

Clathrin light chain B: gene structure and neuron-specific splicing

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ABSTRACT

The clathrin light chains are components of clathrin coated vesicles, structural constituents involved in endocytosis and membrane recycling. The clathrin light chain B (LCB) gene encodes two isoforms, termed LCB2 and LCB3, via an alternative RNA splicing mechanism. We have determined the structure of the rat clathrin light chain B gene. The gene consists of six exons that extend over 11.9 kb. The first four exons and the last exon are common to the LCB2 and LCB3 isoforms. The fifth exon, termed EN, is included in the mRNA in brain, giving rise to the brain specific form LCB2 but is excluded in other tissues, generating the LCB3 isoform. Primary rat neuronal cell cultures express predominantly the brain specific LCB2 isoform, whereas primary rat cultures of glia express only the LCB3 isoform, suggesting that expression of the brain-specific LCB2 form is limited to neurons. Further evidence for neuronal localization of the LCB2 form is provided using a teratocarcinoma cell line, P19, which can be induced by retinoic acid to express a neuronal phenotype, concomitant with the induction of the LCB2 form. In order to determine the sequences involved in alternative splice site selection, we constructed a minigene containing the alternative spliced exon EN and its flanking intron and exon sequences. This minigene reflects the splicing pattern of the endogenous gene upon transfection in HeLa cell and primary neuronal cell cultures, indicating that this region of the LCB gene contains all the necessary information for neuron-specific splicing.

INTRODUCTION

Receptor mediated endocytosis occurs by entrapment of specific macromolecules in coated pits and coated vesicles. Their coat is predominantly composed of clathrin (1) which is noncovalently bound to the vesicles. *In vitro*, it is possible to aggregate clathrin

trimers known as 'triskelions' into closed shell structures. Each of these trimers consists of three molecules of clathrin heavy chain and three molecules of clathrin light chain. There is only one form of clathrin heavy chain, whereas there are two classes of light chains, light chain A (LCA) and light chain B (LCB). In addition, the expression of different clathrin light chain isoforms involves tissue-specific splicing. For LCB there is one ubiquitously expressed form, LCB3, and a brain-specific form (LCB2) which has 54 additional nucleotides not found in LCB3 (2–5). Western blot analysis of various brain parts for LCB2 and immunocytochemical experiments are consistent with the idea that the LCB2 form is expressed predominantly in neurons (6). Although the function of the additional exon in brain is not clear, it has been speculated that it participates in the burst of endocytosis that mediates membrane recycling after neuronal stimulation and synaptic vesicle release (1) and might help in transporting clathrin coated vesicles to the synapse (2).

Alternative splicing is an important mechanism in the generation of protein isoforms from one gene (7–9). A number of genes are known to exhibit brain-specific alternative splicing. Among these genes are the calcitonin/CGRP primary transcript (10), which leads to the production of calcitonin in thyroid C cells and calcitonin gene related peptide (CGRP) in the brain. Other genes studied include the mouse *c-src* gene (11), the preprotachykinin gene that produces substance P and K and related tachykinin peptides (12) and the α -tropomyosin gene (13). The mechanisms that regulate brain-specific alternative splice site selection are still poorly understood. However, several *cis*-elements that are involved in alternative splice site regulation have been identified. These elements include sequences within the 5'- and 3'-splice sites, exon sequences, and sequences located within introns (11, 12, 14–16). In addition, a brain-specific form of the Sm B/B' has been reported (17). To date no cellular factors have been identified that mediate brain-specific alternative splice patterns.

In this paper we have determined the exon/intron organization of the clathrin light chain B gene, the first analysis of a clathrin

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light chain gene. We have identified a cell line that provides a model system to study the alternative splicing of the LCB gene. In addition, using a transient transfection assay, we have determined that the sequences for regulated alternative splicing are confined to sequences within the neuron-specific exon and its flanking intron and exon sequences.

MATERIALS AND METHODS

Standard molecular cloning protocols were followed for most of the experiments (18).

PCR methods and oligonucleotides

PCR reactions were performed in reaction volumes of 50 μ l. The 1 \times buffer contained 16.6 mM (NH₄)₂SO₄, 200 μ M dNTPs, 170

μ g/ml bovine serum albumin, 67 mM Tris-HCl, pH 8.8, at 20°C and the MgCl₂ concentration indicated in Table 2. The PCR reactions were performed using a Gene II (USA Scientific) and a Perkin Elmer thermocycler. The accuracy of the thermocycler was tested using an external device (19). The concentration of the PCR primer was 500 pM for each primer in the final PCR mix that also contained 1 U Taq DNA polymerase/50 μ l. Reverse transcription was carried out using 1 μ g of total RNA, 100 U of H⁻ Superscript MMLV reverse transcriptase (BRL), 10 mM DTT, 1 mM dNTPs, and 50 pmol of the antisense PCR primer. The sequences of the oligonucleotides are shown in Table 1. All oligonucleotides were synthesized at the oligonucleotide facility of the Mount Sinai School of Medicine or the Cold Spring Harbor Laboratory using an Applied Biosystems Synthesizer.

