

Nova autoregulation reveals dual functions in neuronal splicing

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The Nova family of neuron-specific RNA-binding proteins were originally identified as targets in an autoimmune neurologic disease characterized by failure of motor inhibition. Nova-1 regulates alternative splicing of pre-mRNAs encoding the inhibitory neurotransmitter receptor subunits GABA_AR γ 2 and GlyR α 2 by directly binding intronic elements, resulting in enhancement of exon inclusion. Here we identify exon E4 in the Nova-1 pre-mRNA itself, encoding a phosphorylated protein domain, as an additional target of Nova-dependent splicing regulation in the mouse spinal cord. Nova binding to E4 is necessary and sufficient for Nova-dependent exon exclusion. E4 harbors five repeats of the known Nova-binding tetranucleotide YCAY and mutation of these elements destroys Nova-dependent regulation. Furthermore, swapping of the sites from Nova-1 and GABA_AR γ 2 indicates that the ability of Nova to enhance or repress alternative exon inclusion is dependent on the position of the Nova-binding element within the pre-mRNA. These studies demonstrate that in addition to its previously described role as a splicing activator, Nova autoregulates its own expression by acting as a splicing repressor.

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Introduction

RNA-binding proteins are of fundamental importance for the coordination and regulation of post-transcriptional gene expression in eukaryotes, from pre-mRNA splicing to 5'-end capping, cleavage and polyadenylation, nuclear mRNA export, localization, translation and degradation. The contemporary view that the human genome contains approximately 30 000 genes is remarkably different from that anticipated 10 years ago, when numbers of genes higher than 10⁵ were commonly estimated. One of the key mechanisms for creating protein diversity from a limited number of genes is through

alternative splicing of pre-mRNAs. Recent array-based studies suggest that at least 75 % of multi-exon genes undergo alternative splicing (Johnson *et al*, 2003). Both ubiquitous and tissue-restricted RNA-binding proteins are critical to this process (Black, 2003), especially in highly complex systems such as the brain, which has been estimated to be the tissue with the largest total number of tissue-specific alternative splicing events (Xu *et al*, 2002).

The Nova proteins were the first mammalian tissue-specific splicing factors identified. Nova proteins form a family of RNA-binding proteins that are expressed exclusively in neurons of the central nervous system (CNS; Buckanovich *et al*, 1993, 1996; Yang *et al*, 1998). They were identified as the target antigens in paraneoplastic opsoclonus-myoclonus ataxia (POMA), a rare immune-mediated disorder associated with ectopic Nova expression in tumors, and characterized by deficits in inhibitory motor control in the eyes, limbs and trunk (reviewed in Darnell and Posner, 2003).

Nova proteins are sequence-specific RNA-binding proteins that harbor three KH-type RNA-binding domains. Extensive genetic, biochemical and crystallographic studies have demonstrated the ability of Nova to bind RNA containing repeats of the sequence YCAY, both *in vivo* and *in vitro* (Buckanovich and Darnell, 1997; Yang *et al*, 1998; Lewis *et al*, 2000; Jensen *et al*, 2000b). Nova regulates alternative splicing of the GABA_AR γ 2 and GlyR α 2 inhibitory neurotransmitter receptor subunit pre-mRNAs via binding to intronic YCAY-repeat elements (Jensen *et al*, 2000a; Dredge and Darnell, 2003). A putative Nova-binding element was also predicted 8 nt downstream of the alternatively spliced penultimate exon, E4, of Nova-1 (previously termed exon H; Buckanovich *et al*, 1993). Nova fusion protein was shown to bind this sequence with high affinity ($K_d \sim 20$ nM) *in vitro* and native Nova protein in mouse brain lysates co-immunoprecipitates Nova-1 pre-mRNA (Buckanovich and Darnell, 1997). This exon encodes a domain harboring a number of potential sites for phosphorylation by serine/threonine protein kinases.

To further our understanding of Nova function and its role in the regulation of alternative splicing in the brain, we sought to determine whether E4 encodes for a phosphorylated protein domain and whether Nova-1 is capable of autoregulation of alternative splicing of E4. We find that E4 encodes an S/T-rich domain within the Nova-1 protein that is phosphorylated *in vivo*. Moreover, Nova does indeed autoregulate alternative splicing of E4, both in cell transfection assays and *in vivo*; splicing of E4 is altered in the CNS of Nova haploinsufficient (Nova-1^{+/-}) mice compared to wild type (Nova-1^{+/+}). Surprisingly, Nova inhibits rather than activates E4 inclusion, and mapping the Nova-binding site that mediates this action identifies a necessary and sufficient element within the E4 exon itself. Moreover, we demonstrate that the ability of Nova to activate or inhibit exon inclusion is dependent on the position of the Nova-binding site within the regulated pre-mRNA. This work demonstrates that Nova is

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able to function as a splicing repressor in addition to its known role as a splicing activator.

Results

Nova-1 domain 4 is phosphorylated in vivo

The physiological consequence of the inclusion of the peptide sequence encoded by exon 4 (henceforth called 'domain 4') in Nova-1 protein is unknown. E4 encodes a 24-amino-acid protein domain rich in serine and threonine residues (50%), four of which are adjacent to prolines (Figure 1B), which suggests that these residues may be substrates for phosphorylation by serine/threonine protein kinases (Kemp and Pearson, 1990). We tested whether Nova-1 is phosphorylated *in vivo* and whether the two alternatively spliced isoforms are phosphorylated differentially. Plasmids encoding Nova-1_L (E4 included) or Nova-1_S (E4 excluded) were transiently transfected into N2A cells. The cells were then labeled with ³²P-orthophosphate followed by immunoprecipitation for T7-tagged Nova-1 protein. We found that Nova-1_L was robustly phosphorylated, more than 10-fold greater than the level of Nova-1_S phosphorylation (Figure 1A and D).

Analysis of the sequence of domain 4 predicted six residues with the potential for phosphorylation by either protein kinase C (PKC) or glycogen synthase kinase 3 (GSK3) (Figure 1B). To test these predictions, we substituted alanine residues for various serine and threonine residues by site-directed mutagenesis. Phosphorylation was unchanged when sites 5 and 6 were mutated but was markedly reduced, to the baseline level seen for Nova-1_S, when sites 2 and 3, or sites 2, 5 and 6 were mutated, suggesting that site 2 is necessary for phosphorylation (Figure 1C). Moreover, alanine mutation of site 2 alone was sufficient to reduce the phosphorylation to the level of Nova-1_S (Figure 1D). These results indicate that either site 2 is the only site of phosphorylation within domain 4 or that its phosphorylation is required for further phosphorylation to occur, a process termed primed phosphorylation. Primed phosphorylation has been demonstrated for a number of kinases including GSK3 (Fiol *et al*, 1987; Biondi and Nebreda, 2003). Taken together, these data confirm that Nova-1 domain 4 is phosphorylated *in vivo*, and identify the second serine as a major site of phosphorylation.

Nova-1 alternative splicing is altered in Nova-1 heterozygous mice

The exon:intron structure of the Nova-1 gene is shown in Figure 2A. Exon 2 encodes the first KH RNA-binding domain, KH1; KH2 and KH3 are both encoded within exon 5. The penultimate exon, E4 (previously referred to as exon H), is alternatively spliced to generate two isoforms of Nova-1 (Buckanovich *et al*, 1993). The genomic sequence surrounding and including E4 shown in Figure 2A is highly conserved from mouse to human (88% identity, BLAST2) and rich in YCAY repeats: 32 and 28 in mouse and human respectively, compared to 15 expected in a random sequence of this length. Of these, five are present within the 72 nt exon E4; this is significantly higher than the expected frequency of 1.1 for this exon.

To determine whether Nova-1 may play a role in the regulation of E4 splicing *in vivo*, we used semiquantitative RT-PCR to analyze alternative splicing of this exon in the spinal cords of wild-type and Nova-1 heterozygous mice,

