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Research report

Regulation of the neuron-specific exon of clathrin light chain B

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Abstract

Clathrin light chain B (LCB) is a major component of clathrin coated vesicles, which are structures involved in intracellular transport. A neuron-specific isoform of LCB is generated by incorporation of a single exon (EN) using an alternative splicing mechanism that reflects the special demands of neurons, such as axonal transport and synaptic neurotransmission. Here, we demonstrate that this neuron-specific exon is developmentally regulated and is excluded in non-neuronal cells because its 5′ and 3′ splice sites deviate from the mammalian consensus sequences. A gel retardation assay indicated the presence of a developmentally regulated factor in brain that binds to the neuronal exon. In addition, EN usage is repressed by increasing the concentration of htra2-beta1, a splice factor whose isoform expression is influenced by neuronal activity. We propose that a brain-specific factor is involved in EN recognition during development and adulthood. In addition, ubiquitously expressed splicing factors such as htra2-beta1 are involved in regulating EN expression in the adult brain.

Keywords: Alternative splicing; Clathrin light chain B; Transformer-2

1. Introduction

About 30% of mammalian genome expression is dedicated to the brain [10]. Kinetic [9] and clonal [31] analysis indicates that 40–65% of the mRNA expressed in brain is restricted to this tissue. The majority of this mRNA is expressed in neurons and not in glia [59]. In addition, neuron-specific alternative splicing is extensively used in the brain to create additional protein diversity [3,4,34,52,53]. For example, at least 47 Neurexin 1α isoforms are generated by this mechanism [63]. Often, these exons introduce regulatory features, such as stop codons and frameshifts, which lead to truncated messages as well as phosphorylation sites (reviewed in Ref. [53]). Alternative splicing can create soluble receptor forms [37,61], change ligand specificity [30,56], ligand affinity [11,13] or electrophysiological properties [18,25,50].

There are numerous examples of alternative spliced exons that are subject to developmental control [53,65] and in the adult brain, neuron-specific splicing can change in response to neuronal activity [12,64], indicating that it is a mechanism to convey neuronal plasticity.

Clathrin light chain B is an example of an alternatively spliced pre-mRNA. As illustrated in Fig. 1A, clathrin light chain B expresses one neuron-specific isoform, termed LCB2, owing to the use of an alternative exon EN [51]. EN is skipped in all other tissues, generating an isoform termed LCB3. Exon EN encodes a domain that allows calmodulin to bind to assembled clathrin cages [42]. This results in an increased Ca^{2+} sensitivity of neuronal coated vesicles, which probably helps to monitor Ca^{2+} fluxes as a response to neuronal activity [20]. The synaptic localization of vertebrate clathrin light chains [66] and the upregulation of the Aplysia light chain in response to serotonin treatment [19] underline the importance of clathrin in neuronal communication. In addition, the ratio of EN inclusion to EN skipping is influenced by neuronal activity in adult rat brain. Pilocarpine induced neuronal activity in adult rats results in an increase of EN skipping, indicating that the neuron-specific splicing pathways regulating EN usage are subject to control in the adult brain [12].

Neuron-specific splicing has been analyzed in several model systems (reviewed in Ref. [16]), among them the N1

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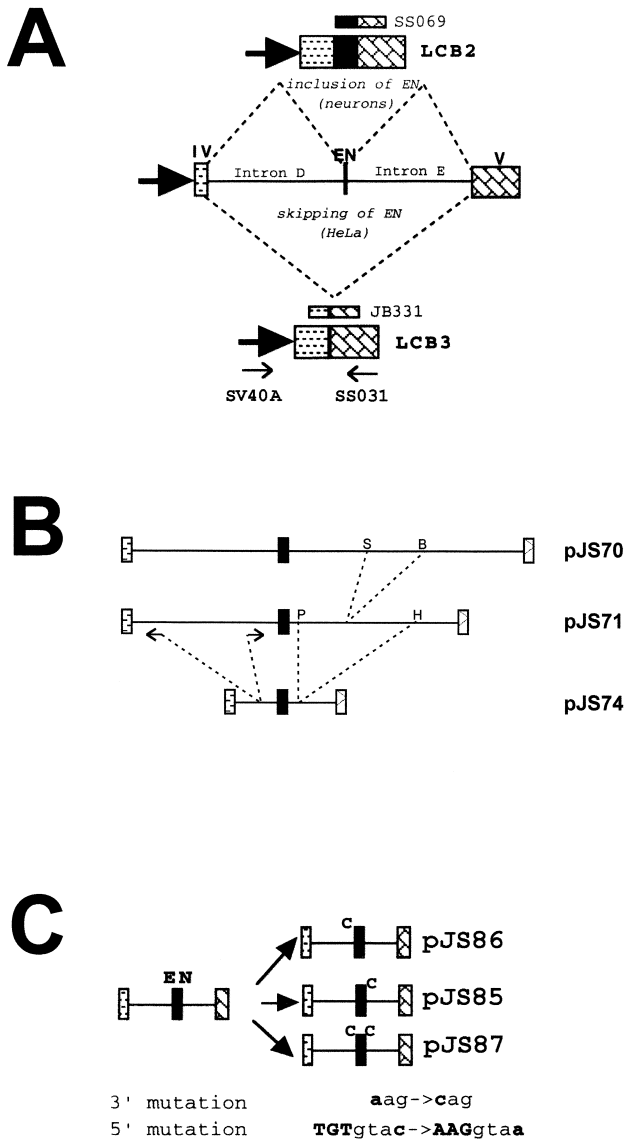

