself (Fig. 4A). The GlyR α2 intron has three distinct UCAU repeats, each separated by two nucleotides (three-fourths are pyrimidines). A fourth UCAU repeat overlaps the first and second in a pattern similar to that seen in RNA selection clone SB31 (Fig. 1B). In the mouse Nova-1 intron, there are two UCAU repeats separated by four pyrimidines, followed by five nucleotides (three of which are pyrimidines) and a 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 α2 exon 3A [35] and 8 bp downstream of Nova-1 exon H [reference 9 and Fig. 4A]). In Nova-1, the UCAU element lies within a 35-nucleotide intronic sequence which is 95% identical between human and mouse. In both the GlyR α2 and Nova-1 pre-mRNAs, the sequence CAGU is present one nucleotide upstream of the first UCAU, and a pyrimidine-rich stretch (seven of eight nucleotides in GlyR α2 RNA, six of eight in mouse Nova–1 RNA, and seven of nine in human Nova-1 RNA) is present downstream of the UCAU element.

We cloned these regions of the GlyR α2 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-bp region containing the UCAU motif (data not shown). Nova-1 bound SB2, GlyR α2, and Nova-1 RNAs in identical manners in the filter binding assay (K_ds of 20, 20, and 15 nM, respectively, in 0.5 M LiCl [Fig. 4B]). Mutation of the GlyR α2 RNA $(UCAU)_3$ repeats to $(UAAU)_3$ eliminated Nova-1 protein binding as did mutation of the Nova-1 pre-RNA $(UCAU)_3$ repeats to $(UACU)_3$ (Fig. 4B). In addition, Nova-1 protein binding to GlyR α2 RNA could be competed by an excess of SB2 RNA but not by the mutant SB2A→U RNA (Fig. 4C). These results demonstrate that Nova-1 binds to both the GlyR α2 and Nova-1 RNAs in vitro with high affinity via the $[UCAU(N)_{0-2}]_3$ motif.

Previously, we had shown that POMA antibodies target the third KH domain of Nova-1 and can disrupt the low-affinity binding (K_d of ~300 nM) of Nova-1 to polyribohomoguanine RNA in vitro (10). 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