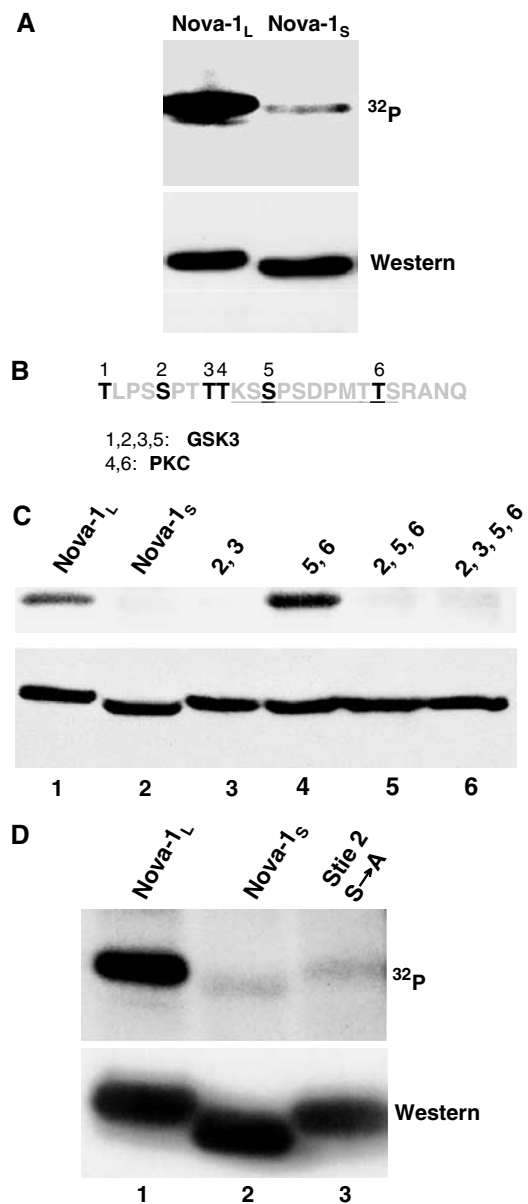


Figure 1 Nova-1 domain 4 is phosphorylated *in vivo*. (A) N2A cells were transiently transfected with T7-tagged Nova constructs with (Nova-1_L) or without (Nova-1_S) inclusion of domain 4, and ³²P-orthophosphate was added to the medium. After 5 h incubation, cells were harvested, lysed and immunoprecipitated with anti-T7-tag antibody. The pellet was treated with RNase prior to separation by SDS-PAGE, transferred to nitrocellulose and exposed to autoradiography film (upper panel). Subsequently, the membrane was probed by Western blot with anti-T7-tag antibody (lower panel). (B) Amino-acid sequence encoded by E4. The residues in bold and numbered 1–6 are phosphorylation sites predicted by Prosite (<http://us.expasy.org/prosite/>) and Scansite (<http://scansite.mit.edu>). The underlined segment is a PEST region predicted by PESTfind (<http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/>). (C) Identification of the phosphorylation site by site-directed mutagenesis. Metabolic phosphorylation assays were performed as in (A). Expression constructs encoding Nova-1_L (lane 1) or Nova-1_S (lane 2), and point mutants in which the indicated residue numbers indicated in (B) were mutated in Nova-1_L to alanines (lanes 3–6) were utilized. (D) Expression constructs encoding Nova-1_L (lane 1), Nova-1_S (lane 2) or Nova-1_L with a serine to alanine mutation at site 2 were utilized for the transfection/metabolic labeling assay as in (A).

which express reduced levels of Nova-1 protein (Jensen *et al*, 2000a). RT-PCR revealed a decrease in E4 exclusion in heterozygous mice to 0.75-fold of the wild-type level (Figure 2B and C). This contrasts with previously studied examples of Nova-dependent splicing regulation in which Nova enhances alternative exon inclusion. To confirm these results, we also performed RNase protection assays (RPA), which revealed a 24.6% decrease in steady-state Nova-1 mRNA level relative to actin in heterozygous mice compared to wild-type levels (data not shown). In 14 littermate pairs spanning postnatal day 7 through 30, E4 inclusion was increased $19 \pm 10\%$ ($P < 0.001$) in heterozygous mice compared to their wild-type littermates (Figure 2B, C, E and F), demonstrating a relationship between Nova-1 expression and E4 inclusion, and suggesting that Nova-1 could directly mediate E4 exclusion.

The magnitude of the effect on E4 splicing in Nova-1 heterozygous mice, while small, was comparable to that seen in GABA_AR γ 2 splicing between wild type and Nova-1 heterozygotes (Figure 2C). Furthermore, up to a 3.9-fold increase in Nova-1 E4 inclusion has been observed in the brains of Nova-2 null mice compared to their wild-type littermates (M Ruggiu, BK Dredge and RB Darnell, unpublished data), strengthening our conclusion that Nova-1 alternative splicing is altered in Nova-deficient mice, and the prediction that both Nova-1 and Nova-2 proteins likely act through direct inhibition of Nova-1 E4 inclusion.

The presence or absence of domain 4 does not influence Nova-1 regulation of alternative splicing of Nova-1 and GABA_AR γ 2 minigenes

To investigate whether Nova is able to act directly on its own transcript to autoregulate E4 splicing, a minigene construct was generated that contains 957 nt of mouse genomic sequence surrounding and including E4. This was flanked at the extreme 5' and 3' ends by donor and acceptor sites derived from the human β -globin gene, which we have previously shown are not themselves subject to Nova-dependent splicing regulation (Dredge and Darnell, 2003). This construct (pE4₈₈₅) was transiently transfected into N2A and 293T cells in the presence or absence of pNova-1_L or pNova-1_S plasmid (that is, with or without domain 4) and spliced products were analyzed by primer extension (Figure 3A). Consistent with the analysis of Nova-1 splicing in Nova-deficient mice, cotransfection of either pNova-1_L or pNova-1_S caused a dose-dependent decrease in E4 inclusion from ≥ 95 to $\leq 12\%$ in both cell lines. No products were detected

in mock-transfected cells (Figure 3A, lanes M). These data confirm that both Nova-1_L and Nova-1_S can repress E4 inclusion by a similar magnitude and indicate that the sequence surrounding and including E4 is sufficient to direct Nova-mediated splicing regulation. Given the number of YCAY motifs in this fragment, these results are consistent with the hypothesis that sequence-specific interactions between Nova-1 and its own pre-mRNA are necessary for splicing autoregulation.

We also tested whether domain 4 influences Nova-dependent splicing of GABA_AR γ 2 and GlyR α 2 minigenes in cell lines since previous analyses have used only pNova-1_L (Jensen *et al*, 2000a; Dredge and Darnell, 2003). In all cases tested, the absence of domain 4, or mutation of the phosphorylation site identified in Figure 1 had no effect on the direction or magnitude of the Nova-1-dependent splicing effect (Figure 3B and data not shown).

Exonic sequence is necessary and sufficient for Nova-dependent splicing autoregulation of exon 4

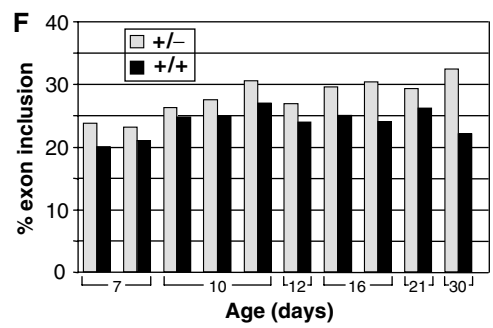
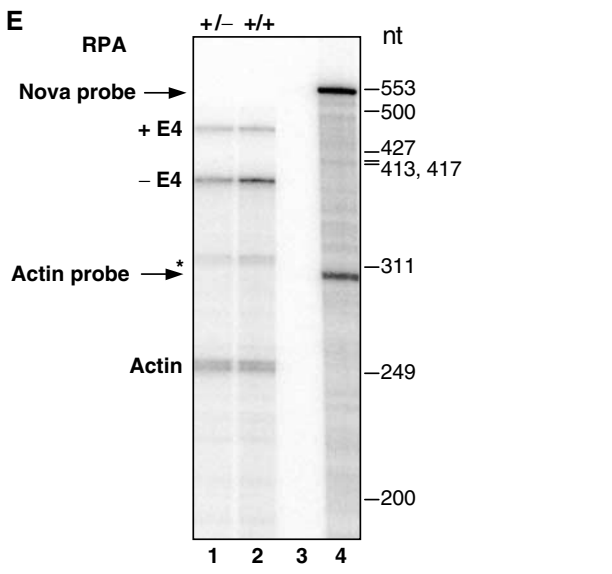
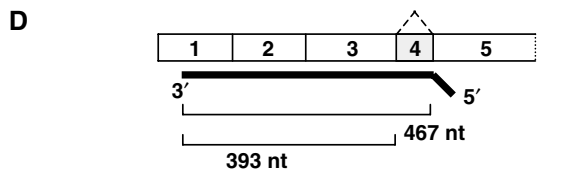
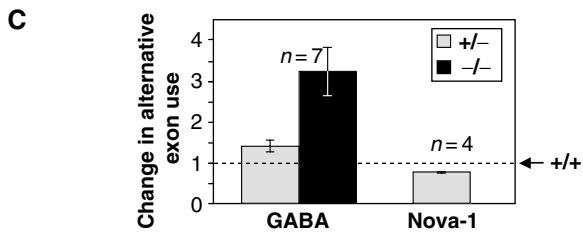
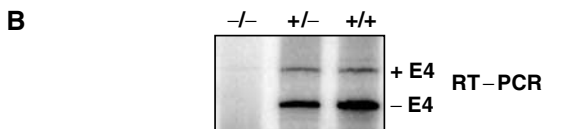
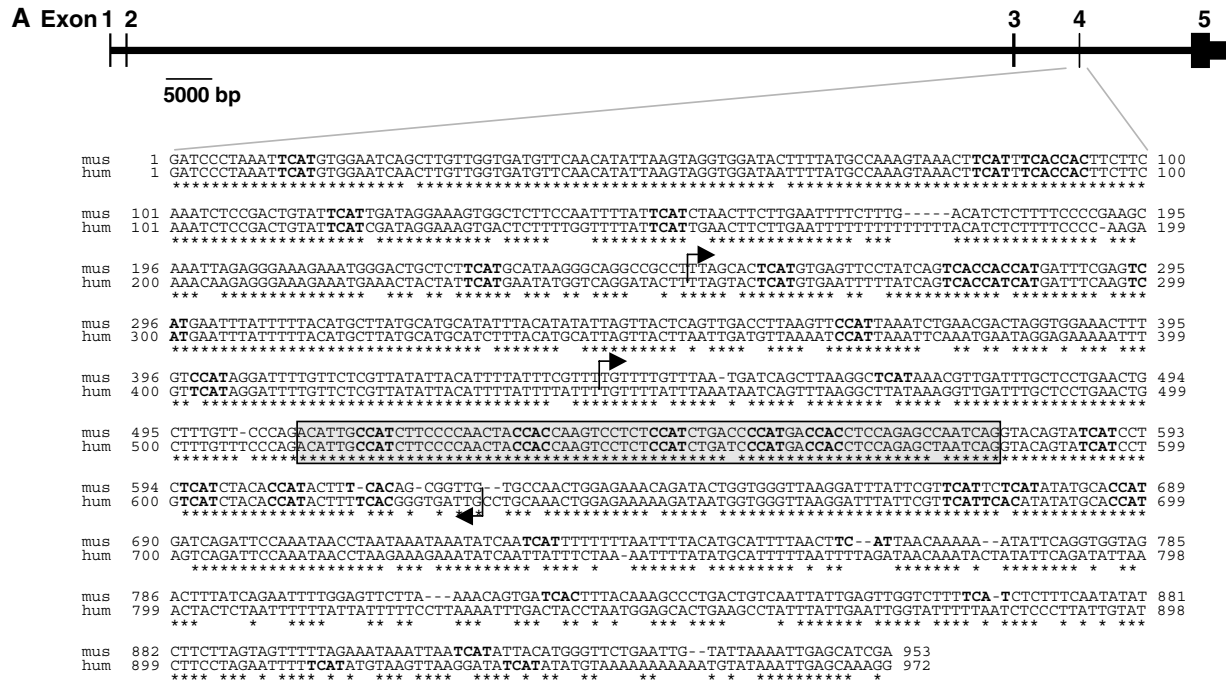
We further analyzed the sequence requirements for Nova-dependent splicing autoregulation within the fragment surrounding and including E4 present in pE4₈₈₅. Initial experiments mutating the four intronic YCAY repeats immediately downstream of E4, predicted earlier to be a Nova-binding site (Buckanovich and Darnell, 1997), showed no effect on Nova-dependent splicing (data not shown). We therefore generated a series of constructs to identify a region necessary and sufficient for Nova-dependent splicing regulation. Figure 4A shows that deletion of the majority of the intronic regions surrounding E4 has no effect on Nova's ability to regulate E4 exclusion (compare constructs pE4₈₈₅, pE4₃₀₇, pE4₁₁₆); pNova-1_L transfection resulted in a decrease in E4 inclusion from almost 100 to 10–21% in both N2A and 293T cell lines. Furthermore, the presence of E4 itself in the minigene was sufficient to mediate a robust Nova-dependent effect on splicing; pNova-1_L transfection resulted in a decrease in E4 inclusion from almost 100 to 42 or 30% in N2A and 293T cells respectively (construct pE4).

