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Brain-specific promoter and polyadenylation sites of the β-adducin pre-mRNA generate an unusually long 3′-UTR

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ABSTRACT

Adducins are a family of membrane skeleton proteins composed of α-, β- and γ-subunits that promote actin and spectrin association in erythrocytes. The α- and γ-subunits are expressed ubiquitously, while the β-subunit is found in brain and erythropoietic tissues. The brain β-adducin protein is similar in size to that of spleen, but the mRNA transcript is a brain-specific one that has not been yet characterized, having an estimated length of 8–9 kb instead of the 3–4 kb of spleen mRNA. Here, we show the molecular basis for these differences by determining the structure of the brain-specific β-adducin transcript in rats, mice and humans. We identified a brain-specific promoter in rodents that, apparently, was not conserved in humans. In addition, we present evidence that the brain-mRNAs are formed by a common mechanism consisting in the tissue-specific use of alternative polyadenylation sites generating unusually long 3′-untranslated region of up to 6.6 kb. This hypothesis is supported by the presence of highly-conserved regions flanking the brain-specific polyadenylation site that suggest the involvement of these sequences in the translational regulation, stability and/or subcellular localization of the β-adducin transcript in the brain.

INTRODUCTION

Adducins are a family of membrane skeleton proteins encoded by three related genes (ADD1 or α, ADD2 or β and ADD3 or γ). All three members undergo alternative splicing of their pre-mRNA potentially generating a variety of protein isoforms, which have not yet been fully characterized (1–4). Adducin is found as hetero-dimer or hetero-tetramer of α/β or α/γ composition in most tissues. Human erythrocytes exclusively contain α/β heterodimers that promote the association of actin and spectrin in the membrane skeleton (5). Both the α- and γ-adducin are expressed ubiquitously, while the β-adducin gene shows a pattern of expression restricted to brain and hematopoietic tissues such as bone marrow and spleen (6–8), suggesting a different transcriptional regulation and function for all three genes.

In the brain, α- and β-adducin expression is found to be highly enriched in regions with high synapse densities of the hippocampus, corpus striatum, cerebral cortex and cerebellum (9,10), and it was shown recently that β-adducin may play a role in the cellular processes underlying synaptic plasticity associated with learning and memory (10). However, both the gene structure of the β-adducin gene and the molecular characterization of the mRNA isoforms are still incomplete. In fact, the brain β-adducin gene product identified by western blot analysis has roughly 110 kDa, only slightly larger than that of 97 kDa detected in spleen (6,11), but the β-adducin mRNA transcript that codes for this protein form is a brain-specific one that has 8–9 kb instead of the 3–4 kb of that one found in spleen (7,12). Although the long brain-specific β-adducin mRNA has been described about 15 years ago (7,12), the molecular basis of this unusually long brain-specific mRNA have not been characterized (1–3,13).

In the present work we determine the molecular structure of the brain-specific β-adducin transcript in rat, mice and humans. The β-adducin gene is found in chromosome 2, 6 and 4 in humans, mice and rats, respectively, in regions of chromosomal synteny, suggesting a common evolution and similar regulatory mechanisms in all three species. However, the brain-specific promoter in rats and mice is not apparently conserved in humans. We show the presence of an 8–9 kb long β-adducin transcript also in human brains and we present evidence of a common mechanism in all three species consisting in the use of tissue-specific polyadenylation sites. In addition, the regions flanking the brain-specific polyadenylation site are highly conserved supporting the hypothesis of a common mechanism regulating the 3′-untranslated region

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(3′-UTR) in rats, mice and humans and highlighting the importance of these sequences in the translational regulation, stability and/or subcellular localization of the β-adducin transcript in brain.

MATERIALS AND METHODS

Animals

The generation of the mice devoid of β-adducin has been described previously (6), the wild-type mice used were C57BL/6. The rats were Sprague–Dawley. All animals used in the experiments described below were males of 3 months of age and were of nearly the same weight.

Protein preparation and western blot analysis

Protein preparations and western blot of erythrocytes and brain were as described (6).

RNA preparation, RT–PCR and northern blot analysis

Total cellular RNA was prepared basically by the guanidinium thiocyanate method (14) from freshly extracted tissues from rat and mice, quantified by measuring absorbance at 260 and 280 nm, and RNA integrity was confirmed by running the samples on 1% agarose formaldehyde gels. Human RNAs were from two commercial suppliers (Ambion and Clonetech). PCR primers used in the present work were prepared by Sigma Genosis. Their sequence and specific use is detailed in Supplementary Table 3. RT–PCRs were performed for 35 cycles.