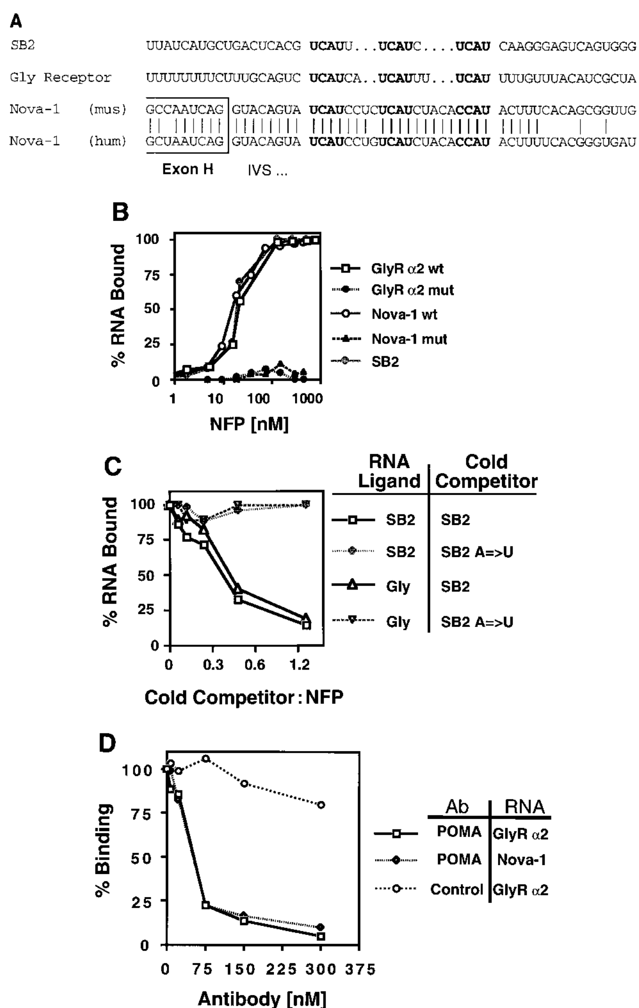

FIG. 4. Nova protein binds to GlyR α2 and Nova-1 RNAs, and binding can be inhibited by paraneoplastic antibodies. (A) Alignment of in vivo $[UCAU(N)_{0-2}]_3$ elements with SB2. UCAU elements present in the rat GlyR α2 (35) and Nova-1 genomic DNA sequences are shown aligned with UCAU elements (boldface) present in RNA selection clone SB2 (from Fig. 1B), with gaps indicated by dots. Nova-1 genomic sequences were determined from human (hum) and mouse (mus) genomic libraries. The Nova-1 UCAU elements are present within an intron (IVS) 8 nucleotides downstream of an alternatively spliced exon (exon H [9]), and the GlyR α2 sequences are present ~80 nucleotides upstream of alternatively spliced exon 3A (35). (B) NFP binds to $[UCAU(N)_{0-2}]_3$ elements in GlyR α2 and Nova-1 RNAs in vitro. Fragments of the GlyR α2 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 GlyR α2 (squares), Nova-1 (open circles), and SB2 RNAs (diamonds) was saturable with a K_d ~20 nM (standard deviation, ~2 nM). Mutation of the UCAU elements in either GlyR α2 (closed circles) or Nova-1 RNAs (triangles) eliminates binding. wt, wild type; mut, mutant. (C) NFP binding to the GlyR α2 can be competed with SB2 RNA. NFP (1.6-pmol) was incubated with 50 fmol of radiolabeled ligand (GlyR α2 or SB2) and increasing amounts of unlabeled competitor RNA (SB2 or SB2A→U). NFP binding to either SB2 (open squares) or the GlyR α2 RNA (open triangles) was 50% competed by a ~1:2 molar ratio of unlabeled competitor to NFP, which corresponds to a ~12-fold molar excess of unlabeled SB2 relative to radiolabeled ligand. An excess of cold SB2A→U mutant RNA failed to compete binding of NFP to either radiolabeled SB2 (closed diamonds) or GlyR α2 (inverted triangle). (D) NFP binding to GlyR α2 and Nova-1 pre-mRNAs can be inhibited by disease antibodies (Ab). 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.

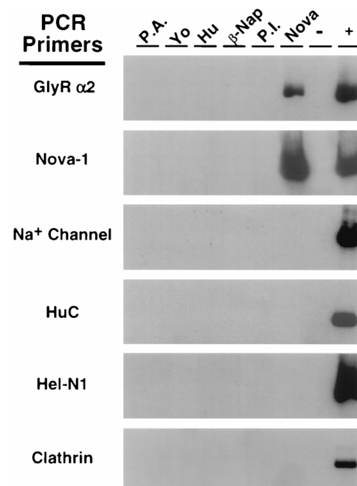

FIG. 5. Nova protein coimmunoprecipitates of with GlyR α2 and Nova-1 pre-mRNAs. Immunoprecipitations of mouse brain were performed as described previously (60) with affinity-purified rabbit anti-Nova-1 antibodies (Nova) or controls, including protein A-Sepharose (P.A.), paraneoplastic cerebellar degeneration serum (Yo), paraneoplastic antiserum directed against the Hu family of n-RBPs (Hu), affinity-purified rabbit anti-β-NAP antibodies, or preimmune serum (P.I.) as indicated. RNA present in the precipitates was phenol extracted as described previously (60) and subjected to RT-PCR amplification with the indicated primers. Nova and GlyR α2 primers bordered the putative Nova-1 binding site, and all PCR products were between 100 and 200 nucleotides in length (see Materials and Methods). RNA present in each immunoprecipitate was also mock amplified without reverse transcriptase (shown only for the Nova immunoprecipitate; −) as a control to ensure the RNA dependence of RT-PCR product. Genomic DNA was amplified (+) to ensure primer fidelity. The fidelity of each immunoprecipitation was confirmed by Western blot analysis (data not shown).