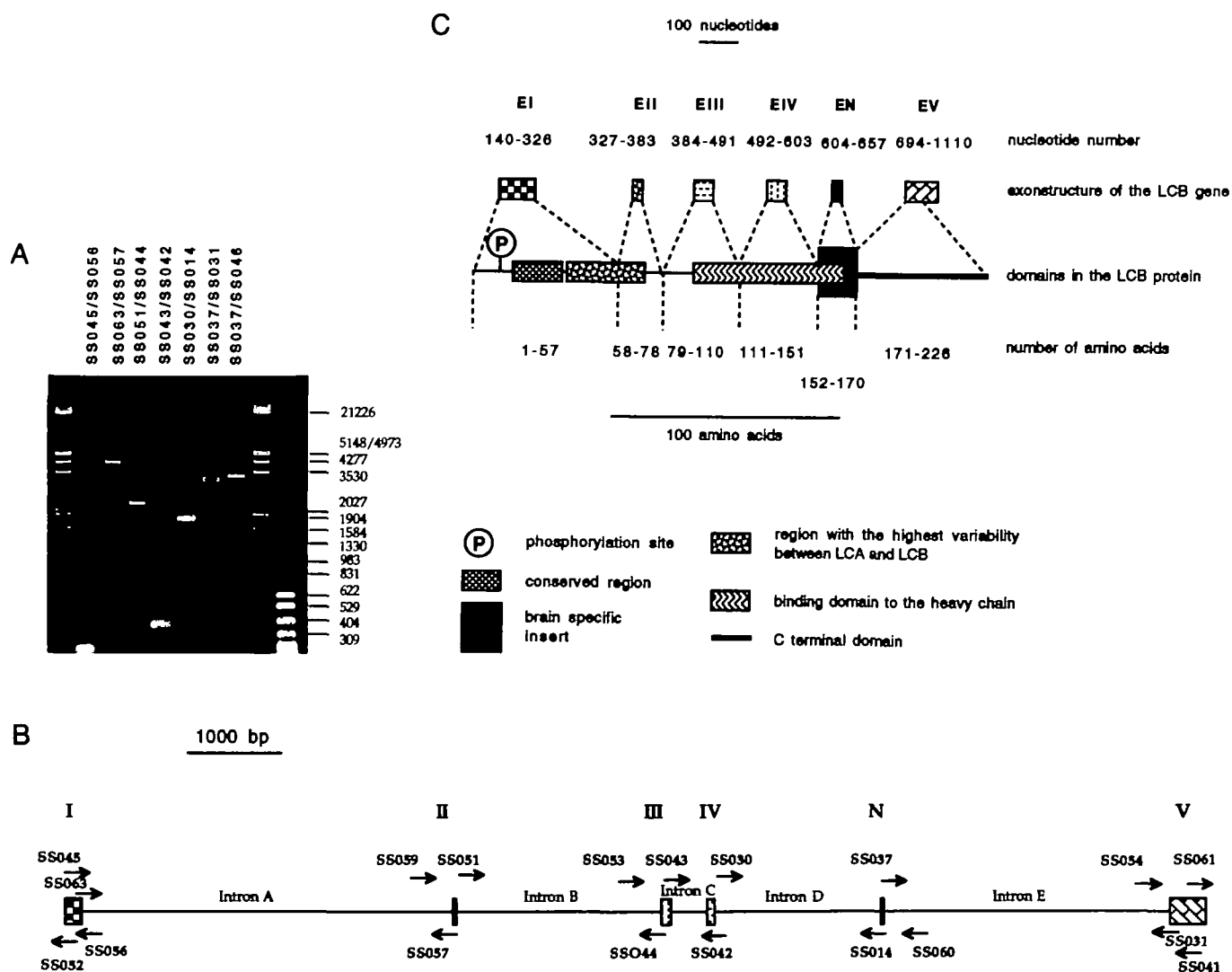


Figure 1 A. Intron length in the LCB gene determined by using PCR. DNA from rat genomic λ clones was amplified using the oligonucleotide combination indicated. The sequences of the oligonucleotides are described in Table 1, their location is shown in Figure 1B. The reaction conditions for the amplification are described in Table 2. The identity of the PCR products was confirmed using Southern Blots with intron primers and direct sequencing of flanking regions of the PCR products using the Maxam-Gilbert method (29). B. Gene structure of the rat clathrin light chain B. The sizes of the introns were determined in a semi-logarithmic plot of the PCR products from Figure 1A. Oligonucleotides used for sequencing and PCR amplification are indicated as arrows (not to scale), pointing towards the 5' end of the oligonucleotide. C. Relation between protein structure and intron/exon boundaries. The exons are shown as shaded boxes as in Fig. 1B. The numbers on top of the exons are the position of nucleotides in the numbering from Ref. (5). Note that in the numbering from Ref. (5) there is a gap between 657 and 694 that accounts for the LCA1 form. The domains of the LCB2 protein are also indicated. Numbers below the protein structure indicate the amino acid position. The numbering begins with the first methionine of LCB2. The six domains of the LCB2 form are indicated as different boxes, according to Ref. (3).