Fig. 1. Minigene analysis and construction. (A) Schematic diagram of the minigene constructs and detection scheme. Minigenes contain EN and its flanking exons IV and V, as well as intron D and E regions. Owing to alternative splicing, they give rise to two forms, LCB2 and LCB3, which include or skip exon EN, respectively. The use of a PCR primer SV40A complementary to the SV40 early promoter (thick arrow) allows the distinction of the transfected from the endogenous gene product. LCB2 and LCB3 forms can be distinguished by size and by their hybridization pattern with ^{32}P-labeled oligonucleotides SS069 and JB331, which hybridize to the parts indicated by shading [6,51]. (B) Intron deletion constructs. pJS70 corresponds to a part of the clathrin light chain B gene [51] and is 4.8 kb in length. Deletion of the *Sal*I–*Bam*HI (S, B) fragment creates pJS71 (3.6 kb). pJS74 (1.3 kb) was created by deleting the *Pst*I–*Hin*dIII (P, H) fragment in intron E and removing parts of intron D with PCR (arrows). (C) Mutagenesis of splice sites. Three exon consensus mutations were generated, having the 3′, 5′ or both splice sites changed into the mammalian consensus. The mutation into the consensus splice site is indicated by a 'C' near the box representing EN. The changes in the 3′ and 5′ splice sites are indicated on the bottom; the changed nucleotides are in boldface. The sequence to the left side of the arrow indicates the wild type.

exon of c-src [33], the 24 nt exon of the GABAa receptor [2], neuronal skipping of the calcitonin/CGRP gene exon four [27], the exon B of DOPA decarboxylase [48] and exons seven, eight and fifteen of the amyloid precursor protein [62,68]. A common feature emerging from the analysis of these systems is the presence of overlapping positive and negative regulatory elements near or within the alternatively spliced exon. In vitro, these regulatory elements have been shown to bind RNA binding proteins, such as the polypyrimidine tract-binding protein [2,8,27], KSRP [33], hnRNPF [32], HRB98DE [49] and U2AF [62]. Since none of these factors is neuron-specific, it is currently debated whether neuron-specific splicing is achieved by modulating the concentration of ubiquitously expressed splicing factors or by the presence of neuron-specific factors.

Evidence for neuron-specific splice factors comes from the observation that the neuron-specific RNA binding protein ELAV can change splice site selection in vivo [24] and that there exist a number of neuron-specific RNA binding proteins such a NOVA [5], Hu-proteins [40] and SmN [47], although their exact role remains to be determined.

Here, we used an in vivo minigene approach to study EN regulation in neuronal and non-neuronal cells. We found that EN inclusion is repressed by its sub-optimal splice sites in non-neuronal cells. Since we observe an EN specific RNA binding activity exclusively in extracts from brain, we postulate an activating factor in neurons. Finally, co-transfection analysis indicated that htra2-beta1, a splicing factor showing activity-dependent regulation in the brain, can repress EN inclusion.

2. Materials and methods

2.1. Minigene construction and cloning

Exon EN and its flanking intron and exon regions were amplified and subcloned as described [51] and subsequently cloned into the expression plasmid pΩ3 [35]. The initial construct pJS70 (4.8 kb) was shortened by deleting the 1.2 kb *Sal*I/*Bam*HI fragment in Intron E (pJS71, 3.6 kb), located approximately 900 nt downstream from the 5′ splice site of EN. This construct was further shortened by removal of the *Hin*dIII/*Pst*I fragment in intron E and deletion of most of intron D using PCR and the primers SSS001/SSS002 (pJS74, 1.3 kb). Eighty-six and 20 nucleotides remain downstream and upstream, respectively, of the original intron sequence surrounding EN. In the pJS74 minigene, approximately 200 nt of intron D and 1000 nt of intron E remained (Fig. 1). DNA was manipulated by standard procedures [45]. Splice site mutations were introduced by PCR using a megaprimer approach. Briefly, the 3′ splice site was changed by amplifying pJS74 in two separate reactions using primer pairs SSMu01/SS031 as well as SV40A/SSMu05. The reac-

tion products were mixed and amplified using SV40A and SS031, generating a point mutation in the 3′ splice site. The resulting PCR fragment was subcloned into pΩ3. The 5′ splice site was changed in a similar way using primers SSMu06 and SSMu02. The point mutations are indicated by bold lettering in the primer list (see Section 2.4). EGFP-CUG and EGFP-rELAV were generated by amplifying the respective cDNAs with CUGf and CUGrev, as well as rELAVfor and rELAVrev, followed by subcloning into pEGFPC2 (Clontech). EGFP-htra2-beta1 and -beta3 have been described earlier [38].

2.2. Minigene analysis and splice reporter assays

Minigenes were analyzed essentially as described [39]. Briefly, using the calcium phosphate method, 2 μg of the reporter gene (pJS70, pJS71, pJS85–87) were co-transfected with 0, 1, 2, 3 and 4 μg of EGFP-tagged splice factor into HEK293 or HeLa cells. The amount of transfected DNA was balanced by including 4, 3, 2, 1 and 0 μg of empty EGFP-C2. RNA was prepared 16 h after transfection using RNAse easy columns (Qiagen). RNA amounting to 1 μg was reverse transcribed using 250 ng SS031 and 1.25 U MMLV H^- reverse transcriptase. One-tenth of this reaction was amplified using primers SS031 and SV40A. Denaturation was 30 s at 94°C, annealing 30 s at 57°C and extension 45 s at 72°C for 25 cycles.

2.3. Primary neuronal cultures

Primary neuronal cell culture from E16 rat embryo midbrains was essentially performed as described [7] in chemically defined medium. These cultures consist predominantly of neurons [52] and were transfected after 1 week in culture using lipofectin (Gibco).