To determine whether the YCAY repeats within E4 are necessary for Nova-dependent repression of this exon, we mutated the first two (m1, 2; Figure 4B), last three (m3, 4, 5) or all five (m1–5) YCAY repeats to YAAY, a sequence that does not bind to Nova (Buckanovich and Darnell, 1997; Jensen *et al*, 2000a; Lewis *et al*, 2000). These mutations did not change the baseline level of inclusion of the exon (Figure 4B), indicating that sequences required for the recognition and

Figure 2 Change in alternative splicing of Nova-1 E4 in Nova-1 mouse spinal cord. (A) Exon structure of the mouse Nova-1 gene, adapted from <http://genome.ucsc.edu/>. Scale bar represents intronic lengths, and exons are to approximate scale only. Below is a comparison of the mouse (mus) and human (hum) sequence surrounding alternatively spliced E4 (gray box) by ClustalW alignment (MacVector). YCAY motifs are in boldface type, and arrows indicate regions included in minigene constructs pE4₃₀₇ and pE4₁₁₆ (see Figure 4). (B) RT-PCR. Spinal cord RNA isolated from P17 Nova-1 null, heterozygous and wild-type mouse littermates was analyzed by semiquantitative RT-PCR using primers to exons 3 and 5. The reverse primer was ³²P-labeled. (C) Comparison of the magnitude of splicing defects in heterozygous mice analyzed by RT-PCR. The RT-PCR data from panel B together with additional samples were plotted as the change in alternative exon use (with the ratio of alternative exon included:excluded form from the wild type set to 1) for comparison with previously published RT-PCR data; Jensen *et al*, 2000a). Error bars indicate the standard deviation, n = number of litters assayed. (D) Schematic representation of the ³²P-labeled antisense RNA probe used for RPA and the resulting product sizes used for quantitation. An antisense RNA probe specific for β -actin mRNA was also made. (E) RPA. Spinal cord RNA isolated from P16 Nova-1 heterozygous mouse and wild-type littermate was analyzed by RPA. The specific activities of the probes were adjusted to give protected fragments of approximately equal intensities for each message. Lane 3: yeast RNA control; lane 4: undigested probes. The asterisk marks an unidentified product that was protected by the Nova-1 probe. (F) Quantitation of the data presented in (E) together with additional RNA samples. Products were quantitated by phosphorimager and corrected for the expected number of ³²P-labeled uridines per protected RNA fragment.

inclusion of this exon were not disrupted. However, all of the mutations had a dramatic effect on Nova's ability to repress exon inclusion. In both cell lines, mutation of all five YCAY repeats in construct pE4 or pE4₁₁₆ completely abolished the ability of Nova to regulate E4 splicing (Figure 4B, m1-5, and

data not shown). In N2A cells, cotransfection of pNova_L resulted in a decrease in E4 inclusion from almost 100 to 43% (pE4) compared to a decrease from 100% to only 92% (m1, 2) or 87% (m3, 4, 5) upon partial mutation of the exon. Similarly, in 293T cells, addition of pNova_L decreased E4