Screening and cloning

To obtain genomic clones for the LCB gene we screened 600,000 pfu of a Charon 4A rat genomic library (20) using the ³²P end labeled oligonucleotide JB181. As a probe for hybridization we used 10⁶ cpm of push column (Stratagene) purified ³²P end labeled JB181 in 5×SSC, 50 mM Na-Phosphate buffer pH 6.8, 1 mM Na₄P₂O₇ and 5×Denhardt's Solution (1×Denhardt : 0.2% Ficoll, 0.2% BSA, and 0.2% polyvinylpyrrolidone, M.W. 40,000). The filters were washed with a final stringency of 68°C in 2×SSC/0.1% SDS. This screen resulted in five positive clones, one of which (λ HaeIII-3) contained Exon EII, EIII, EIV, EN, and EV of the LCB gene. This λ clone was subcloned into the plasmid Bluescript SK(+) (Stratagene) using a KpnI site in intron C and the EcoRI sites of the Charon 4A polylinker. This resulted in two subclones, lam-4 and lam-5. Lam-5 contained exon EIV, EN, EV, and the 3' flanking part of the gene. Lam-4 contained exon EII and EIII. The other four λ clones obtained contained only exon EN and EV. To obtain the 5' end of the gene we screened an EMBL3B library kindly provided by R.Hynes. This screen was performed using a PCR probe generated with oligonucleotides SS043 and SS042 (see Figure 1B). The probe was synthesized with 25 μl 800 Ci/mmol dCTP as the only dCTP present in a 50 μl PCR reaction that contained 100 ng λHaeIII-3 DNA, 200 μM dATP, dTTP and dGTP each, the 1×buffer specified above and 1.75 mM MgCl₂. The extension time at 72°C was 4 minutes. We routinely obtain full length probes using this protocol (21). The probe was purified by precipitation and heat denatured. 500,000 pfu of the EMBL3B library were probed in 50% formamide, 50 mM Hepes pH 7.0, 1×Denhardt's solution, 3×SSC, 160 μg/ml salmon sperm DNA and 20 μg/ml yeast tRNA. The filters were washed with a final stringency of 65°C in 0.1×SSC/0.1% SDS for one hour. One positive clone was obtained (λEM3). This clone contained the 5' end of the gene, exons EI to EN and parts of intron E. A KpnI fragment of λEM3 that contained exon EI was subcloned into Bluescript SK(+).

Construction of a LCB minigene

A minigene containing the alternatively spliced exon EN and parts of exons EIV and EV was constructed by amplifying the genomic region spanned by the oligonucleotides SS030 and SS031 using 1 ng λ HaeIII-3 DNA as template. The 4.9 kb fragment could be amplified by using a four minute extension time. EcoRI linkers were ligated to the fragment which was subcloned into Bluescript SK(+). This clone pJS40 was further shortened by removal of a 800 bp BamHI/SalI fragment in intron E. The truncated insert was subcloned into the expression vector pΩ3 (22) generating pJS71 (Fig. 5). pΩ3 contains the SV40 early promoter and an SV40 T poly A site.

Cell culture

Standard procedures were used for cell culture and cell maintenance (23). Primary neuronal cultures of dissociated rat embryo mesencephalon were cultured in serum free medium as described (24). P19 embryonal carcinoma (EC) cells were cultured and induced as described (25,26). HeLa cells and primary mesencephalic cultures were transfected using lipofectin (BRL). We plated approximately 800,000 mesencephalic cells per 35 mm culture dish for each transfection. The cells were transfected between 3–7 days after they were plated. For transfection, a solution of 10 μg lipofectin/ml serum free culture medium was mixed with an equal volume of a solution of 10

μg/ml DNA in serum free culture medium, giving a final volume of 1 ml. Both solutions were mixed prior to transfection and added to the cells. Primary neuronal cultures remained in this medium for two days. The medium for HeLa cells was changed after an overnight transfection. RNA was harvested after two days and prepared using the NP40 lysis method as described (27).