2.4. Oligonucleotides

Nucleotides changed in mutagenesis are indicated by boldface

SV40A: TGAGCTATTCCAGAAGTAGT (Minigene analysis, Fig. 1A)

SS031: GGGGTCTCCTCCTTGGATTCT (Minigene analysis, Fig. 1A)

SSS001: TTGCTGTCATGATCTTCCC (Minigene construction, Fig. 1B)

SSS002: TCCTCCCTGTGCCTCACT (Minigene construction, Fig. 1B)

SSMu01: GTTTTCCTC**C**AGGATCGCTG (Minigene construction)

SSMu05: ATCCT**G**GACCTTTTCCGTTA (Minigene construction)

SSMu02: ACCATTGGCTA**AAG**GTA**A**GTGCCTCCT**C** (Minigene construction)

SSMu06: AC**T**TAC**CTT**TAGCCAATGGT (Minigene construction)

SS069: CTTCCGATGCCACATAGCCAATGG (Minigene analysis, Fig. 1A)

JB331: TCGAGTCTTCCGATGCCCTGTTGTTGAT (Minigene analysis, Fig. 1A)

SSRNA01: ACAAAGCGUUCUACCAGCAGCCAGAUGCUG (Gel shift, Fig. 5C)

SSRNA02: ACAAAGCGUUCUACCAGCAGCCAGAU (Gel shift, Fig. 5C)

SSRNA03: AGCGUUCUACCAGCAGCC (Gel shift, Fig. 5C)

SS034: CGATCACATAGCCAATGGTATCAGCATCTGGCTGCTGGTAGAACGCTTTGTCAGCGATCCTAC (construction of pJS84)

SS035: CGGTAGGATCGCTGACAAAGCGTTCTACCAGCAGCCAGATGCTGATACCATTGGCTATGTGAT (construction of pJS84)

SS009: CAACAGGATCGCTGACAAAGCGTTCTACCAGCGCCAGATGCTGATACCATTGGCTATGTGAGC (construction of pJS26)

SS010: CACATAGCCAATGGTATCAGCATCTGGCTGCTGGTAGAACGCTTTGTCAGCGATCCTGTTGGATC (construction of pJS26)

CUGf: GGAATTCACCATGAACGGCACCCTGGACCACCCAG (construction of CUG minigene)

CUGrev: GGGATCCTCAGTAGGGCTTGCTGTCATTCTTCG (construction of CUG minigene)

rELAVf: CCGAATTCATGGAAACACAACTGTCTAATGGGCCAAC (construction of ELAV minigene)

rELAVrev: CCGGATCCTTTGTTTGTCTTAAAGGAGACCTGCAGTAC (construction of ELAV minigene)

2.5. Western blot analysis

Protein extracts from rat brains at various developmental stages were obtained and analyzed by Western blot as described [52]. The antibody 7H12 [23] was used to detect clathrin light chains LCB2 and LCB3.

2.6. Gel shift analysis and in vitro RNA transcription

Nuclear extracts from embryonic (E15 and E20) young, adult rat brain (8 weeks postnatal) and HeLa cells were essentially prepared as described [14]. SS034 and SS035 were cloned into the *Cla*I site of pBluescript creating pJS84. This EN probe consists of 54 nucleotides of exon EN and nine nucleotides of flanking cDNA. The shorter probe pJS26 was created by cloning SS009 and SS010 into the *Sac*I and *Kpn*I sites of pBluescript. RNA was prepared using ^{32}P αUTP (800 Ci/mmol) as the only UTP nucleotide. The gel shift was performed in 10 mM HEPES, pH 7.9, 300 mM KCl, 0.025% NP40, 1 mM DTT, 10%

Glycerol, 30 ng/μl BSA, 1 μg/μl tRNA, 0.26 μg/μl nuclear extract and 10,000 c.p.m. radiolabeled RNA on a 5% non-denaturing Tris–glycine acrylamide gel. Tar RNA used as a positive control was generated after subcloning nts +1 to +65 of HIV [36] under T7 promoter control in pBluescript. As an unspecific control, the polylinker (95 nt) of *Sac*I linearized pBluescript was transcribed using T7 RNA polymerase.

3. Results

3.1. Sub-optimal splice sites block the neuron-specific exon EN in non-neuronal cells

The analysis of the endogenous splicing pattern of clathrin light chain B reveals that one exon, termed EN, is exclusively used in neurons [51]. We could previously demonstrate that fibroblast cells skip EN in a minigene consisting of exon EN and its flanking exons and intron regions, whereas primary neuronal midbrain cultures include EN in this construct [51]. This indicates that the regulatory sequences for neuron-specific splicing of clathrin light chain B reside within exon EN and its flanking exon and intron borders.

In order to identify nucleotide sequences involved in alternative splice site selection in more detail, we now constructed a series of minigene intron deletions and analyzed them in transiently transfected cells. The RNA generated from these minigenes was assayed by RT-PCR, using an antisense LCB primer (SS031) for reverse transcription. SS031 was then used with an SV40 promotor-derived primer (SV40A) for PCR amplification. The PCR products differ by 54 nt in size, depending on whether EN was included or skipped. In addition, the identity of the reaction products was further confirmed by Southern blot analysis, using exon specific primers as probes (Fig. 1A). The introns surrounding EN are 1750 nt and 3200 nt in size [51], which is larger than the average vertebrate intron size of 1127 nt [17]. Using internal restriction sites and PCR, we therefore deleted intronic sequences from the original minigene (Fig. 1B). Inspection of the splice sites surrounding EN shows that both splice sites deviate from the mammalian consensus splice site sequence [55]. The deviation of the 3′ splice site is located in the nucleotide position −3 where an A is used instead of the consensus C or T. The 5′ splice site deviates in four positions from the consensus. We therefore mutated the splice sites to match the mammalian consensus (Fig. 1C).

These minigenes were transfected into HeLa cells. As shown in Fig. 2A, intron shortening did not affect EN inclusion. However, when we tested the minigenes with mutated splice sites, we found that each splice site consensus mutation alone could activate EN inclusion in HeLa cells (Fig. 2B). As shown in Fig. 2A and B, EN usage was a minor form when the 3′ splice site was improved (pJS86), but it was the dominant form when the 5′ splice site was changed into the consensus (pJS85, Fig. 2A, C). Upon longer exposure, EN skipping could still be detected using this minigene (data not shown). If both splice sites were changed into the consensus sequence, skipping of exon EN was no longer detectable in HeLa cells (pJS87, Fig. 1C), even after prolonged exposure (data not shown). Together, these results indicate that EN is not used in HeLa cells, because its splice sites deviate from the consensus and are not recognized.