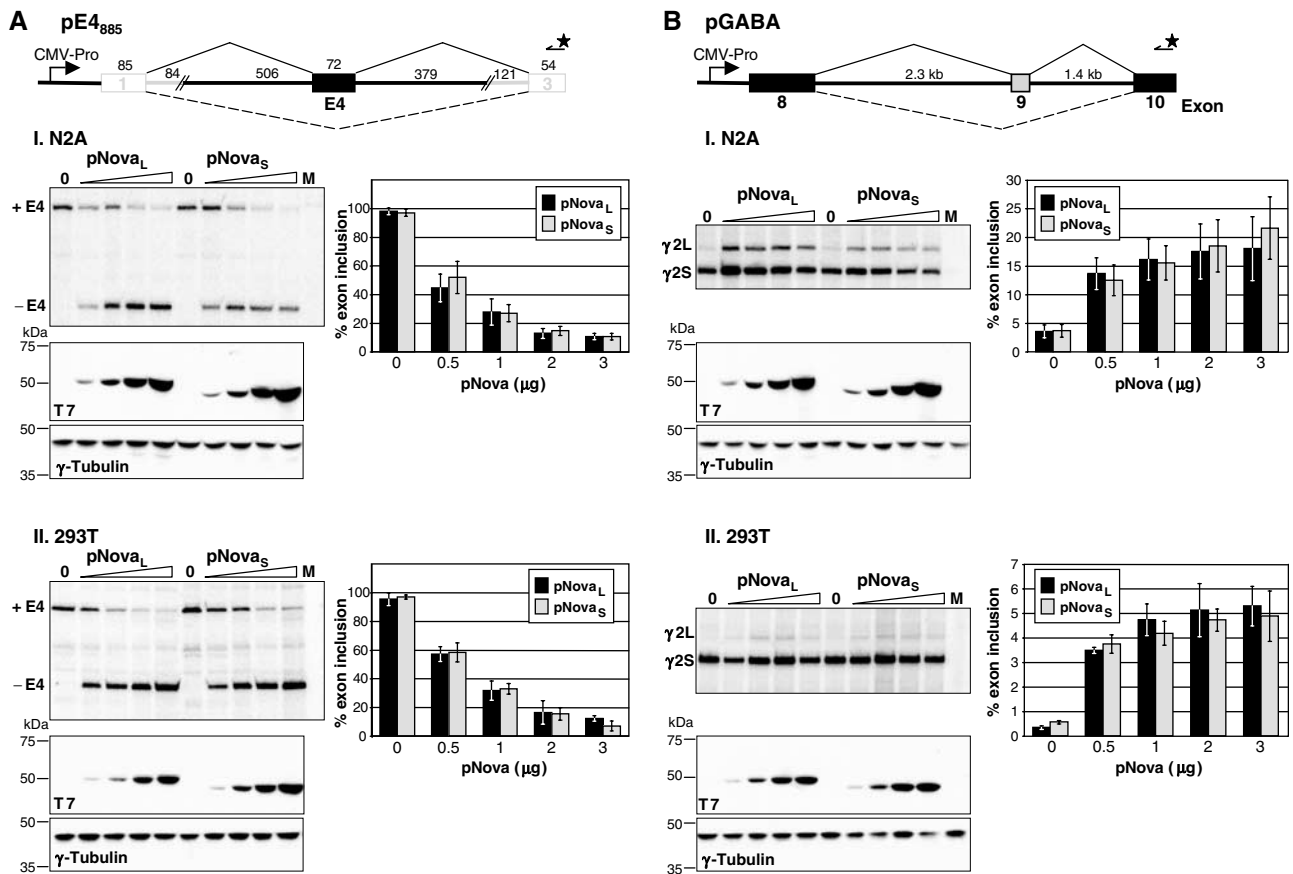


Figure 3 Nova-1 regulates alternative splicing of Nova-1 and GABA_AR γ 2 in cell lines independent of domain E4 inclusion. **(A)** Nova-1 minigene construct used for transient transfection assays harboring 957 of Nova-1 mouse genomic sequence (black lines, black filled box; sequence is shown in Figure 2) flanked by donor and acceptor sites derived from the human β -globin gene (gray lines, empty boxes). The sizes (in base pairs) are shown above each element. The starred arrow depicts the ³²P-labeled primer used for primer extension analysis. Distances are not to scale. RNA was isolated from N2A (I) or 293T (II) cells transiently transfected with pE4₈₈₅ and increasing amounts of pNova-1_L or pNova-1_S (0, 0.5, 1, 2 and 3 μ g) and analyzed by primer extension (upper panels). Mock transfections were performed using no plasmid DNA (lanes M). Below are Western blots using anti-T7 antibody (middle panels) showing the titration of T7-tagged Nova-1 protein levels. The blots were stripped and reprobbed with anti- γ -tubulin antibody (lower panels) as a loading control. The graphs depict quantitation of three independent transfection experiments, displayed as the average percent central exon inclusion [Phosphorimager counts in the exon included product/(counts in the exon included product + counts in the exon excluded product) \times 100], \pm standard deviation. **(B)** Transient transfection assays were performed and analyzed as in (A) using a GABA_AR γ 2 (pGABA) minigene containing the full mouse intronic regions surrounding and including alternatively spliced exon 9 plus shortened exons 8 and 10.

inclusion from 100 to 32% (pE4) versus 92% (m1, 2) or 82% (m3, 4, 5). We also tested mutation of each YCA Y motif individually in 293T cells and found that in a single experiment, Nova reduced E4 inclusion from 100 to 31% (unmutated), 70% (m1), 59% (m2), 47% (m3), 50% (m4) or 72% (m5) upon cotransfection of 2.0 μ g of pNova1_L (data not shown). Taken together, these data demonstrate that the YCA Y repeats in E4 are necessary and sufficient for Nova-dependent splicing regulation of this exon and that all five YCA Y motifs contribute to the magnitude of the effect.

The magnitude of Nova's effect on splicing of construct pE4 was smaller than that seen when 47 bp of adjacent downstream Nova-1 intronic sequence was also present in the minigene (Figures 4A and 5), implying that the intronic sequence may add to the Nova-dependent exon repression. Indeed, when the four intronic YCA Y repeats downstream of E4 in construct pE4₁₁₆ were mutated to YAAY, the resulting

minigene (pE4₁₁₆mi) behaved almost identically to one lacking Nova-derived intronic sequence (pE4) (Figure 5). However, inclusion of the intronic regions only, surrounding a heterologous exon (pGlo2 Δ b₁₁₆), decreased basal exon inclusion to 74–79%, but was not sufficient to support significant Nova-dependent E4 repression. We conclude that the predicted intronic Nova-binding site immediately downstream of E4 is neither necessary nor sufficient for robust Nova-dependent splicing regulation in this context, but may enhance the ability of Nova-1 to repress E4 inclusion.

To determine which regions of this RNA sequence are necessary for high-affinity Nova-1 binding, the 5' and 3' boundaries of the smallest RNA that retains high affinity to Nova-1 were determined. *In vitro*-transcribed RNA bounding E4 (Figure 6) was labeled with ³²P at either the 5' or 3' end and subjected to mild alkaline hydrolysis. The RNA was then incubated with Nova-1_L fusion protein (NFP), and bound RNAs captured by filtration through nitrocellulose, eluted

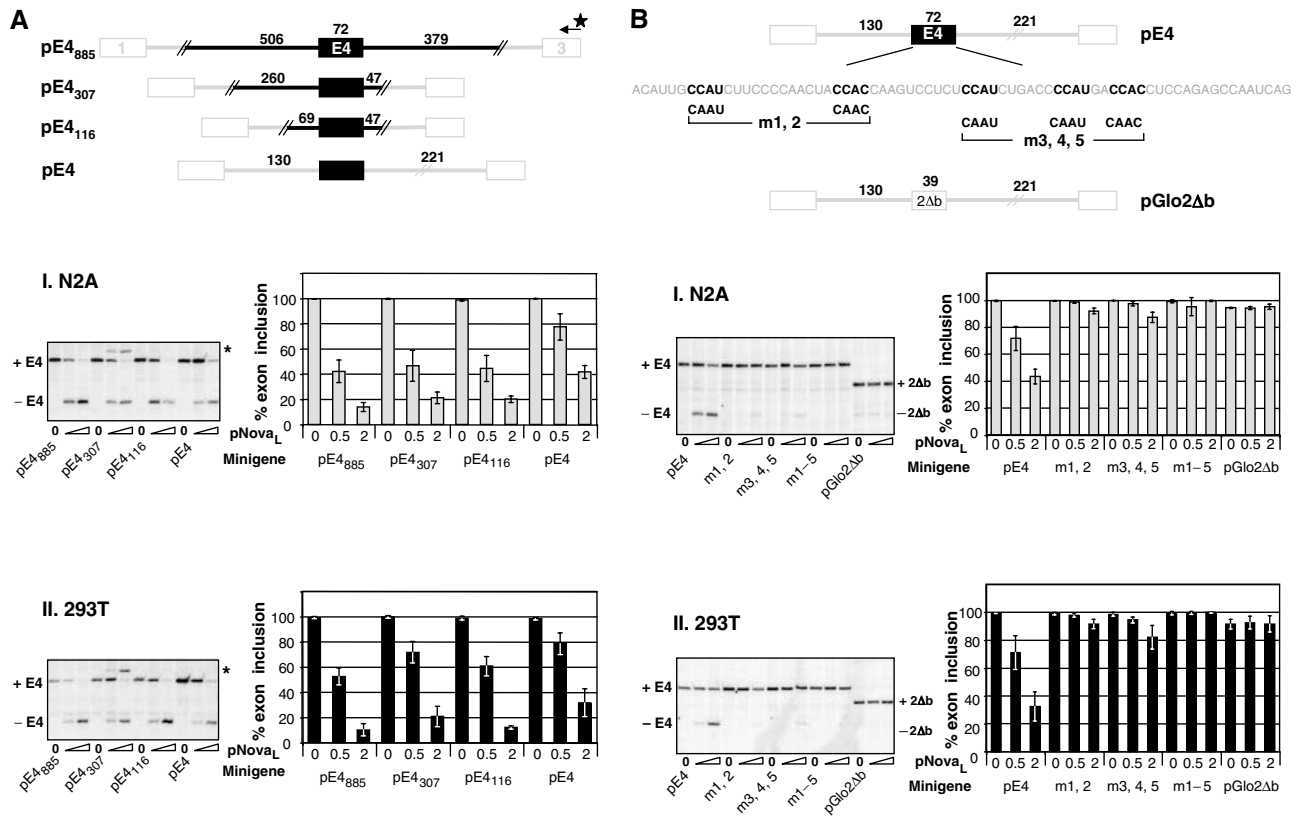


Figure 4 Exon E4 is necessary and sufficient for regulation of alternative splicing by Nova-1. (A) The minigenes depicted were cotransfected into N2A (I) or 293T (II) cells with increasing amounts of pNova-1_L (0, 0.5 and 2.0 μg); spliced products were measured by primer extension and phosphorimage analysis as in Figure 3 and titration of transfected pNova-1_L expression was monitored by Western blotting (not shown). The asterisk denotes an uncharacterized product consistently seen with construct pE4₃₀₇. (B) Mutagenesis of the YCAAY repeats in E4 abrogates Nova-dependent regulation of alternative splicing. YAAAY mutations were made in the first two (m1, 2), last three (m3, 4, 5) or all five (m1-5) YCAAY motifs of E4 in construct pE4. Plasmid pGlo2Δb was used as a Nova-independent control. The minigenes were transfected and analyzed as in (A).

and analyzed by denaturing PAGE. Two boundaries were detected in each case; when the RNA was 5' labeled, the 3' boundaries mapped 1 nt downstream of the third and second YCAAY repeats of E4 (Figure 6A (I), 1° and 2°, respectively). When the RNA was 3' labeled, the 5' boundaries mapped 1 nt upstream of the seventh (Figure 6A (II), 1°) or 2 nt upstream of the eighth (Figure 6A (I), 2°) YCAAY repeat of the test RNA such that three or two YCAAY repeats were contained within the smallest RNA bound (Figure 6A (II), 1° and 2°, respectively). In both cases, the secondary boundary was only apparent at a higher protein concentration, suggesting that NFP may bind these shorter RNAs with lower affinity than it does the longer sequences. The results suggest that Nova-1 is able to bind RNA sequences either derived from exon E4 or from the intronic region immediately downstream *in vitro*. Thus, this experiment did not define a single high-affinity Nova-binding site—rather we can conclude that a minimum of two to three YCAAY repeats are necessary for high-affinity Nova binding.