RESULTS

Gene structure of rat clathrin light chain B

Two partially overlapping rat genomic clones, λHaeIII-3 and λEM3, were obtained from screening a Charon 4A and an EMBL3B library. These clones were used to determine the gene structure. The exon/intron structure of the LCB gene was determined using a PCR approach. Based on the cDNA sequence (5) we designed oligonucleotides that were complementary to exon sequences. The sequences of the oligonucleotides are shown in Table 1. Using the isolated clone DNA as templates, we determined the size of the introns. The gene structure is shown in Figure 1B. The LCB gene consists of at least six exons that are separated by five introns of approximately 4, 2.2, 0.4, 1.8, and 3.1 kb in size. The sequence of the exon/intron boundaries of the gene were determined using subcloned fragments. The sequences are shown in Table 3. The exon/intron boundaries were compared to the consensus sequence for vertebrate splice sites (7). The 3'-splice site of Exon EIV is unusual as it contains four consecutive guanosine nucleotides within the polypyrimidine stretch. Both the 3'- and 5'-splice sites of the alternatively spliced exon EN are also unusual. The 3'-splice site contains an adenosine nucleotide prior to the AG. This position is usually occupied by a pyrimidine nucleotide, mainly a cytosine. The 5'-splice site contains three nucleotides that differ from the consensus sequence, two of them are pyrimidine bases instead of purine bases.

Analysis of the alternative splicing patterns of LCB gene in various cell types

We analyzed several cell lines for the expression of the two isoforms LCB2 and LCB3. RNA from HeLaS3 (ATCC CCL # 22), AtT20 (ATCC CCL # 89) and PC12 cells (ATCC CRL

Table 1. Oligonucleotides used in this study. Restriction sites in the oligonucleotides are underlined.

name	L	sequence 5' → 3'	location
SS014	21	TCAGGATCCGGCTGCTGGTAG	ExonN, antisense
SS030	21	TGCCTCGAAGGTGAACCGAAC	ExonIV, sense
SS031	21	GGGGTCTCCTCTTGGATTCT	Exon V, antisense
SS037	17	GATCGCTGACAAAGCGT	Exon N, sense
SS041	21	TCCCAGAGACTCAAGAGGAAC	Exon V, antisense
SS042	19	TTTGGCCTTCTCCCGCCC	Exon IV antisense
SS043	20	GGACAGGTTGACTCAGGAGC	Exon III sense
SS044	19	TCGACGCTAGCCATCGGC	Exon III, antisense
SS045	20	ATGGCTGAGGACTTCGGCTT	Exon I, sense
SS051	20	GTTCGGAGGACATGGGGACT	Exon II, sense
SS052	20	CGCTCTCCGACGACGAGAAG	Exon I, antisense
SS053	21	GTGCTCTGGTTTGAGGGCTAT	IntronB, sense
SS054	21	TTCTGGCTAACGTCGCCAGGGT	IntronE, sense
SS056	21	CTCGGAGTCCGGGCTGCGCA	Exon I, antisense
SS057	20	AAACACATCTCCATTGACTGT	Exon II antisense
SS059	19	GCAAAGACAGGAGCGTTCT	Intron A, sense
SS060	20	TTGGCTGCGAGAGACAAAG	Intron E, antisense
SS061	19	TTACCCACCCTGTCCTTC	Exon V, sense
SS063	19	TGCGCAGCCCGGACTCGCG	Exon I, antisense
JB181	43	TCGAGTGGTATCAGCATCTGGCTGCTGGT- AGAACGCTTGTCA	Exon EN,
JB331	28	TCGAGTCTTCCGATGCCCTGTTGTTGAT	Exon EIV/EV,

1721) was reverse transcribed and amplified using SS030 and SS031. We also analyzed RNA from neuroblastoma NB41A3 (ATCC, # CCL 173) and neuro-2a cells (ATCC, # CCL 131) because these cells are probably derived from the neuronal crest and might exhibit neuronal properties. Since the light chain B RNA is not abundantly expressed in most tissues (3) we used a PCR assay to detect LCB isoforms. RNA from different cell types was reverse transcribed using the primer SS031 and PCR amplified using the primers SS030 and SS031 (Table 1). The primer SS030 is complementary to sequences in exon EIV, and SS031 is complementary to sequences in exon EV. The RNA from the LCB3 form generated a PCR fragment of 152 bp. The PCR product generated by LCB2 RNA is 206 bp long, since it contains the brain-specific exon EN. Both PCR products can be distinguished by their size and their hybridization patterns. The PCR product generated by LCB2 RNA hybridizes with the oligonucleotide JB181 that contains sequences complementary to exon EN, whereas the PCR product generated by LCB3 hybridizes with the oligonucleotide JB331 that contains sequences complementary to the flanking parts of exon EIV and EV. From all cell types examined, only primary neuronal cultures from rat cortex, mesencephalon or superior cervical ganglion expressed an amount of LCB2 that was detectable in an ethidium bromide stained agarose gel (Figure 2). In HeLa, ArT-20, neuroblastoma NB41A3, neuro-2a, PC12 and primary glial culture the LCB3