high-affinity GlyR α2 or Nova-1 RNA ligand. Preincubation of Nova-1 protein with affinity-purified POMA antibodies strongly inhibited Nova-1 binding to both RNAs; a threefold molar excess of antibody reduced RNA binding 80% (Fig. 4D). Nova-1 binding to GlyR α2 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 α2 and Nova-1 pre-mRNAs in vivo, we immunoprecipitated crude 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 α2, Nova-1, or a series of abundantly expressed neuronal pre-mRNAs. Nova-1-immunoprecipitated RNA gave strong RT-dependent PCR products with both GlyR α2 and Nova-1 primers but not with control primers (Fig. 5). Control immunoprecipitation with antiserum that recognizes a different family of abundantly expressed n-RBPs (Hu) yielded no detectable bound GlyR α2 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-β-NAP [45]) failed to immunoprecipitate neuronal RNAs. These immunoprecipitations demon-

strate that Nova-1 binds specifically to the GlyR α2 and Nova-1 pre-mRNAs in vivo and offer an opportunity to identify additional in vivo ligands.

DISCUSSION

Nova-1 is a sequence-specific KH-type RBP. We have shown that Nova-1, a neuron-specific KH-type RBP (9, 10), 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 have identified relatively long sequence-specific pyrimidine-rich RNAs that bind 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 threefold increase in binding affinity but is not essential for binding. 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 Rev-responsive element (27, 31) and between the U1-A protein and U1A RNA (51, 52, 64) and the interactions between the L32 RNA (68) and Epstein-Barr virus RNA 1 (17, 63) ligands and their ribosomal proteins (L32 and L22, respectively). In addition, the two in vivo Nova-1 RNA ligands that we have identified are both surrounded by potential stem structures, as assessed by the Zuker RNA folding algorithm ($\Delta G \cong -15$ [data not shown and reference 71]).

The length of the loop sequence element identified for Nova-1 compares favorably with those of core consensus RNA ligands identified for other RBPs, which typically consist of six to nine nucleotides (12, 29, 36, 62). 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 in both the in vitro RNA selection clones and the in vivo Nova-1 and GlyR α2 pre-mRNAs, suggesting a Nova-1 RNA binding consensus sequence of $[UCAU(Y)_{0-2}UCAU(Y)_{0-4}NCAU]$, although a more conservative consensus eliminating the pyrimidine bias is $[UCAU(N)_{0-4}]_3$. 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 (~3×10^9 nucleotides), 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 that we have identified.

KH domains are thought to be involved in RNA binding (10, 11, 56). A large number of KH-type RBPs have been identified since the motif was recognized in 1993 (57). These include the mammalian hnRNP proteins (e.g., hnRNP K) that are thought to be important for the processing of RNA (19) and FMR-1, the fragile-X gene, for which loss of function mutations lead to severe mental retardation (58). However, none have previously been demonstrated to bind RNA with sequence specificity. The functions of a number of KH domain-containing proteins in yeast and invertebrates have been described. MER-1 is a yeast RBP that mediates meiosis-specific alternative splicing of the *MER-2* gene, and PSI is a *Drosophila* protein that inhibits formation of a functional splice variant of the P-element transposase in somatic tissues (54). More recently, the mammalian splicing factors SF-1 (2) and KSR (41) have been cloned and found to contain one and four KH domains, respectively. In addition, the *Drosophila* Bicaudal-C (Bic-C) and the *Caenorhabditis elegans* MEX-3 KH domain-containing proteins have been suggested to have roles in RNA localization. In several of these instances, the KH domains themselves are thought to play critical roles in the function of these RBPs. A point mutation (I367N) within the second KH domain of FMR-1 results in severe mental retardation, and mutations within the conserved regions of KH domains of *gld-1*, a gene required for oocyte development in *C. elegans*, MEX-3, and Bic-C lead to loss of protein function (18, 32, 39). It is thought that such mutations within the KH domain alter exposed residues within a flexible loop that may directly contact RNA (42, 44). Our observations that the N3 Nova-1 construct containing only the third KH domain of Nova-1 is sufficient to mediate binding to SB2 and that a point mutation (analogous to the FMR-1 mutation) within KH3 abrogates this binding (Fig. 2A) demonstrate that the Nova-1 KH3 domain mediates sequence-specific RNA binding. However, our results do not rule out the possibility that additional residues present in our N3 construct outside the 36- to 38-amino-acid KH3 domain also interact with RNA ligands or that residues absent from our N3 construct (e.g., in the spacer region upstream of KH3) are involved in high-affinity RNA binding (Fig. 2A).