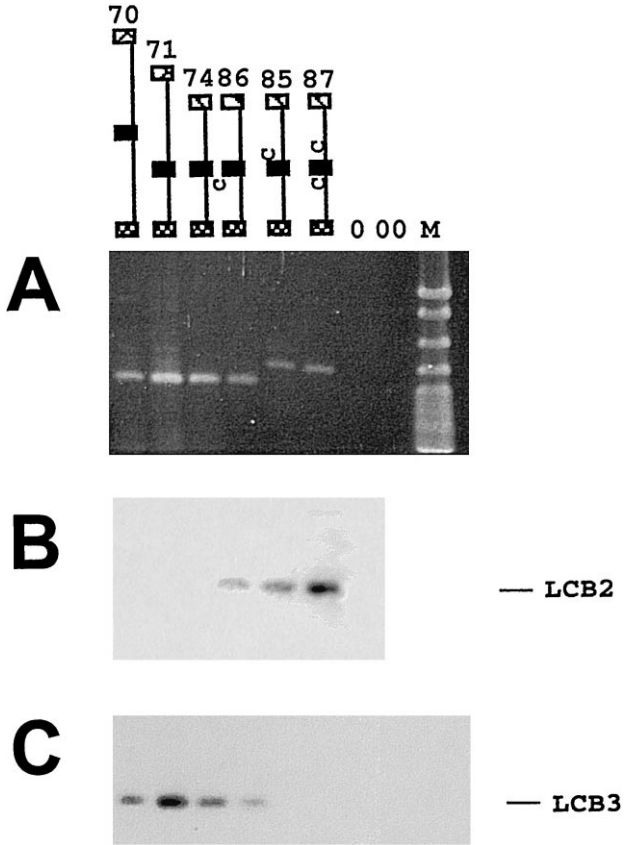

Fig. 2. Splicing of the minigenes in HeLa cells. (A) Splicing of the minigenes in HeLa cells. Minigenes indicated on top were transfected into HeLa cells. Cartoons schematically indicate the minigenes, exon are boxes, introns are lines (pJS70: 4.8 kb, pJS71: 3.6 kb and pJS74 and pJS85–87: 1.3 kb). The constructs pJS85–87 have the 3′, 5′ and both splice sites changed into the consensus. Splicing products were detected with ethidium bromide staining. M.pBRMspI marker, 0: RNA without RT, 00: PCR reaction without any template. (B) Detection of exon EN use in HeLa cells. The gel shown in panel C was transferred to nitrocellulose and probed with SS069, which recognized EN-inclusion (LCB2 form). (C) Detection of exon EN use in HeLa cells. The same filter as in D was stripped and reprobed using JB331, which recognizes exon EN-skipping (LCB3 form). Upon longer exposure, a signal can be observed in the pJS85 lane (data not shown).

3.2. Optimal splice sites are not necessary in primary neuronal cultures

An extensive search of neuronal cell lines revealed that only primary neuronal cultures faithfully reflect the splicing pattern of clathrin light chain B observed in the brain

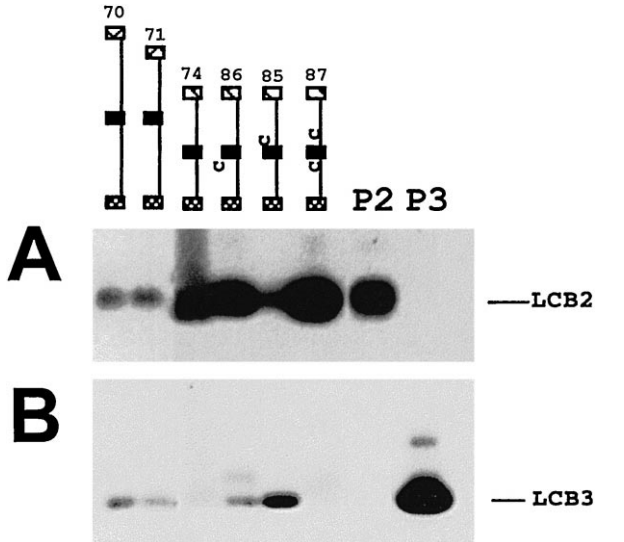

Fig. 3. Splicing of minigenes in primary neuronal cells. (A) Detection of exon EN use in primary mesencephalic cultures. Primary midbrain cultures were transfected with the minigenes indicated on top. RNA was analyzed by RT-PCR. PCR products were separated on agarose and transferred to nylon filters. The filter was probed with SS069, an oligonucleotide that detects EN inclusion (LCB2 form). P2 and P3: plasmid controls for the amplification of LCB2 and LCB3. (B) Detection of exon EN skipping in primary neuronal cultures. The filter from panel A was stripped and reprobed using JB331, an oligonucleotide that detects EN skipping (LCB3 form).

[51]. Therefore, to test the influence of intron length and splice site composition on splicing of EN in a neuronal context, we transfected the same minigenes into primary neuronal cultures. As in the HeLa context, no influence of intron length on EN usage could be detected (Fig. 3A, B). EN usage is the predominant form in neuronal cultures. Improvement of the splice sites further increased the use of EN in neuronal cells. Similar to HeLa cells, changing both splice sites into the consensus sequence abolishes EN skipping in a neuronal context (Fig. 3, pJS87). Reproducibly, we obtained stronger signals when we transfected the shorter minigenes pJS74 and pJS85-87 into neuronal cultures, which might reflect a higher transcription efficiency of these minigenes in neurons. We conclude that neurons can include EN into mature mRNA either because they express a specific factor that recognizes EN or because their splicosomal proteins are specifically composed to allow for EN recognition.