form could be detected using the ethidium bromide stain. If the PCR products were further analyzed using Southern Blot with ^{32}P end labeled JB181 as a probe, and long exposure time, all these cells show weak expression of LCB2 (data not shown). This expression pattern indicates that primary neuronal cultures, like brain tissue, include the brain specific exon EN in the mRNA of LCB. It is also worth noting that since the clathrin light chains A and B are similar and share sequence homology, one might expect to detect a LCB1 form related to the LCA1 form that contains a second brain-specific exon (3–5). However, with our sensitive PCR approach we could not detect a band of the expected size (241 bp) for a predicted LCB1 form using JB 181 as a probe, which is expected to identify both LCB2 and the hypothetical LCB1 form.

Induction of the brain specific form of clathrin light chain B in P19 teratocarcinoma cells

The expression of LCB2 and LCB3 RNA in P19 mouse teratocarcinoma cells was examined because these cells can be induced to differentiate into a neuronal phenotype in the presence of retinoic acid (25). We analyzed the appearance of LCB2 after different times of retinoic acid treatment. LCB2 mRNA was detected after four days of retinoic acid treatment (Figure 3). We found that undifferentiated P19 murine embryonal carcinoma cells do not contain detectable levels of the brain-specific light chain

Table 2. Reaction conditions for the PCR reactions that were used to determine the intron sizes in the LCB gene.

#	oligonucleotide	MgCl ₂ conc. [mM]	elongation time [min]	annealing temp. [°C]	product length
1	SS045/056	3.0	1.5	55	200
2	SS063/057	3.5	4.0	50	4020
3	SS051/044	2.0	3.0	57	2210
4	SS042/042	3.0	2.0	55	370
5	SS030/014	1.5	3.0	55	1790
6	SS037/031	2.0	4.0	55	3143
7	SS037/041	3.5	4.0	55	3250

Table 3. Sequences of the Exon/Intron boundaries. The sequences were submitted to GenBank, the accession numbers are L01560–L01565.

Exon	Intron	A	G	G	T	A	A	G	T	consensus
62	77	100	100	60	74	84	50			% usage
g	G	G	T	g	A	G	T			ExonI → Intron A
A	G	G	T	A	A	G	c			ExonII → Intron B
g	G	G	T	g	A	G	g			ExonIII → Intron C
A	G	G	T	g	A	G	T			ExonIV → Intron D
g	t	G	T	A	c	G	T			ExonN → Intron E

A. 5' Splice sites in the gene of the clathrin light chain B. The consensus splice site for vertebrates is indicated (6). Nucleotides that differ from the consensus sequences are indicated by small letters. The numbers indicate the per cent usage of a particular nucleotide in the consensus sequence.

Intron	Py	Py	Py	Py	Py	Py	Py	Py	Py	Py	N	C	A	G	G	consensus
	78	81	83	89	85	82	81	86	91	89		78	100	100	55	% usage
T	C	C	T	T	g	C	T	C	T	T	C	C	A	G	G	Intron A → ExonII
T	G	T	g	C	T	T	T	g	T	T	G	t	A	G	G	Intron B → ExonIII
T	C	T	g	C	g	g	g	C	T	C	A	C	A	G	A	Intron C → ExonIV
A	A	C	g	g	T	T	T	C	T	C	a	A	G	G	A	Intron D → ExonN
C	T	C	T	C	T	C	T	a	C	C	T	T	C	A	G	Intron E → ExonV

B. 3' Splice sites in the gene of the clathrin light chain B.

LCB2 mRNA. However, upon induction with retinoic acid the cells express light chain LCB2 mRNA. To further confirm the correlation between induction of neuronal cell type and LCB2 mRNA expression, cells were induced with DMSO. We were unable to detect LCB2 mRNA in these cells, consistent with their muscle cell phenotype. To test whether LCB2 is simply induced in response to retinoic acid or as a result of neuronal cell-type formation, we treated cells in monolayer with retinoic acid. Under