Identification of in vivo Nova-1 RNA ligands. We have found evidence that the Nova-1 protein interacts with GlyR α2 and Nova-1 pre-mRNAs in vivo by coimmunoprecipitation and RT-PCR analysis. In previous studies, protein-RNA coimmunoprecipitation has been used to purify and identify the RNA ligands of numerous autoimmune target antigens (e.g., snRNPs, Ro, and La [60]). This approach has also been used to identify DNA elements bound by transcription factors (e.g., ultrabiothorax, thyroid hormone receptor, and Myc/Max DNA binding elements [7, 23, 24]). Presumably as a result of the low-abundance ligands bound to Nova-1, we were unable to directly identify bound RNA ligands (8a) but were able to analyze candidate RNA ligands by RT-PCR analysis.

The biology of the candidate Nova-1 RNA ligands (GlyR α2 and Nova-1 pre-mRNAs) are consistent their binding Nova-1 protein in neurons. Both RNAs are expressed in Nova-1-expressing cells. Expression studies demonstrate that GlyR α2 mRNA is expressed in many brain regions which coexpress Nova-1, including midbrain, brainstem, and spinal cord in embryonic development and continuing into the adult (4). Single-cell studies reveal that Nova-1 protein and GlyR α2 mRNA are coexpressed within motor neurons in the spinal cord (9, 46, 63a). An interaction between Nova-1 protein and its own pre-mRNA would suggest that Nova-1, like many other RBPs in *Drosophila* and mammals (6, 8, 47), may regulate posttranscriptional processing of its own RNA. Our finding of specific polypyrimidine-rich RNA elements to which Nova-1 binds in vivo suggests potential functions for the Nova-1 protein. While polypyrimidine binding proteins have been implicated in the regulation of polyadenylation, translation, and stability (43), their best-characterized role is in the regulation of pre-mRNA splicing. The ubiquitous polypyrimidine tract binding protein U2AF is an essential splicing factor that binds to polypyrimidine tracts upstream of exon splice acceptors to recruit the U2 snRNP to the splice acceptor site (67). In *Drosophila*, the *sxl* protein binds to a specific polypyrimidine-rich RNA that is interspersed with several purine residues (55). Poor polypyrimidine tracts upstream of alternate splice sites in the tra pre-mRNA bind relatively weakly to U2AF, which is displaced by *sxl*, leading to alternate splice site selection (67). Similarly, polypyrimidine tract binding protein is believed to function as

a splice inhibitor by binding polypyrimidine tracts (37). It is noteworthy that the $(UCAUY)_3$ elements in both the GlyR α2 (35) and Nova-1 (9) pre-mRNAs are adjacent to exons that undergo alternative splicing in neurons and that a number of KH domain-containing RBPs in invertebrates and mammals act as alternative splicing regulators. Taken together, these data suggest that one role of Nova-1 may be to regulate alternative splicing in neurons.

Nova-1 and neurologic disease. The interaction of Nova-1 with an inhibitory glycine neurotransmitter receptor RNA may be important in the development of the paraneoplastic neurologic disorder. The neurologic symptoms of POMA are attributable to a loss of motor inhibition (15, 16, 38). Naturally occurring mutations of members of the glycine receptor family in both humans and mice (49, 50) lead to myoclonic neurologic symptoms similar to those seen in POMA. In hereditary hyperekplexia, a human myoclonic neurologic disorder, various point mutations have been found in GlyR α1 (53). Similarly, the spastic and spasmodic mice have a myoclonic phenotype and mutations within glycine receptor genes; spasmodic mice have point mutations in GlyR α1, and spastic mice have splicing defects due to transposable element insertion within a splice junction of GlyR 2B (49, 50). In addition, expression of a wild-type GlyR 2B transgene in spastic mice rescues the myoclonic phenotype (26). These observations suggest the possibility that the neurologic disease in POMA results from aberrant regulation of glycine receptor expression.