Nitrocellulose filter-binding assays were performed to determine the affinity of Nova-1 binding to its own pre-mRNA. NFP bound the 133 nt Nova-1 RNA bounding E4 with high affinity ($K_d = 2.7$ nM), approximately five-fold higher affinity than binding to a 159 nt RNA derived from the Nova binding sequence in GABA_ARγ2 ($K_d = 13.3$ nM; Figure 6B; Dredge and Darnell, 2003). An irrelevant β-glo-

bin-derived RNA of similar length (glo, 175 nt) showed no detectable binding (Figure 6B). Thus, Nova-1 binds to its own transcript with physiologically relevant affinity.

Binding site position determines whether Nova enhances or represses alternative exon inclusion

We recently described a Nova-dependent intronic splicing enhancer (NISE) necessary and sufficient for regulation of GABA_ARγ2 alternative splicing by Nova (Dredge and Darnell, 2003). Aside from harboring repeats of the tetranucleotide YCAAY over a similar length of sequence, this site has little in common with the Nova-dependent exonic splicing silencer (NESS) defined here. The NISE element harbors seven YCAAY repeats, which span 65 nt of sequence located 80 nt upstream of the downstream splice acceptor site. Of these seven repeats, five are UCAU, one CCAU and one UCAC. This contrasts with the NESS element, which is composed of three CCAU and two CCAC motifs over 50 nt of exonic sequence. We sought to determine whether Nova's ability to enhance or repress exon inclusion was dependent on the sequence of the Nova-binding site or its position within the pre-mRNA. Figure 7 schematizes the constructs made. We replaced exonic sequences in pE4 (now renamed p-eNESS) with the NISE element to generate plasmid p-eNISE (Figure 7A). Alternatively, we replaced intronic sequences in pGlo2Δ with the NISE or NESS elements to generate the

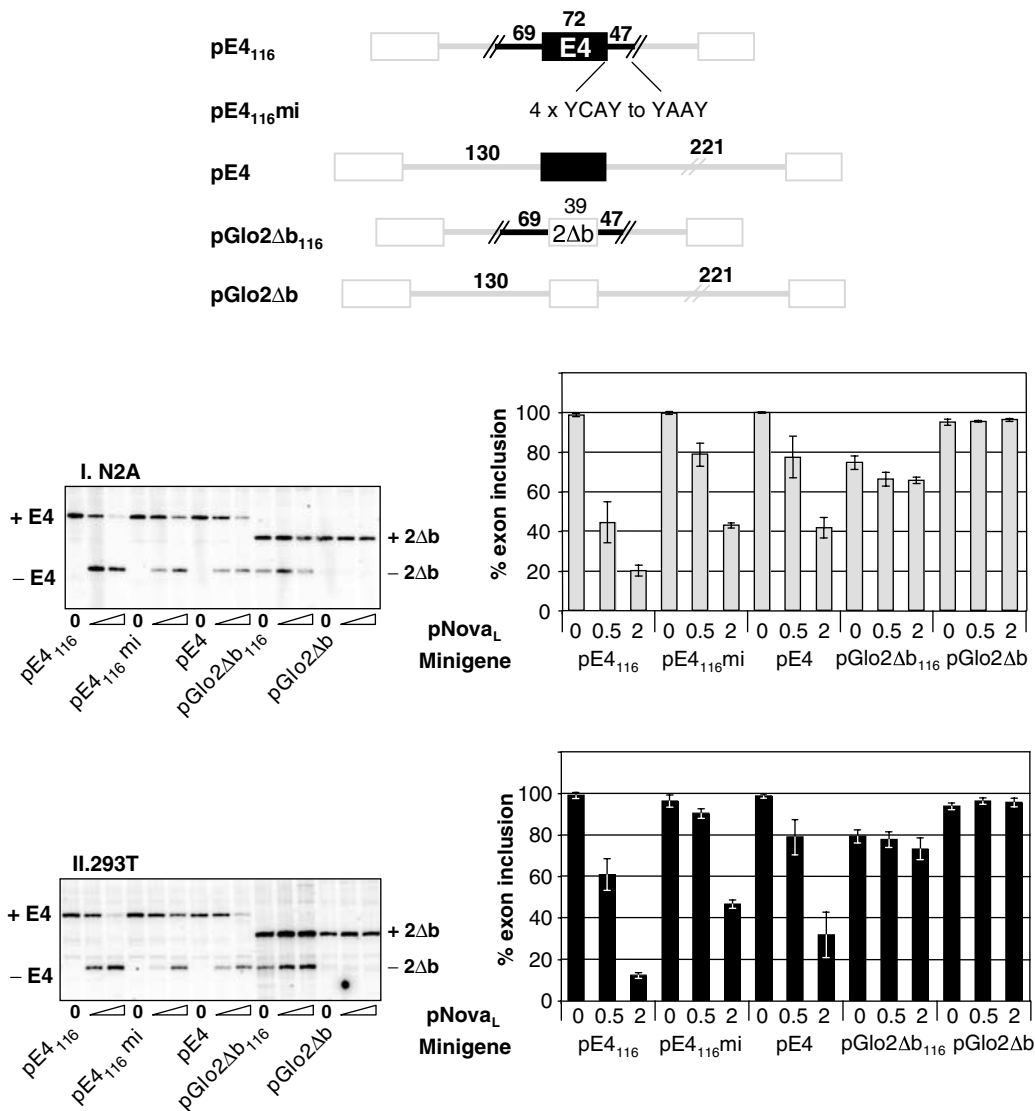


Figure 5 Downstream intronic sequences enhance Nova- dependent splicing of E4, but are neither necessary nor sufficient for the effect. Four YCAY repeats within the Nova-1 intronic sequence downstream of E4 in construct pE4₁₁₆ were mutated to YAAY to generate construct pE4₁₁₆mi. Intronic sequences in pGlo2Δb were replaced by the Nova-1 intronic sequences present in pE4₁₁₆ to generate construct pGlo2Δb₁₁₆. Constructs pE4 and pGlo2Δb were used as Nova-dependent and -independent controls, respectively. Minigenes were cotransfected into N2A (I) or 293T (II) cells with increasing amounts of pNova-1_L (0, 0.5 and 2.0 μg); spliced products were measured by primer extension and phosphorimage analysis as in Figure 3 and titration of transfected pNova-1_L expression was monitored by Western blotting (not shown).

plasmids p-iNISE and p-iNESS, respectively (Figure 7B). Corresponding mutant constructs harboring YCAY to YAAY mutations in the introduced segments were also generated (designated with an 'm').

When either p-eNISE or p-eNESS was cotransfected with pNova-1_L, exon inclusion was repressed (Figure 7A). Conversely, when either Nova-binding site was placed in the intron downstream of the regulated exon, Nova enhanced alternative exon inclusion (Figure 7B). Mutation of the YCAY repeats to YAAY abolished the Nova-dependent effect in all cases. Taken together, these data demonstrate that Nova's ability to enhance or repress exon inclusion is dependent upon the position of the Nova-binding site within the pre-mRNA and upon intact YCAY repeats. Interestingly, introduction of GABA_ARγ2-derived sequence into Nova-1 E4 (p-eNISE and p-eNISEm) reduced the baseline level of exon inclusion

in both cell lines. Furthermore, mutation of the YCAY repeats abolished baseline inclusion of the central exon of p-eNISE. These observations suggest that introduction of the YAAY mutations into the exonic sequences of p-eNISE may have inadvertently generated a splicing silencer, and will be highlighted further in the discussion.

A second putative NESS has been identified in exon 27 (E27) of neogenin by CLIP, a technique developed recently in our laboratory to purify protein-RNA complexes (Ule *et al*, 2003). Increased levels of E27 inclusion were found in the brains of Nova-2 null mice and E27 is sufficient to confer Nova-dependent exon repression in minigene assays (Ule *et al*, 2003, and data not shown). In order to test whether this element can act in a position-dependent manner, as an intronic splicing enhancer, we cloned the neogenin CLIP tag into the intron of pGlo2Δ (p-iNEO), and found that the

element now conferred Nova-dependent exon inclusion (Figure 7C). Thus, two entirely distinct Nova-responsive exonic elements can be functionally switched to intronic enhancer elements, strongly suggesting that Nova's activity in alternative splicing is directly related to the position of the Nova target elements within the pre-mRNA.

Nova CLIP tags have also been identified in introns 7 and 14 of gephyrin pre-mRNA, and splicing of exon 9 was shown to be enhanced in Nova-2 null mice (Ule *et al*, 2003). In addition, a Nova-bound CLIP tag was identified, which corresponds to an intronic region of c-jun N-terminal kinase

2 (JNK2), downstream of an alternative exon, 6B, which is spliced in a mutually exclusive manner with exon 6A. Inclusion of exon 6B was shown to be enhanced relative to 6A in Nova-2 null mice (Ule *et al*, 2003). In both of these cases, Nova appears to bind to intronic sequences to repress exon inclusion, in contrast to the paradigm presented here. However, when these tags were placed into the intron of pGlo2Δ, both mediated Nova-dependent exon inclusion, demonstrating a clear positional effect on alternative splicing in this assay (plasmids p-iGEPH, p-iJNK2; Figure 7C). We conclude that the position of these elements within the native transcripts and possibly additional unidentified sequences in gephyrin and JNK2 pre-mRNAs are likely to be important in mediating Nova's repressive effect on splicing *in vivo*, and, more generally, that Nova binding sequences confer effects on exon splicing in a position-dependent manner.