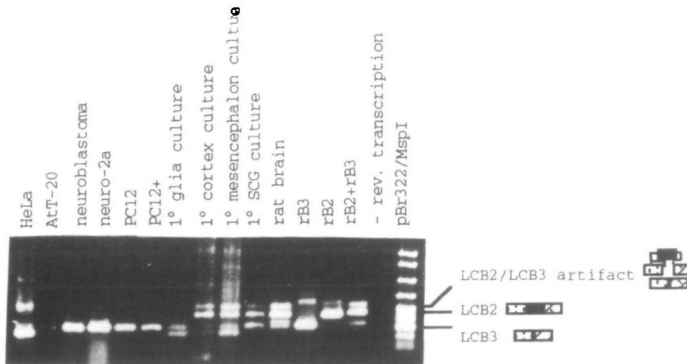


Figure 2. Distribution of LCB in various cell cultures. 1 μ g RNA from the different cell cultures was reverse transcribed using SS031 and amplified using SS030 and SS031 in 1.5 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 200 μ M dNTPs, 170 μ g/ml BSA, 67 mM TrisHCl, pH 8.8, at 20°C. 30 cycles were used (1 minute 94°C, 1 minute 55°C, and 2 minutes 72°C). One tenth of the PCR products was separated on a 2% agarose gel (TBE). Only primary neuronal cultures express the brain specific form LCB2 with about the same intensity as brain. As positive controls plasmids rB2 and rB3 (30) containing cDNA from LCB2 and LCB3 were amplified, producing the expected bands of 206 and 154 bp length. If the two plasmids were mixed prior to amplification, a heterodimer PCR artifact probably consisting of one strand LCB2 and one strand LCB3 appears. This artifact can also be seen in the PCR products from brain and 1° neuronal cell cultures. These artifacts have also been described for other alternatively spliced genes (31). The LCB2 and LCB3 form and the artificial heterodimer form are schematically indicated. The nature of the bands above LCB2 in HeLa cells and below LCB3 in HeLa and primary glial cultures is not known. These bands are probably PCR artifacts, since they do not hybridize with the internal primers JB181 or JB331 (data not shown). SCG: RNA from superior cervical ganglion. - rev. transcription: negative control using RNA from primary cultures from mesencephalon without reverse transcriptase.

these culture conditions EC cells differentiate into fibroblast like cells with no significant numbers of neuronal cells (25). Here, we did not observe any LCB2 mRNA induction (Figure 4). This contrasts with the above experiments where, after four days of retinoic induction in aggregates, the cells express LCB2 mRNA. The expression of the LCB2 isoform in P19 teratocarcinoma cells can be only detected when the PCR products are analyzed using the sensitive Southern blot method. The induction is barely visible if the PCR products are stained with ethidium bromide. These findings demonstrate that the expression of the LCB2 form is concomitant with the expression of the neuronal phenotype in the P19 teratocarcinoma cell system, which is also indicated by the accumulation of LCB2 during the time course of induction. However, the major form of LCB expressed in the retinoic acid induced neuron-like P19 teratocarcinoma cells is the peripheral form LCB3, which is in contrast to the splicing pattern of primary neuronal cultures. This may indicate differences between the splicing machinery in neurons and neuron like P19 teratocarcinoma cells.

The expression of a minigene construct in HeLa and primary neuronal cultures reflects the tissue-specific splicing behavior of the clathrin light chain B gene

In order to define the necessary cis-acting elements that are involved in the regulation of the brain specific exon EN, we constructed a minigene, pJS71, that contained the flanking parts of exon EIV and EV as well as intron D, exon EN and parts of intron E (Figure 5). The minigene pJS71 was transfected into HeLa cells and 1° rat mesencephalon cultures. RNA from these cells was recovered and analyzed by reverse transcription using SS031 and PCR using the primers SV40A and SS031. The SV40A primer is complementary to the sequences from the SV40 sequences in pW3. The use of the primer SV40A made it possible to discriminate between RNA from the transfected construct and endogenous RNA. The PCR products derived from these transfection experiments were analyzed in a Southern Blot using JB181 and JB331 as a probe for LCB2 and LCB3. As shown in Figure 5, HeLa cells express predominantly the peripheral form LCB3, whereas primary rat mesencephalon cultures express predominantly the brain specific form LCB2. These results show that the minigene has the same cell type specific regulation like the endogenous gene.