The observation that Nova-1 binding to GlyR α2 RNA in vitro is abrogated by POMA disease antibodies (Fig. 4D) suggests a potential mechanism of neuronal dysfunction in these patients: binding of POMA antibody to Nova-1 protein might disrupt the interaction between the Nova-1 protein and GlyR α2 pre-mRNA. Since Nova-1 recognizes GlyR α2 pre-mRNA sequences upstream from the alternatively spliced exon 3A, disruption of Nova-1 function could result in altered ratios of the mutually exclusive exons 3A and 3B. Although no specific function has been assigned to these alternatively spliced exons, they encode highly related 22-amino-acid domains present on the extracellular region of the receptor, suggesting that these domains may modify receptor-ligand interactions. Such a mechanism presupposes that anti-Nova antibodies are able to gain access to neurons, a process for which there is no direct evidence. However, Nova antibodies are present in high titer in the cerebrospinal fluid of POMA patients (9, 38). Moreover, some data have suggested that autoantibodies can penetrate living cells, including reports that antiribonucleoprotein antibodies may reach intranuclear antigens and that antineuronal antibodies can penetrate neurons (reviewed in references 1 and 16).

ACKNOWLEDGMENTS

We thank Nat Heintz, Titia de Lange, and Magda Konarska for helpful and stimulating discussions and Nat Heintz, Titia de Lange, and members of our laboratory for critical reading of the manuscript. We also thank Yolanda Yang for assistance developing the filter binding assays.

This work was supported by the NINDS (grant RO1 NS34389) and a Hirschl Career Scientist Award to R.B.D. R.J.B. was supported by NIH MSTP training grant 5T32 GM07739.