Discussion

Nova autoregulates its own splicing

Nova was the first mammalian neuron-specific splicing factor to be identified and modeled in a genetic system (Jensen *et al*, 2000a). Nova has been shown to regulate the alternative splicing of a number of transcripts *in vivo*, including the GABA_ARγ2 and GlyRα2 receptor subunit pre-mRNAs and gephyrin pre-mRNA (Jensen *et al*, 2000a; Dredge and Darnell, 2003; Ule *et al*, 2003). Here we have used genetic, biochemical and transient transfection approaches to identify an additional target of Nova-dependent splicing regulation, the Nova-1 pre-mRNA itself. Given that Nova expression is restricted to neurons (Buckanovich *et al*, 1993), and our demonstration here of altered E4 inclusion in Nova-1 heterozygous mouse spinal cord, we conclude that Nova regulates its own transcript in neurons.

A number of splicing factors have been shown to be capable of autoregulation. For example, *Drosophila* Sex lethal (Sxl) binds on both sides of an alternatively spliced exon within its own pre-mRNA to induce exon skipping (reviewed in Black, 2003). Similarly, hnRNP A1 binds either side of alternatively spliced exon 7B in its own transcript, resulting in exon skipping (Blanchette and Chabot, 1999). Our results represent the first instance of splicing autoregulation in mammals to be validated in a genetic system.

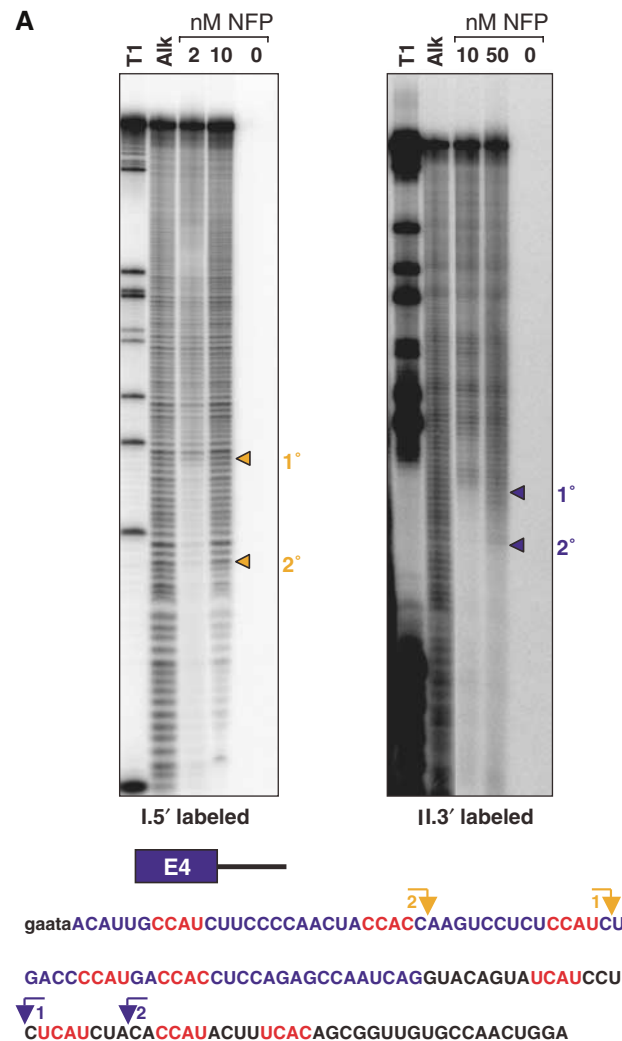


Figure 6 Nova-1 binds with high affinity to its own RNA *in vitro*. (A) Mapping the boundaries of Nova-1 protein binding to Nova-1 E4 plus downstream intronic RNA. RNA was labeled with ³²P at either the 5' or 3' end and subjected to mild alkaline hydrolysis (Alk). The RNA was then incubated with Nova-1 fusion protein (NFP) at a final concentration of 2, 10 or 50 nM and protein:RNA complexes were captured by filtration through nitrocellulose. Bound RNAs were eluted and analyzed by denaturing PAGE. RNase T1 digestion (T1) was used to generate size standards. Boundaries are highlighted by arrows and indicated on the transcribed RNA sequence shown at the bottom. A schematic representation of the RNA transcribed is shown above the sequence; exonic sequence is shown in blue, intronic sequence in black and YCAY motifs are highlighted in red. Small letters represent sequences derived from primers. (B) Nitrocellulose filter-binding assays were performed using Nova-1 RNA as in (A) or control RNAs. GABA: 159 nt RNA corresponding to the GABA_ARγ2 NISE element (Dredge and Darnell, 2003); glo: 175 nt RNA derived from human β-globin, which spans regions of exon 1 and intron 1 and contains no YCAY tetranucleotides.