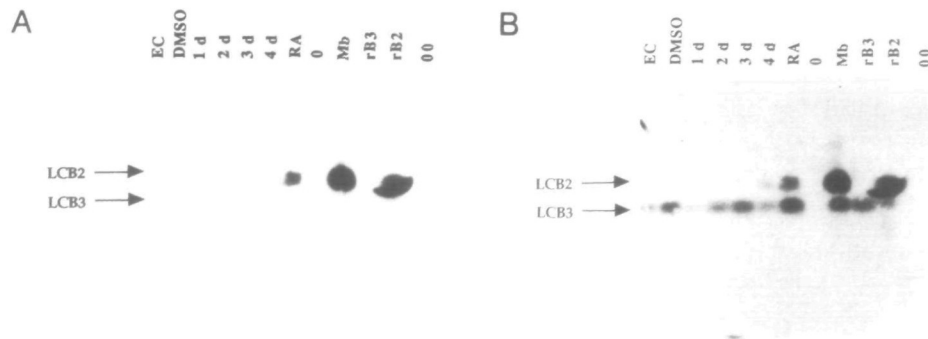


Figure 3. Retinoic acid induction of LCB2 in P19 teratocarcinoma cells. PCR products were separated on 2% SeaKem agarose and transferred to genescreen. A. Filters were probed with JB181 which hybridizes to LCB2. B. After being stripped of label, filters were reprobed with JB 331 that detects LCB3. The autoradiographs were then superimposed. EC, uninduced cells; DMSO, cells induced for seven days with DMSO; 1d,2d,3d,4d, cells induced for one, two, three, and four days with retinoic acid; RA, cells were induced for four days with retinoic acid and then cultured for three more days in the absence of retinoic acid. MB, mouse brain; rB2 and rB3, PCR products generated from cloned cDNA templates; 0, negative control of PCR mix without template; 00, negative control using mouse brain RNA that was not reverse transcribed.

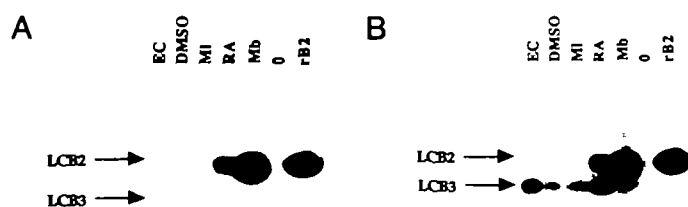


Figure 4. LCB2 is only expressed in P19 teratocarcinoma cells after neuronal phenotype formation. **A.** Filters were probed with JB181 which hybridizes to LCB2. **B.** After the label was removed, the filters were reprobed with JB 331 that detects LCB3. The autoradiographs were then superimposed. EC, uninduced cells; DMSO, cells induced for seven days with DMSO; MI, EC cells induced in monolayer with retinoic acid for four days followed by 3 days growth without retinoic acid; RA, cells were induced for four days with retinoic acid and then cultured for three more days in the absence of retinoic acid; MB, mouse brain.

DISCUSSION

Gene structure

Protein structure is reflected in the gene structure. The clathrin light chain proteins can be separated into six domains (3). The determination of the gene structure reveals that some of these domains are represented by exons in the LCB gene, as shown in Figure 1C. The six domains in the LCB protein are: (i) a phosphorylation site, (ii) a region that is conserved between the two light chain forms LCA and LCB, (iii) a region that has the highest variability between LCA and LCB, (iv) a domain that binds to the heavy chain, (v) a brain specific domain, and (vi) a C terminal domain (9). Exon EI contains the phosphorylation site, the region that is conserved the most between LCA and LCB and a part of the region with the highest variability. Exon EII contains the rest of the region with high variability. The heavy chain binding domain contains exons EIII and EIV. The brain specific insert is encoded by exon EN, whose function is yet to be elucidated, and the carboxy-terminal domain and the 3' untranslated region is produced by exon EV.

The 5'- and 3'-splice sites of the alternative spliced exon EN are not in consensus. Inspection of the 5'- and 3'-splice sites of the LCB gene shows that the 5'-splice site of exon EN is divergent from the vertebrate consensus sequence (Table 3A). This might be important in the regulation of the alternative splice of exon EN as it is in the preprotachykinin gene (12). In the case of the preprotachykinin gene a weak 5'-splice site reduces the ability of the U1 snRNP to bind to the 5'-splice site thereby limiting the ability of splicing factors to interact with exon EN. The 3'-splice site of the exon EN also differs from the vertebrate consensus sequence; the nucleotide prior to the AG nucleotides is usually a C (Table 3B). Only 2% of all vertebrate genes have an adenosine residue in this position (7). Thus, the combination of unusual 5'- and 3'-splice sites might contribute to the exclusion of exon EN in nonbrain tissue. Why the exon EN is retained in the mRNA in brain remains to be determined.

Distribution of the splice forms in different cell types

Only primary neuronal cultures reflect the splicing pattern that is observed in brain. We examined a number of cell cultures to find a model that mimics the splicing pattern of LCB. From all cells examined, only primary neuronal cultures from either