REFERENCES

1. **Alarcon-Segovia, D., A. Ruiz-Argüelles, and L. Llorente.** 1996. Broken dogma: penetration of autoantibodies into living cells. Immunol. Today **17:**163–164.
2. **Arning, S., P. Grüter, G. Bilbe, and A. Kramer.** 1996. Mammalian splicing factor SF1 is encoded by variant cDNAs and binds to RNA. RNA **2:**794–810.
3. **Bartel, D. P., M. L. Zapp, M. R. Green, and J. W. Szostak.** 1991. HIV-1 Rev regulation involves recognition of non-Watson-Crick base pairs in viral RNA. Cell **67:**529–536.
4. **Bechade, C., C. Sur, and A. Triller.** 1994. The inhibitory neuronal glycine receptor. Bioessays **16:**735–744.
5. **Bell, L., E. Maine, P. Schedl, and T. Cline.** 1988. Sex-lethal, a Drosophila sex determination switch gene, exhibits sex-specific RNA splicing and sequence similarity to RNA binding proteins. Cell **55:**1037–1046.
6. **Bell, L. R., J. I. Horabin, P. Schedl, and T. W. Cline.** 1991. Positive autoregulation of sex-lethal by alternative splicing maintains the female determined state in Drosophila. Cell **65:**229–240.
7. **Bigler, J., and R. N. Eisenman.** 1994. Isolation of a thyroid hormone-responsive gene by immunoprecipitation of thyroid hormone receptor-DNA complexes. Mol. Cell. Biol. **14:**7621–7632.
8. **Boelens, W., E. Jansen, W. van Venrooij, R. Stripecke, I. Mattaj, and S. Gunderson.** 1993. The human U1 snRNP-specific U1A protein inhibits polyadenylation of its own pre-mRNA. Cell **72:**881–892.
8a. **Buckanovich, R. J., and R. B. Darnell.** Unpublished data.
9. **Buckanovich, R. J., J. B. Posner, and R. B. Darnell.** 1993. Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. Neuron **11:**657–672.
10. **Buckanovich, R. J., Y. Y. Yang, and R. B. Darnell.** 1996. The onconeural antigen Nova-1 is a neuron-specific RNA binding protein, the activity of which is inhibited by paraneoplastic antibodies. J. Neurosci. **16:**1114–1122.
11. **Burd, C. G., and G. Dreyfuss.** 1994. Conserved structures and diversity of functions of RNA-binding proteins. Science **265:**615–621.
12. **Burd, C. G., and G. Dreyfuss.** 1994. RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. EMBO J. **13:**1197-1204.
13. **Caceres, J. F., S. Stamm, D. M. Helfan, and A. R. Krainer.** 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science **265:**1706–1709.
14. **Cullen, B. R.** 1994. RNA-sequence-mediated gene regulation in HIV-1. Infect. Agents Dis. **3:**68–76.
15. **Darnell, R. B.** 1994. Paraneoplastic syndromes, p. 137–141. *In* E. Feldmann (ed.), Current diagnosis in neurology. Mosby-Year Book, Inc., Philadelphia, Pa.
16. **Darnell, R. B.** 1996. Onconeural antigens and the paraneoplastic neurologic disorders: at the intersection of cancer, immunity and the brain. Proc. Natl. Acad. Sci. USA **93:**4529–4536.
16a. **Darnell, R. B.** Unpublished data.
17. **Dobbelstein, M., and T. Shenk.** 1995. In vitro selection of RNA ligands for the ribosomal L22 protein associated with Epstein-Barr virus-expressed RNA by using randomized and cDNA-derived RNA libraries. J. Virol. **69:**8027–8034.
18. **Draper, B. W., C. C. Mello, B. Bowerman, J. Hardin, and J. R. Priess.** 1996. MEX-3 is a KH domain protein that regulates blastomere identity in early C. elegans embryos. Cell **87:**205–216.
19. **Dreyfuss, G., M. J. Matunis, S. Piñol-Roma, and C. G. Burd.** 1993. hnRNP proteins and the biogenesis of mRNA. Annu. Rev. Biochem. **62:**289–321.
20. **Ellington, A., and J. Szostak.** 1990. In vitro selection of RNA molecules that bind specific ligands. Nature **346:**818–822.
21. **Engebrecht, J. A., K. Voelkel-Meiman, and G. S. Roeder.** 1991. Meiosis-specific RNA splicing in yeast. Cell **66:**1257–1268.
22. **Famulok, M., and J. W. Szostak.** 1992. Stereospecific recognition of tryptophan agarose by in vitro selected RNA. J. Am. Chem. Soc. **114:**3990–3991.
23. **Gould, A. P., J. J. Brookman, D. I. Strutt, and W. R.A.H.** 1990. Targets of homeotic gene control in Drosophila. Nature **348:**308–311.
24. **Grandori, C., J. Mac, F. Siebelt, D. E. Ayer, and R. N. Eisenman.** 1996. Myc-max heterodimers activate a DEAD box gene and interact with multiple E box-related sites *in vivo*. EMBO J. **15:**4344–4357.
25. **Green, R., A. D. Ellington, D. P. Bartel, and J. W. Szostak.** 1991. *In vitro* genetic analysis: selection and amplification of rare functional nucleic acids. Methods (Orlando) **2:**75–86.
26. **Hartenstein, B., J. Schenkel, J. Kuhse, B. Besenbeck, C. Kling, C. M. Becker, H. Betz, and H. Weiher.** 1996. Low level expression of glycine receptor beta subunit transgene is sufficient for phenotype correction in spastic mice. EMBO J. **15:**1275–1282.
27. **Heaphy, S., C. Dingwall, I. Ernberg, M. J. Gait, S. Green, J. Karn, A. D. Lowe, M. Singh, and M. A. Skinner.** 1990. HIV-1 regulator of virion expression (rev) protein binds to an RNA stem-loop structure located within the rev responsive element region. Cell **60:**685–693.
28. **Hedley, M. L., and T. Maniatis.** 1991. Sex-specific splicing and polyadenylation of *dsx* pre-mRNA requires a sequence that binds specifically to *tra-2* protein in vitro. Cell **65:**579–586.
29. **Heinrichs, V., and B. S. Baker.** 1995. The Drosophila SR protein RBP1 contributes to the regulation of doublesex alternative splicing by recognizing RBP1 RNA target sequences. EMBO J. **14:**3987–4000.
30. **Inoue, K., K. Hoshijima, H. Sakamoto, and Y. Shimura.** 1990. Binding of the Drosophila sex-lethal gene product to the alternative splice site of transformer primary transcript. Nature **344:**461–463.
31. **Jensen, K. B., L. Green, S. MacDougal-Waugh, and C. Tuerk.** 1994. Char-