3.3. Developmental regulation

A number of exons that are alternatively spliced in the brain are developmentally regulated and often these exons are surrounded by sub-optimal splice sites [51]. Therefore, we evaluated the temporal pattern of exon EN inclusion in the developing rat brain (Fig. 4). Using Western blot analysis, we determined EN expression in whole homogenates of the developing rat brain. EN expression can be first detected at embryonic day E16 and expression increases as development proceeds. We conclude that EN usage is developmentally controlled. Therefore, the expression of a neuron-specific factor or a change in spliceosomal protein composition allowing for EN inclusion should be under developmental control as well.

3.4. Gel mobility shift analysis of exon EN RNA

To evaluate whether there are different activities recognizing EN in neuronal and non-neuronal cells, we performed gel shift analysis. We prepared nuclear extracts derived from adult, embryonic day E15 and E20 rat brains and HeLa cells. Using radiolabeled RNA corresponding to EN (Fig. 5A), we observed a shift in nuclear extract prepared from adult rat brain, but no EN-specific binding activity was detectable in HeLa or embryonic brain nuclear extracts. Using a different RNA (tar) we could observe tar-RNA binding to the HeLa cell extract, demonstrating that this extract was intact. Using the EN probe, the shift could be observed after mixing HeLa and brain nuclear extract, indicating the absence of a binding inhibitor in HeLa nuclear extract. This shift was not observed in brain extracts when we used a synthetic polylinker RNA

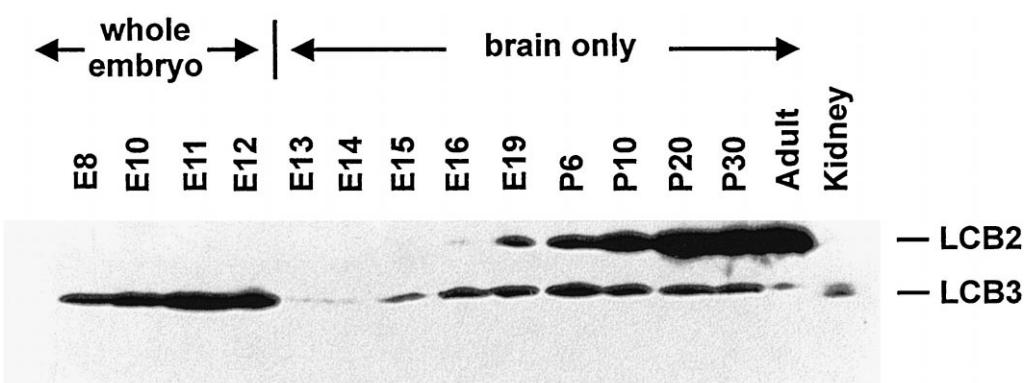

Fig. 4. Developmental regulation of EN. The Western blot was performed using protein extracts from whole embryos (E8–E12) and embryonic and postnatal brains (E13 to adult). The antibody (7H12) used detects both the LCB2 and LCB3 form. Inclusion of EN is first detectable at embryonic day 16 (E16).