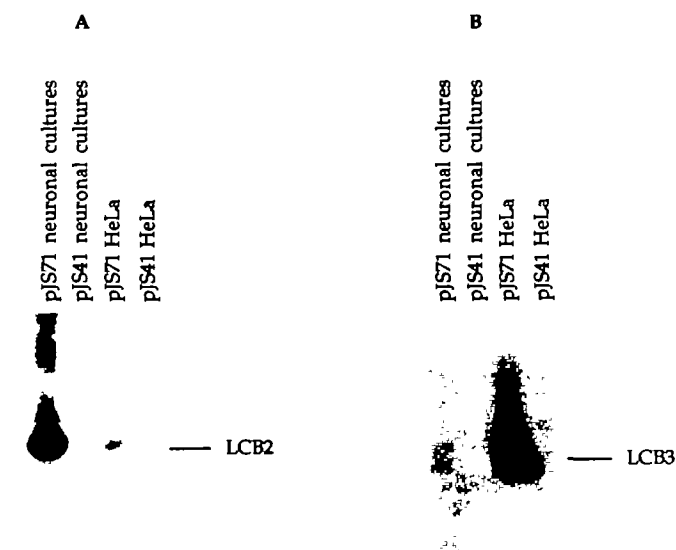
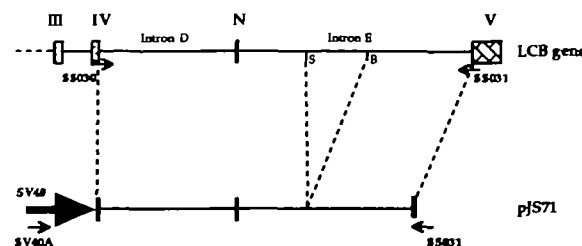


Figure 5. Expression of the minigene pJS71 in HeLa cells and primary cultures from rat mesencephalon. The construction of the minigene pJS71 from the genomic DNA is indicated. The minigene was generated using an SS030/SS031 PCR fragment in which the BamHI/Sall (B, S) fragment in Intron E was deleted. The location of the oligonucleotide SV40A in the SV40 promoter (thick arrow) is indicated. Plasmid DNA of pJS71 was transfected into HeLa cells and primary rat mesencephalon cultures. The plasmid pJS41 that contained the minigene insert of pJS71 in the antisense orientation was transfected as a control. The RNA of the transfected cells was amplified using SV40A and SS031. **A.** Southern blot of the PCR products using JB181 as a probe. The brain specific form LCB2 is expressed in primary rat mesencephalon cultures, not in HeLa cells. **B.** Reprobing of the same filter using JB331. The peripheral form LCB3 is expressed predominantly in HeLa cells, upon longer exposure a band corresponding to LCB3 can also be seen in primary neuronal cultures.

mesencephalon, cortex or superior cervical ganglion reflected the splicing pattern found in brain. The finding that primary glial cultures express only the LCB3 form indicates that the LCB2 form is specific for neurons. Data supporting this theory have also been found using an antibody that is specific for sequences encoded in the brain-specific exon (6). The presence of the brain-specific LCB2 form in cultures from cortex and superior cervical ganglion indicates that this form is expressed in both the central and peripheral nervous system. The brain-specific form LCB2 was found to be expressed in P19 teratocarcinoma cells when these cells are treated with retinoic acid and develop into a neuronal phenotype. However, when the cells are treated with retinoic acid under conditions that do not promote the formation of a neuronal phenotype, no LCB2 form is detectable (Figure 4). These data further suggest that LCB2 is a neuron-specific isoform. It is interesting that by contrast immortalized cells that retain

neuronal properties such as Neuro 2a cells and neuroblastoma NB41A3 cells mainly express the peripheral form LCB3. Even in the P19 system the peripheral form LCB3 is predominant in cells that have been induced to a neuronal phenotype. A possible explanation is that immortalized cells could have lost their ability to include the neuron-specific exon due to their transformation, indicating that the neuron specific splicing of LCB is only required when morphological or functional synapses are made. The interconnection of neurons is a basis for storage and retrieval of information. This function prohibits further division of neurons (28). Contrary to neurons, transformed cell lines like Neuro 2a divide and do not express the neuron specific LCB2 form. Thus, it may be possible that the neuron specific isoform is necessary for the communication of neurons that can not take place in transformed cell lines. The real function of exon N, however, is not clear.

The information in the splicing cassette of exons EIV to EV is sufficient for cell specific regulation

In order to define the *cis*-elements necessary for the brain-specific alternative splicing and to test whether primary neuronal cultures can serve as a cellular model in transfection assays, we transfected a minigene into neuronal and HeLa cells. The splicing pattern observed in tissue is mimicked by transfection of the minigene pJS71 in primary neuronal cultures and HeLa cells. Primary neuronal cultures that contained predominantly neurons and some glia (24) preferentially included the neuron specific exon EN, whereas HeLa cells preferentially skipped this exon reflecting the situation in brain and peripheral tissue. These results indicate that all the necessary elements for the neuron-specific splice site selection are located in the alternative spliced exon EN, and its flanking intron and exon sequences. Furthermore, we demonstrated that primary neuronal cultures can be used in analyzing the splicing pattern of minigenes containing brain-specific exon. Experiments are in progress to further define the *cis*-elements involved in neuron-specific splice site selection.

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