acterization of an in vitro-selected RNA ligand to the HIV-1 Rev protein. J. Mol. Biol. **235:**237–247.
32. **Jones, A. R., and T. Schedl.** 1995. Mutations in *gld*-1, a female germ cell-specific tumor suppressor gene in *Caenorhabditis elegans*, affect a conserved domain also found in Src-associated protein Sam68. Genes Dev. **9:**1491–1504.
33. **Kamma, H., D. S. Portman, and G. Dreyfuss.** 1995. Cell type-specific expression of hnRNP proteins. Exp. Cell Res. **221:**187–196.
34. **Kjems, J., M. Brown, D. D. Chang, and P. A. Sharp.** 1991. Structural analysis of the interaction between the human immunodeficiency virus Rev protein and the Rev response element. Proc. Natl. Acad. Sci. USA **88:**683–687.
35. **Kuhse, J., A. Kuryatov, Y. Maulet, M. L. Malosio, V. Schmieden, and H. Betz.** 1991. Alternative splicing generates two isoforms of the α2 subunit of the inhibitory glycine receptor. FEBS Lett. **283:**73–77.
36. **Levine, T. D., F. Gao, P. H. King, L. G. Andrews, and J. D. Keene.** 1993. Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. Mol. Cell. Biol. **13:**3494–3504.
37. **Lin, C.-H., and J. Patton.** 1995. Regulation of alternative 3′ splice site selection by constitutive splicing factors. RNA **1:**234–245.
38. **Luque, F., H. Furneaux, R. Ferziger, M. Rosenblum, S. Wray, S. Schold, M. Glantz, K. Jaeckle, H. Biran, M. Lesser, W. Paulsen, M. River, and J. Posner.** 1991. Anti-Ri: an antibody associated with paraneoplastic opsoclonus and breast cancer. Ann. Neurol. **29:**241–251.
39. **Mahone, M., E. E. Saffman, and P. F. Lasko.** 1995. Localized *Bicaudal-C* RNA encodes a protein containing a KH domain, the RNA binding motif of FMR1. EMBO J. **9:**2043–2055.
40. **Malim, M., J. Hauber, S. Y. Le, J. Maizel, and B. Cullen.** 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature **338:**254–257.
41. **Min, H., C. W. Turck, and D. L. Black.** A new regulatory protein, KSR, mediates exon inclusion through an intronic splicing enhancer. Submitted for publication.
42. **Morelli, M. A. C., G. Stier, T. Gibson, C. Joseph, G. Musco, A. Pastore, and G. Trave.** 1995. The KH module has an αβ fold. FEBS Lett. **358:**193–198.
43. **Morris, D. R., T. Kakegawa, R. L. Kaspar, and M. W. White.** 1993. Polypyrimidine tracts and their binding proteins: regulatory sites for post-transcriptional modulation of gene expression. Biochemistry **32:**2931–2937.
44. **Musco, G., G. Stier, C. Joseph, M. A. C. Morelli, M. Nilges, T. Gibson, and A. Pastore.** 1996. Three-dimensional structure and stability of the KH domain: molecular insights into the fragile X syndrome. Cell **85:**237–245.
45. **Newman, L. S., M. O. McKeever, H. J. Okano, and R. B. Darnell.** 1995. β-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein. Cell **82:**773–783.
46. **Racca, C., A. Gardiol, and A. Triller.** 1997. Dendritic and postsynaptic localizations of glycine receptor α subunit mRNAs. J. Neurosci. **17:**1691–1700.
47. **Rongo, C., E. R. Gavis, and R. Lehmann.** 1995. Localization of oskar RNA regulates oskar translation and requires oskar protein. Development **121:**2737–2746.
48. **Ruskin, B., P. D. Zamore, and M. R. Green.** 1988. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. Cell **52:**207–219.
49. **Ryan, S. G., M. S. Buckwalter, J. W. Lynch, C. A. Handford, L. Segura, R. Shiang, J. J. Wasmuth, S. A. Camper, P. Schofield, and P. O'Connell.** 1994. A missense mutation in the gene encoding the alpha 1 subunit of the inhibitory glycine receptor in the spasmodic mouse. Nat. Genet. **7:**131–135.
50. **Saul, B., V. Schmieden, C. Kling, C. Mulhardt, P. Gass, J. Kuhse, and C. M. Becker.** 1994. Point mutation of glycine receptor alpha 1 subunit in the spasmodic mouse affects agonist responses. FEBS Lett. **350:**71–76.