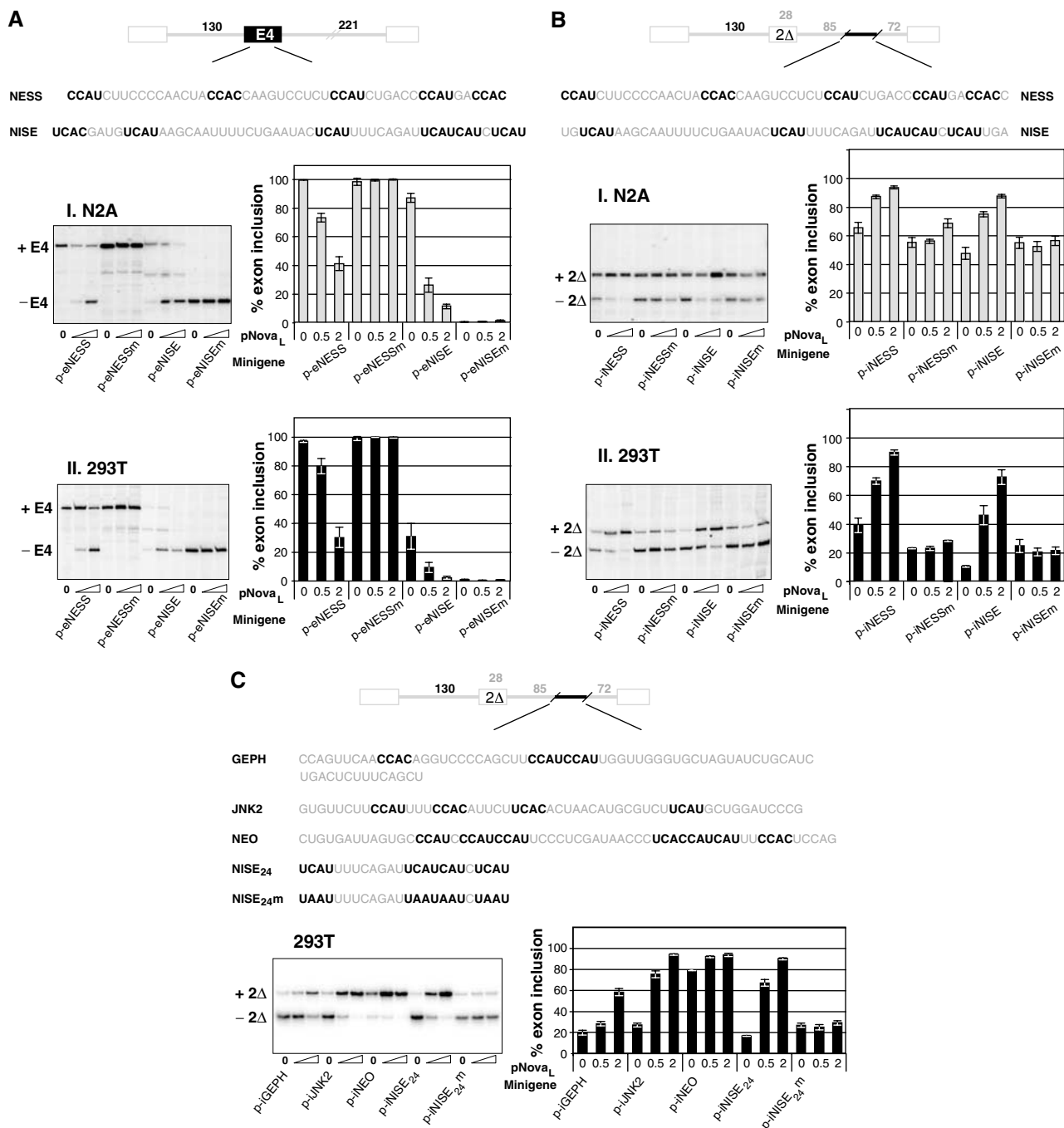


Figure 7 Nova-dependent regulation is dependent on the position of the Nova-binding site within the pre-mRNA. **(A)** A 50 nt stretch of Nova-1 E4 (NESS) containing all five YCAY repeats was replaced in pE4 (renamed p-eNESS) by a 54 nt stretch of the GABA_AR γ 2 intron 9 NISE region (NISE) encompassing six UCAY repeats to generate plasmid p-eNISE. Corresponding constructs with YCAY to YAAY mutations in the introduced segments (p-eNESSm and p-eNISEm) were also tested. These constructs were cotransfected into N2A (I) or 293T (II) cells with increasing amounts (0, 0.5 and 2.0 μ g) of pNova-1_L; spliced products were measured by primer extension and phosphorimager analysis as in Figure 3 and titration of transfected pNova-1_L expression was monitored by Western blotting (not shown). **(B)** A total of 51 nt from Nova-1 E4 or GABA_AR γ 2 NISE, each harboring five YCAY repeats, were cloned into pGlo2 Δ 72 nt upstream of the downstream splice acceptor site (plasmids p-iNESS and p-iNISE). Corresponding constructs with YCAY to YAAY mutations in the introduced segments (p-iNESSm and p-iNISEm) were also tested. These constructs were transfected and analyzed as in (A). **(C)** Three additional putative Nova target sequences in gephyrin, JNK2 and neogenin identified by CLIP (Ule *et al*, 2003) were cloned into pGlo2 Δ (plasmids p-iGEPH, p-iJNK2 and p-iNEO). Construct p-iNISE₂₄ harboring a 24 nt stretch of the GABA_AR γ 2 NISE and a corresponding construct with YCAY to YAAY mutations (p-iNISE_{24m}) were used as Nova-dependent and -independent controls, respectively (Dredge and Darnell, 2003). These minigenes were transfected and analyzed as in (A).

For Nova-1, splicing of E4 results in the addition of a phosphorylation domain into the protein product. The physiological consequences of inclusion and/or phosphorylation of domain 4 are unclear. Both hyper- and hypophosphoryla-

tion have been shown to inhibit the ability of SR proteins to regulate splicing (Prasad *et al*, 1999). However, in transfection assays, the presence of domain 4 had no effect on Nova's ability to regulate alternative splicing of GABA_AR γ 2, GlyR α 2

or Nova-1 minigenes. This implies that domain 4 has no discernable effect on the recognition and binding of these RNAs, nor on protein–protein interactions required for their regulation in tissue culture cells.

Nova-1 domain 4 also harbors a predicted PEST domain. PEST motifs serve as proteolytic signals to target protein for degradation by the 26S proteasome (Rechsteiner and Rogers, 1996). Although we have not directly measured the half-life of the two isoforms in a pulse–chase experiment, we have no indication that Nova-1_L is less stable than Nova-1_S since the steady-state level of protein after transfection was the same as detected by Western blot against the T7 tag (Figure 3, lower panels). Thus, we do not yet understand the significance of E4 phosphorylation.

Nova as a dual function splicing factor: the NESS element

We have shown that Nova-1 E4 is necessary and sufficient for Nova-dependent repression of alternative exon inclusion, and therefore can be defined as a NESS. This contrasts with previously described examples of Nova regulation of alternative splicing in which Nova enhances exon inclusion via sequence-specific interactions with intronic regions (Jensen *et al*, 2000a; Dredge and Darnell, 2003). Thus, Nova is a dual function splicing factor, able to both enhance and repress exon inclusion. SR proteins have been shown to function in a similar manner, acting as either positive or negative regulators of alternative splicing depending on the position of the relevant binding site within the pre-mRNA. For example, an SR-protein-binding site positioned just upstream of a branch point causes splicing inhibition in an adenovirus pre-mRNA by blocking access of U2snRNP. The same element can act as an exonic splicing enhancer if it is placed downstream of the 3' splice site (Kanopka *et al*, 1996). Interestingly, this inhibition is dependent on phosphorylation of the SR proteins; inhibition is relieved upon dephosphorylation of the bound SR proteins (Kanopka *et al*, 1998).

We demonstrated that Nova's action on alternative splicing is position dependent by swapping the positions of the Nova-binding sites derived from the Nova-1 and GABA_AR γ 2 pre-mRNAs (Figures 7A, B and 8A), and by transforming an exonic element in neogenin E27 to a splicing enhancer by moving it to an intronic location (Figure 7C). Furthermore, the position of the Nova-binding site within an intron also appears to confer important information, as the gephyrin and JNK2 Nova CLIP tags, which we hypothesize to act as intronic splicing silencers, act as enhancer elements when placed in a location within an intron equivalent to that of the GABA_AR γ 2 NISE. Thus, we can predict that Nova-binding sites within alternatively spliced exons are likely to confer Nova-dependent exon exclusion; however, more examples are needed to define the relationship between the position of Nova-regulatory regions within introns and the outcome of Nova:RNA binding. We note that the single CLIP tags isolated from JNK2 and gephyrin do not preclude the possibility of additional Nova-binding sites in these transcripts.

Interestingly, the sequences of the Nova-1 E4 and GABA_AR γ 2 Nova regulatory regions are quite different, including a high incidence of Cs (seven of a possible 10) in the first and last positions of the YCAY tetranucleotide in the NESS and a high incidence of Us (12 of a possible 14) in these positions in the NISE. This may simply reflect the tendency

for exons to have a higher GC content than introns (reviewed in Bernardi, 1995). However, when the E4 NESS sequence was replaced by GABA_AR γ 2 NISE sequence, the inclusion of the resulting exon was reduced (construct p-eNISE; Figure 7A) or abolished (construct p-eNISEm) even in the absence of Nova protein and despite an increase in length of 4 nt. We hypothesize that the NESS element contains positive signals such as ESEs, which are not present in the central exon of p-eNISE and are blocked by the binding of Nova. Alternatively, the NISE sequence may introduce additional Nova-independent negative sequences. ESE prediction algorithms based on bioinformatic predictors (Fairbrother *et al*, 2002) and SR protein SELEX results (Cartegni *et al*, 2003) have been used successfully to predict regulatory elements within exons. However, our attempts to correlate our results with the patterns of ESEs predicted using these programs were unsuccessful. Both the work presented here and that on GABA_AR γ 2 (Dredge and Darnell, 2003) highlight the difficulty of predicting Nova (and other RBPs) regulatory sites bioinformatically, even when many details regarding the sequence specificity and structural details of Nova interactions with RNA have been well established.

Mechanism of Nova action to regulate alternative splicing

Several mechanisms have been proposed to explain exon skipping via exonic splicing silencers (ESSs). In a few cases, exonic sequences are thought to be involved in RNA secondary structures, which prevent access of necessary components of the splicing machinery to splice sites. Chicken β -tropomyosin exon 6B forms RNA–RNA base pairs with the upstream intron, preventing accessibility of this exon in myoblasts and resulting in inclusion of a mutually exclusive exon, 6A (Libri *et al*, 1991). However, the majority of ESS elements that have been described function through *trans*-acting protein factors, which bind them.

High-affinity Nova-binding sites exist throughout the exon and extend into the intron downstream of E4. While the intronic elements themselves are not sufficient to mediate Nova-dependent E4 inhibition, when they are present adjacent to E4 we see an enhancement of Nova's action on splicing inhibition. This suggests that Nova may multimerize from an obligate exonic element into the intronic sequences, much like hnRNP A1, initially binding within HIV-1 Tat exon 3, is thought to multimerize to adjacent intronic sequence and block U2AF65 intronic binding (Zhu *et al*, 2001), and as seen in Rev-RRE multimerization (Malim and Cullen, 1991).

Nova binding to these sites could inhibit splicing in a number of ways. For example, binding of Nova could sterically inhibit the binding and/or assembly of critical spliceosome components such as U1-associated factors at the splice junction(s) of E4, or interfere with bridging interactions across the exon (Figure 8B). This mode of inhibition has been proposed for negative factors such as hnRNP A1 and PTB (reviewed in Cartegni *et al*, 2002; Black, 2003). Alternatively, Nova binding could competitively inhibit the binding of a positive factor, such as an SR protein, to E4 (Figure 8C). Antagonism between SR proteins such as SF2/ASF, and hnRNP proteins such as hnRNP A1 has been shown to influence splicing of a number of transcripts including HIV-1 *tat* (Mayeda and Krainer, 1992; Zhu *et al*, 2001). Evidence