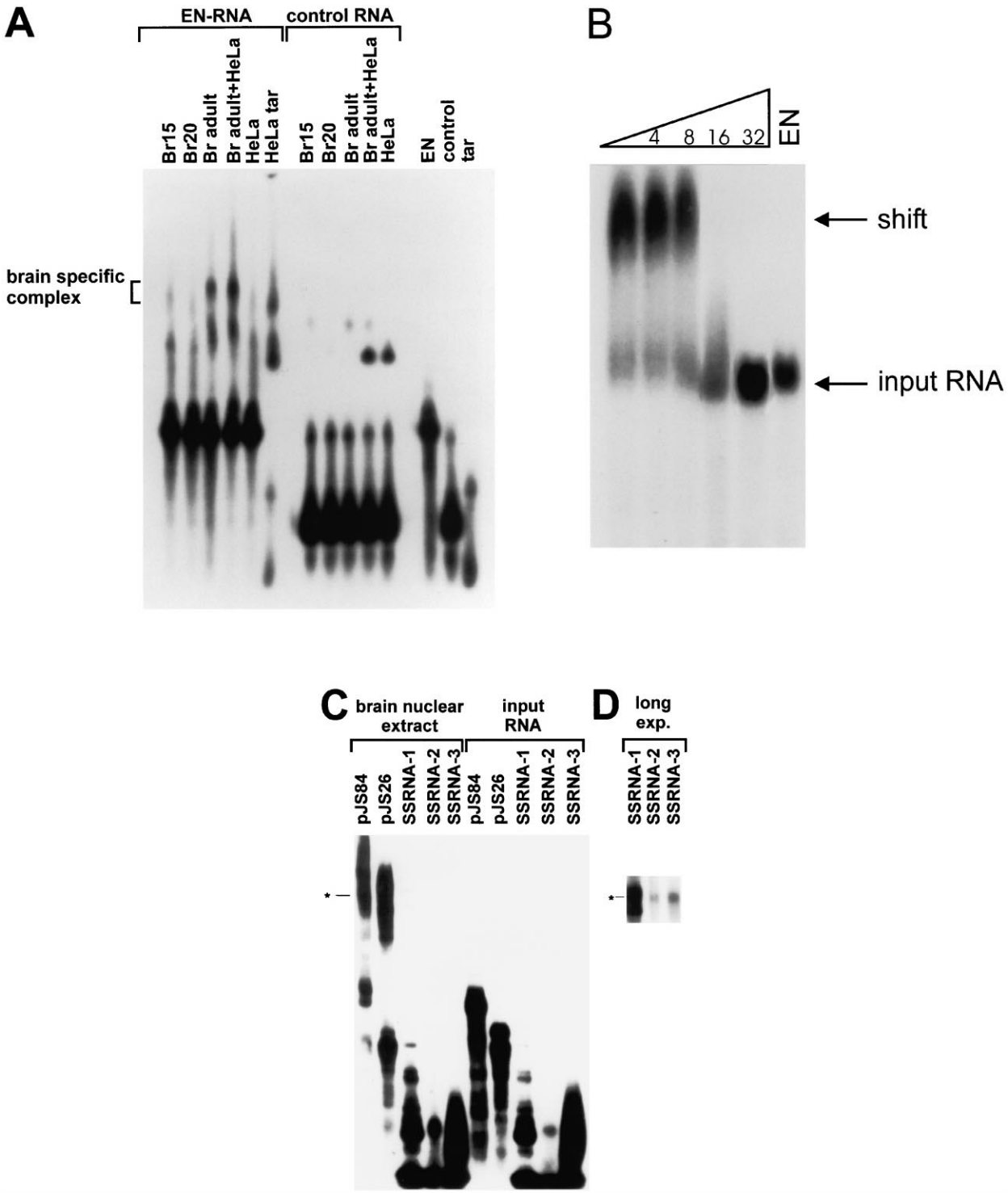

Fig. 5. Gel shift analysis of an EN binding activity in brain and HeLa extracts. (A) Formation of a brain-specific EN–RNA protein complex. EN–RNA made from pJS84 was subjected to gel shift in brain and HeLa nuclear extracts. An RNA shift was observed in adult brain nuclear extract, but not in embryonic and HeLa cell extracts. The tar control RNA shifts in HeLa cells extract, control-RNA: RNA made from pBluescript polylinker. Due to secondary structures, this RNA migrates with two different mobilities in native gels. Br15: nuclear extract from embryonic brains E15, Br20: nuclear extract from embryonic brains E20, Br adult: nuclear extract from adult brains, HeLa: nuclear extract from HeLa cells. EN, control and tar: RNAs without extract added. (B) Competition of the gel mobility shift with unlabeled RNA. EN–RNA made from pJS84 was subjected to gel shift in brain nuclear extracts. The numbers indicate the molar excess of unlabeled EN–RNA that was used as competitor. (C) Formation of RNA–protein complex with shorter EN probes. RNA derived from pJS84 (63 nt), pJS26 (54 nt) and synthetic oligonucleotides SSRNA-1 to 3 (30, 26, 18 nt) was subjected to gel shift in nuclear extracts from adult brain. (D) Longer exposure of panel B. The exposure time was about five times longer than in B, only the shifts derived from SSRNA1–3 are shown.

(Fig. 5A). Instead, we detected a shift of this probe in HeLa nuclear extract. This indicates that EN RNA binds to a component that is specific for brain nuclear extract.

An excess of unlabeled EN RNA abolished the mobility shift, again demonstrating its specificity (Fig. 5B). The gel mobility shift analysis described above was performed using a probe (pJS84, 63 nt clathrin sequence) that spans the entire exon EN and several nucleotides of the exons flanking EN. In order to determine the minimum size of RNA that was necessary for the mobility shift to occur, we