51. **Scherly, D., W. Boelens, N. A. Dathan, W. J. van Venrooij, and I. W. Mattaj.** 1990. Major determinants of the specificity of interaction between small nuclear ribonucleoproteins U1A and U2B and their cognate RNAs. Nature **345:**502–506.
52. **Scherly, D., W. Boelens, W. J. van Venrooij, N. A. Dathan, J. Hamm, and I. W. Mattaj.** 1989. Identification of the RNA binding segment of human U1 A protein and definition of its binding site on U1 snRNA. EMBO J. **8:**4163–4170.
53. **Shiang, R., S. G. Ryan, Y. Z. Zhu, A. F. Hahn, P. O'Connell, and J. J. Wasmuth.** 1993. Mutations in the alpha 1 subunit of the inhibitory glycine receptor cause the dominant neurologic disorder, hyperekplexia. Nat. Genet. **5:**351–358.
54. **Siebel, C. W., A. Admon, and D. C. Rio.** 1995. Soma-specific expression and cloning of PSI, a negative regulator of P element pre-mRNA splicing. Genes Dev. **9:**269–283.
55. **Singh, R., J. Valcarcel, and M. R. Green.** 1995. Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. Science **268:**1173–1176.
56. **Siomi, H., M. Choi, M. Siomi, R. Nussbaum, and G. Dreyfuss.** 1994. Essential role for KH domains in RNA binding: impaired RNA binding by a mutation in the KH domain of FMR1 that causes fragile X syndrome. Cell **77:**33–39.
57. **Siomi, H., M. J. Matunis, W. M. Michael, and G. Dreyfuss.** 1993. The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. Nucleic Acids Res. **21:**1193–1198.
58. **Siomi, H., M. Siomi, R. Nussbaum, and G. Dreyfuss.** 1993. The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell **74:**291–298.
59. **Stamm, S., M. Q. Zhang, T. G. Marr, and D. M. Helfman.** 1994. A sequence compilation and comparison of exons that are alternatively spliced in neurons. Nucleic Acids Res. **9:**1515–1526.
60. **Steitz, J.** 1989. Immunoprecipitation of ribonucleoproteins using autoantibodies. Methods Enzymol. **180:**468–481.
61. **Szostak, J. W., and A. D. Ellington.** 1993. In vitro selection of functional RNA sequences. The RNA world. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
62. **Tacke, R., and J. L. Manley.** 1995. The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. EMBO J. **14:**3540–3551.
63. **Toczyski, D. P., and J. A. Steitz.** 1993. The cellular RNA-binding protein EAP recognizes a conserved stem-loop in the Epstein-Barr virus small RNA EBER 1. Mol. Cell. Biol. **13:**703–710.
63a. **Triller, A., and R. B. Darnell.** Unpublished data.
64. **Tsai, D. E., D. S. Harper, and J. D. Keene.** 1991. U1-snRNP-A protein selects a ten nucleotide consensus sequence from a degenerate RNA pool presented in various structural contexts. Nucleic Acid Res. **19:**4931–4936.
65. **Tuerk, C., and L. Gold.** 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science **249:**505–510.
66. **Tuerk, C., and S. MacDougal-Waugh.** 1993. In vitro evolution of functional nucleic acids: high-affinity RNA ligands of HIV-1 proteins. Gene **137:**33–39.
67. **Valcarcel, J., R. Singh, P. Zamore, and M. Green.** 1993. The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. Nature **362:**171–175.
68. **Vilardell, J., and J. R. Warner.** 1994. Regulation of splicing at an intermediate step in the formation of the spliceosome. Genes Dev. **8:**211–220.
69. **Zamore, P. D., J. G. Patton, and M. R. Green.** 1992. Cloning and domain structure of the mammalian splicing factor U2AF. Nature **355:**609–614.
70. **Zapp, M., and M. Green.** 1989. Sequence-specific RNA binding by the HIV-1 rev protein. Nature **342:**714–716.
71. **Zuker, M.** 1989. On finding all suboptimal foldings of a RNA molecule. Science **244:**48–52.