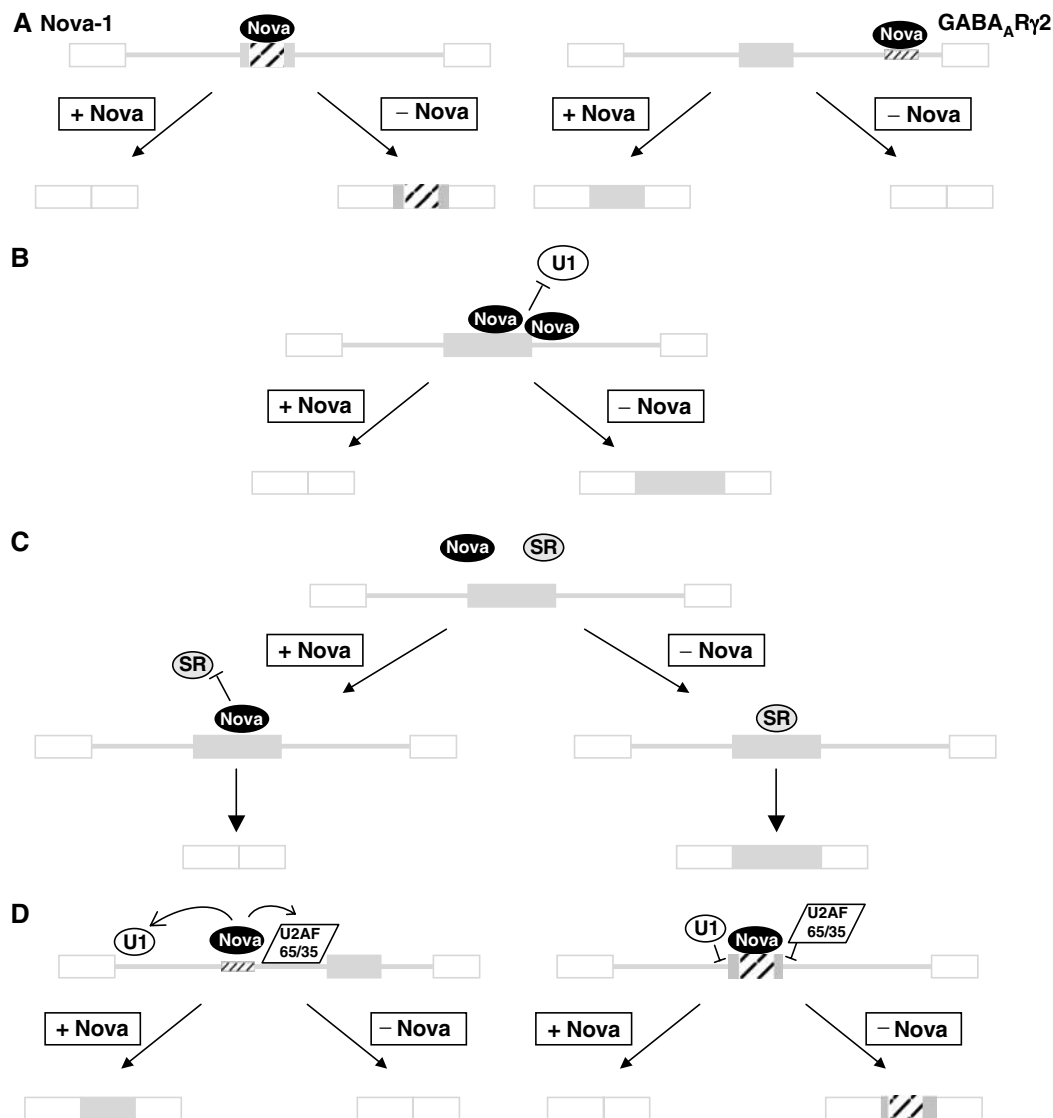


Figure 8 Models for Nova's action on alternative splicing. (A) Position-dependent action of Nova on alternative splicing. Nova binding to a high-affinity site within an alternative exon represses exon inclusion. In contrast, Nova binding within the intron downstream of an alternative exon enhances exon inclusion. (B) Nova-1 binding to exon E4 may inhibit binding and/or assembly of constitutive splicing factors such as U1 as shown, or sterically inhibit bridging interactions across the exon (not shown). (C) Nova-dependent inhibition may occur through competitive inhibition of nonessential splicing factors such as SR protein(s). (D) Nova may inhibit exon inclusion via incorrect recruitment of splicing factors. Empty boxes represent constitutively included exons, and filled boxes represent alternative exons. Hatching represents high-affinity Nova-binding sites. Note that while Nova protein is represented as a single oval, a number of Nova molecules are likely to be bound at each binding site.

for this model comes from our observation that when the E4 NESS sequence was replaced by GABA_AR γ 2 NISE sequence (construct p-eNISE; Figure 7A), the inclusion of the resulting exon was reduced.

An alternative possibility is that Nova binding to E4 could lead to recruitment of constitutive factors, as has been suggested for regulation of GlyR α 2 and GABA_AR γ 2 alternative splicing (Dredge and Darnell, 2003), but to the wrong position relative to the RNA and thus in a manner inhibitory for exon inclusion. Nova's orientation to a bound RNA is highly directional, as evidenced by the cocrystal structure of Nova KH3 with a target RNA (Lewis *et al*, 2000). If Nova interactions with other protein factors are also directional, Nova could dictate the position of these factors relative to the RNA (Figure 8D).

Conclusions

Nova-1 is an important regulator of alternative splicing in the mammalian brain. In addition to previously defined targets, Nova-1 regulates alternative splicing of its own transcript in mouse spinal cord. Nova acts through an exonic splicing enhancer to inhibit inclusion of a 72 nt exon, which encodes a phosphorylation domain. This is the first demonstration that Nova can act directly through an exonic element to mediate exon skipping. Moreover, we have established that the action of Nova to repress or enhance alternative exon inclusion is dependent on the position of the Nova-binding site within the pre-mRNA. Elucidation of the mechanism of Nova-dependent splicing regulation through identification of splicing targets is yielding insight into the general mechanisms of alternative

splicing regulation and the exquisite control and complexity of gene expression in the brain.

Materials and methods

Metabolic phosphorylation assay

N2A cells were grown in 10 cm dishes in DMEM supplemented with 10% fetal bovine serum (FBS) and transfected with 5 µg of vector DNA encoding T7-tagged Nova-1 using 15 µl of Fugene (Roche) according to the manufacturer's instructions. When cells reached 70% confluence, usually after 40 h incubation, the medium was substituted with phosphate-free DMEM supplemented with dialyzed FBS. After 1 h incubation in phosphate-free medium, ³²P-orthophosphate to a final 0.5 mCi/ml was added. After 5 h incubation at 37°C, the cells were harvested and lysed in 1 ml lysis buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 0.15 M NaCl, 0.01 M Na phosphate pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM Na₂OV₄ and 1 × complete protease inhibitor cocktail (Roche)). Nova-1 was immunoprecipitated with 5 µg anti-T7-tag monoclonal antibody (Novagen) and treated with RNaseA and T1. Following SDS-PAGE, the products were transferred to nitrocellulose and exposed to autoradiography film. The identity of the bands observed by autoradiography was confirmed by Western blot.

RNase protection assays and RT-PCR

RNA preparation and RNase protection assays were performed as described previously (Dredge and Darnell, 2003). Antisense probes were *in vitro* transcribed using T7 RNA polymerase from pGEM T-Easy plasmid backbones (Promega) containing PCR products generated with primers Ri28 (5'-CGCCGGACTCGCGAAAAG) and exH-R (5'-CTGATTGCTCTGGAGG), and linearized with *Nde*I; or from plasmid pTRI-β-Actin-Mouse antisense control template (Ambion). Probes were labeled by incorporation of [α -³²P]UTP, and the specific activities were adjusted by incorporation of unlabeled UTP to give protected fragments of approximately equal intensities for each message (ratios of unlabeled:labeled UTP were 3:1 and 400:1 for Nova-1 and actin probes, respectively). RT-PCR was performed as previously described (Jensen *et al*, 2000a) using primers G6 (5'-GGGAGGACAGACAATTGTT) and mRi4 (5'-GCCCTGACTGCTCCATTA).

Plasmid constructs

Plasmids pNova-1_L, pGABA and pGlo2Δ have been described previously (Dredge and Darnell, 2003). Plasmid pNova-1_S is

identical to pNova-1_L, except that exon 4 has been deleted by PCR. Plasmid pE4₈₈₅ was derived from pGlo2Δ with the insertion of an ~1 kb *Bam*HI-*Pst*I fragment of mouse Nova-1 genomic sequence. Further chimeric minigenes and mutations were generated by PCR. All DNA segments generated by PCR were sequenced in their entirety. Plasmid DNA for transfection into cell lines was prepared by a modified cesium chloride method (Sambrook *et al*, 1989), and dialyzed against 1 × TE.

Cell transfection and primer extension

N2A and 293T cells (ATCC) were transfected with 3.25 or 2.25 µg of total DNA, comprised of 0.25 µg of the appropriate minigene and variable amounts of pNova-1 and empty pCI-neo vector (Promega), using Fugene6 (Roche) as described by the manufacturer. After 40 h, the cells were washed with 1 × PBS, collected by scraping and halved for RNA extraction and protein extraction. Primer extension was performed using 5 µg of total RNA purified using the RNeasy Mini kit system (Qiagen), 0.5 pmol γ -³²P-ATP-labeled primer E3R (5'-CAGCACACAGACCAGCACGTTG, β-globin) or mrp2 (5'-TTGAATGGTTGCTGATCTGGGACC, GABA_ARγ2) and SuperScript III reverse transcriptase (Invitrogen), at 55°C. Western blots were performed using anti-γ-tubulin monoclonal antibody GTU-88 (Sigma) or HRP-conjugated anti-T7-tag monoclonal antibody (Novagen) and detected by chemiluminescence (Perkin-Elmer).

Boundary mapping and nitrocellulose filter-binding assays

Fusion protein synthesis, boundary mapping and nitrocellulose filter-binding assays were performed as described previously (Dredge and Darnell, 2003). Nova-1 RNA was *in vitro* transcribed using SP6 RNA polymerase from a gel-purified PCR product generated using primers SP6-exH-F (5'-TCCCGATTAGGTGACAC TATAGAATACATTGCCATCTTCCCC) and inh-R (5'-TCCAGTTGGCA CAACCGC). GABA and glo RNAs were generated as described previously (Dredge and Darnell, 2003).

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