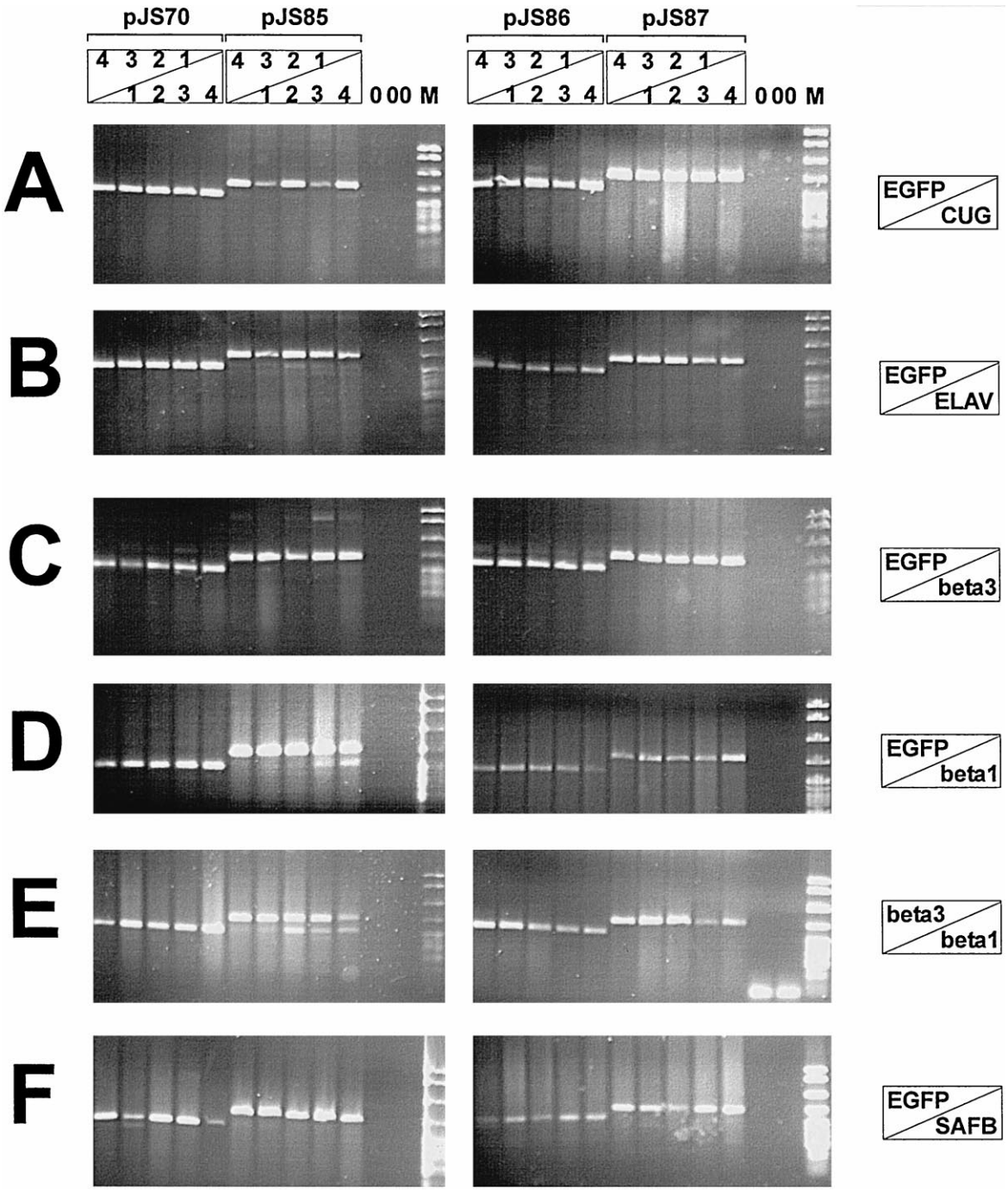

Fig. 6. Influence of RNA binding proteins on EN regulation in vivo. In vivo splicing assays were performed with minigenes pJS70 and pJS85–87 in the presence of increasing amounts of the EGFP-tagged cDNAs coding for factors involved in pre-mRNA processing indicated. The amount of transfected cDNA was balanced using empty vector (EGFP-C2), micrograms transfected are indicated in the top panels. With the exception of E, the DNA amount was normalized by the addition of EGFP-C2. In E, the amount of transfected DNA was kept constant by titrating EGFP-htra2-beta1 against EGFP-htra2-beta3. The cDNAs used for transfection are indicated on the right, the minigenes used are indicated on top. (A) CUG-binding protein, (B) rat ELAV-like protein, (C) htra2-beta3, (D) htra2-beta1, (E) htra2-beta1 and beta3, (F) scaffold attachment factor B. 0: RNA without RT, 00: PCR reaction without any template.

used shorter RNA substrates in another gel mobility shift analysis (Fig. 5C). An in vitro labeled RNA (pJS26, 54 nt) that spans just EN, without flanking clathrin light chain sequence, yielded a shift similar to the one observed with pJS84. In order to rule out contributions from the polylinker region, we used synthetic oligonucleotides in gel shift analysis. We could detect a similar shift using end-labeled *O*-methylated RNA oligonucleotides (SSRNA-1, 30 nt; SSRNA-2, 26 nt and SSRNA03, 18 nt). However, since the end labeling procedure results in a lower specific activity, the intensity of the signal is lower. Furthermore, the intensity of the shift decreased with the decrease in length of the oligonucleotide. With the shorter oligonucleotides SSRNA-2 and -3, a signal could only be seen after longer exposure (Fig. 5D). Using the longer in vitro RNA probes pJS84 and pJS26, three bands could be observed after short exposure. Only the middle band (indicated by a '∗') could be seen when we used the short synthetic oligonucleotides SSRNA-2 and SSRNA-3. The simplest explanation for the observed gel mobility shift is the presence of an EN binding activity expressed in adult brain, but not in HeLa cells. Assuming that binding to EN aids its spliceosomal recognition, this could result in EN inclusion in adult brain, but not in HeLa cells. We observed a small amount of EN usage in the embryo (Fig. 4), starting at embryonic day E16. The lack of an EN gel mobility shift in extracts made from embryonic day 15 and 20 could be due to insufficient amounts of the binding activity at this stage.

3.5. Co-transfection experiments

Several alternatively spliced exons were shown to be influenced by the concentration of SR proteins in co-transfection experiments. EN inclusion was shown to be slightly enhanced by an increase of SF2/ASF concentration in vivo [6]. We obtained similar results with other members of the SR family of proteins, such as X16 (SRp20), SC35 and SRp40 (data not shown). We therefore tested several other components of spliceosome for a concentration dependent influence on EN usage. We used pJS70, which represses EN in fibroblasts, as well as the shorter minigenes pJS85-87, which bear splice site mutation activating EN usage in fibroblasts. We reasoned that the combination of exon repression and activation mutants would permit detection of an influence on EN regulation.

First, we tested the CUG binding protein [60], which has been implicated in controlling the splicing regulation of the human cardiac troponin T gene [41]. Since there are several CUG clusters in intron D about 50 nt upstream from exon EN (Fig. 1A), we tested EGFP-CUG with our minigenes pJS70 and pJS85–87. As shown in Fig. 6, CUG-bp neither activated or blocked EN usage in any of the minigenes tested (Fig. 6A).

Similarly, EGFP-rELAV, a neuron-specific protein [21] whose *Drosophila* counterpart has been shown to change [24] splice sites in vivo, did not show any regulatory effect (Fig. 6B).

The developmentally regulated neuronal expression of EN suggests similar properties for its regulatory factor. We recently cloned htra2-beta3 [38], a variant of a human homologue of the *Drosophila* splicing factor transformer-2. Htra2-beta3 lacks the first SR-domain of htra2-beta1 and its expression is confined to brain, liver, kidney and testis. In brain, the expression of the beta3 isoform is developmentally controlled. Expression could be detected at and after embryonic stage 20, but not at E18. Increasing the concentration of EGFP-htra2-beta3 had no effect on any of our minigenes (Fig. 5C). However, when we tested htra2-beta1 in a similar way, we observed repression of EN usage in pJS85, a minigene that contains the consensus 5′ splice site (Fig. 5D). A similar effect was observed when EGFP-beta3 was titrated against EGFP-beta1 (Fig. 5E), indicating that the concentration of htra2-beta1 influences EN regulation.

Finally, we tested scaffold attachment factor-B, a protein that has been shown to be complexed with htra2-beta1 [39] as well as htra2-beta3 [38]. Whereas SAF-B can change the alternative splicing pattern of an E1A minigene [39], we see no effect of this protein on splicing of any clathrin light chain B minigenes analyzed.

Together, this analysis demonstrates that of all factors tested, only htra2-beta1 has a strong influence on EN regulation in vivo indicating that in addition to a neuron-specific factor, the cell specific concentration of various spliceosomal proteins might influence EN usage.

4. Discussion

4.1. Splice sites

In this study we analyzed *cis* elements regulating the alternatively spliced exon EN of clathrin light chain B. We have previously shown that it is regulated in a tissue-specific manner and now demonstrate that, in addition to its neuronal specificity, it is regulated in a temporal manner. The exon is used only in neurons and is expressed for the first time at embryonic day 16 in the brain. The expression increases until adulthood is reached. In order to determine the mechanism of EN regulation, we performed a mutational and biochemical analysis of EN expression.

Analysis of minigenes transfected into fibroblasts demonstrated that EN is not recognized in HeLa (Fig. 2) and HEK293 (Fig. 6) cells and most likely in all non-neuronal cells, because its splice sites deviate from the consensus and are probably not recognized by the splicing machinery. Improvement of 5′ splice sites has been shown to often [26,57] but not always [43] activate exon usage by

facilitating U1 binding [26]. In addition, the 3′ splice site of EN deviates from the consensus at the −3 position, where an A is used instead of the usual C. A statistical analysis of neuron-specific alternatively spliced exons demonstrates that 20% of these exons have a 3′ splice site with an unusual A at the −3 position [53] compared to 4% in constitutively spliced exons. Some of these exons, such as exon 6 of *tau* [1], the alternative exon of the GABAa receptor γ2 subunit [22] and the alternative exon of nervous system specific RNP protein-1 [44] are under developmental control as well. Furthermore, a natural mutation that changes a T to C at the −3 position increases exon 5a usage in the human *pax6* gene [15] which underlines the importance of this position. However, the exact role of the C at the −3 position is unclear. In the model of RNA interactions in the spliceosome, the conserved AG of the 3′ splice site pairs with the C_9U_{10} nucleotides of U1snRNA [54]. The nucleotide at the −3 position could pair with G_{11} of U1snRNA, which would strongly favor a C over an A. Therefore, it is likely that using an A at this position is a more general mechanism to prevent exon usage. In order to repress exon EN usage in HeLa cells fully, both the 5′ and 3′ splice site of EN have to deviate from the consensus. The unusual splice sites could be a means to ensure that EN is not expressed in cells other than neurons, perhaps to ensure that clathrin-coated vesicles are maintained calmodulin insensitive [42] in non-neuronal tissues. Since about one-third [53] of neuron-specific, alternatively spliced exons are surrounded by two weak splice sites, exon repression by two weak splice sites is probably a more general mechanism.

4.2. Gel shift

In order to determine how neurons and fibroblasts differ in their ability to recognize exon EN, we compared the ability of extracts made from HeLa cells and brain at various developmental stages to bind to EN RNA. We found that only extracts made from adult rat brain bind to EN in a gel retardation assay. In contrast, no binding activity could be observed in extracts made from embryonic brain and from HeLa cells. From the developmental expression profile of EN (Fig. 4), we expected a small amount of EN binding activity at embryonic stages E20. The absence of this activity in Br20 extracts could be due to detection limits in the gel shift assay or could indicate a regional concentration of the binding activity in the brain that is diluted upon homogenization. However, since we can detect the activity in adult brain, our results demonstrate a tissue and developmentally specific binding activity that correlates with the expression pattern of EN.

4.3. Co-transfection

Using the wild-type gene and three activation mutants, we next tested several proteins involved in pre-mRNA processing for their influence on EN regulation. The only protein that had a considerable effect on EN regulation in vivo was htra2-beta1. An increased amount of htra2-beta1 represses EN usage of a 5′ splice site consensus mutant (pJS85) in fibroblasts. Interestingly, when both splice sites are optimized (pJS87), no influence of htra2-beta1 could be detected, indicating that EN then behaves like a constitutive exon. In the presence of an activating factor in neurons, it seems likely that an increase of htra2-beta1 can repress EN usage in neurons, where EN usage is the default pathway. Interestingly, we previously demonstrated [12] that pilocarpine-induced neuronal activity represses EN usage in rat hippocampus and cortex in vivo. This change in splicing pattern is concomitant with a shift from htra2-beta3 to htra2-beta1 isoform [12]. Therefore, the ratio of htra2-beta1 to beta3 is subject to control in vivo and might influence the splicing pattern of various alternatively spliced exons, including EN.

Furthermore, our transfection results show for the first time a functional difference between htra2-beta1 and htra2-beta3 in an in vivo assay. Htra2-beta3 lacks the first SR domain of htra2-beta1, and is therefore structurally related to the *Drosophila* tra^{179} variant [29] that also deletes the first SR domain due to an alternative splicing mechanism. This alternative splicing mechanism is tissue-specific both in mammals, where it occurs in brain, liver, testis and kidney, and in *Drosophila*, where it is restricted to the male germline. It is likely that these isoforms reflect a regulatory splicing module that has been conserved from *Drosophila* to mammals [29,38]. Several other splicing factors, among them human suppressor-of-white-apricot [46] and hnRNPA1 [6], have been shown to stimulate exon skipping using in vivo co-transfection experiments. The difference between htra2-beta1 and htra2-beta3 in our in vivo assay shows for the first time that the ability of a mammalian splicing factor to cause exon skipping can be regulated by tissue-specific alternative splicing of this factor. The influence of htra2-beta1 on EN is surprising, since EN and its surrounding exon sequences do not contain GAA repeats [51], sequences that are preferentially targeted by htra2-beta1 in vitro [58]. This might indicate differences between the in vivo situation and the in vitro SELEX procedure that selects the thermodynamically strongest interactions. However, htra2-beta1 is abundantly present in HeLa nuclear extract [12] and we did not observe binding of EN RNA to components of HeLa nuclear extract. It is therefore possible that the effects of htra2-beta1 on EN repression are indirect, through interference with the SR-protein mediated protein network that assembles at splice sites [28,67].

Together, our data indicate that EN is not used in non-neuronal cells because of its weak splice sites. In neurons, we found an EN binding activity and postulate that it activates EN usage. In addition, EN usage is regulated by the concentration of SR-proteins, such as htra2-beta1, which might be used by neurons to repress EN expression in response to outside stimuli.

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