For northern blot analysis, 20 μg of RNA was loaded in 1.2% agarose formaldehyde gels, transferred to nylon membranes, and hybridized with the appropriate DNA probe labeled with 32-αP-dCTP.

RNase protection and primer extension assays

For the RNase protection assay, overlapping fragments covering the entire rat β-adducin cDNA sequence were subcloned from a full-length rat cDNA clone into pBS-KS plasmid. The RNase protection assay was performed essentially as described (15,16). Each of the DNA subclones was linearized, the antisense strand synthesized radioactively with either T7 or T4 RNA polymerase and purified through denaturing acrylamide gels. The probe and β-adducin RNA hybrids were digested, run in denaturing acrylamide gels and exposed for autoradiography.

The primer extension assays were performed essentially as described (15). Briefly, the oligonucleotide 5′βNC, complementary to the end of the 99 bases rat exon 2 was annealed to rat spleen and brain RNA and the RT reaction was performed in the presence of radioactive 32-αP-dCTP. The primer extension products were run in denaturing acrylamide gels and exposed for autoradiography.

Database analyses and bioinformatics

The search for alternative exons in the β-adducin gene required analysis of both cDNA and expressed sequence tag (EST) sequences in the Genbank. To identify candidate first exons or alternative internal exons, we used each of the β-adducin exon sequences as probes for BLAST species-specific searches of Genbank. The candidate sequences that were found contiguous to β-adducin exons were checked for proper splicing and their chromosomal location was determined by BLAST analysis against the complete genome using the NCBI browser (http://www.ncbi.nlm.nih.gov/BLAST/). The alignment of the ESTs with the β-adducin genomic sequence and the detection of the ESTs containing poly(A) tails were analyzed manually with the help of the UCSC Genome Browser (http://genome.ucsc.edu/). The coordinates of the chromosomal position of the exons and introns listed in Supplementary Table 1 refer to that used in the ENSEMBL browser (July 2005) developed by the EMBL, EBI and Sanger Institute (http://www.ensembl.org/index.html). In order to confirm experimentally that these sequences were bona fide exons in the β-adducin gene, RT–PCRs were performed from brain and spleen total RNA and the results are shown in Figure 3 and Supplementary Figure 2.

Promoter analysis was performed with both the DNAstar Lasergene program and the TESS browser from the University of Pennsylvania (http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME). A selection of the predicted transcription factor binding sites that displayed a high score and were present in both analyses is shown in Supplementary Figure 1.

RESULTS

The reported full-length cDNAs for β-adducin (ADD2) in mouse, rat and humans have 3119, 3115 and 3938 bp, respectively and the molecular weight of the encoded proteins [(6,7,12), ENSEMBL and Supplementary Table 1] is on line with the presence of 5′- and 3′-UTR of average length (17). In addition, the β-adducin pre-mRNA undergoes alternative splicing and northern blot analysis of erythropoietic tissues using a β-adducin probe detects the expected β-adducin bands in the range of 3.5–4.5 kb in all three species (Figure 1A, Lanes 2, 4 and 7). In fact, more than one band was seen when spleen RNA was used in the northern blot experiment and it was suggested that they might be the result of alternative splicing of the β-adducin pre-mRNA (1–3) generating the β-add97 and β-add63 families of transcripts.

Interestingly, northern blot studies showed that the β-adducin mRNA from brains of rats and mice had ∼8–9 kb (Figure 1A, Lanes 1 and 3) while the protein band in the same tissue had an apparent molecular weight of 110 kDa, only slightly higher than that of 97 kDa observed in spleen (Figure 1B, Lanes 8 and 9) (6,7,11,12). This is consistent with previous reports that showed that brain adducin cross-reacts immunologically with erythrocyte adducin having at least 50% of antigenic sites in common (11). The size differences between the brain and erythrocyte β-adducins might be due to post-translational modifications, but, to our knowledge, there is no report showing neither the reasons for substantial differences between the mRNA and protein size nor the complete identity of the erythroid and brain subunits.

In addition, we show in Figure 1A that the same size difference between brain and bone marrow mRNA was also present in humans. In fact, an 8–9 kb band was also observed by northern blot analysis of human brain RNA (Figure 1A, Lane 5). It is interesting to note that the bands observed in humans were slightly bigger than those of mice and rats. Regrettably, we were not able to detect a clear signal of

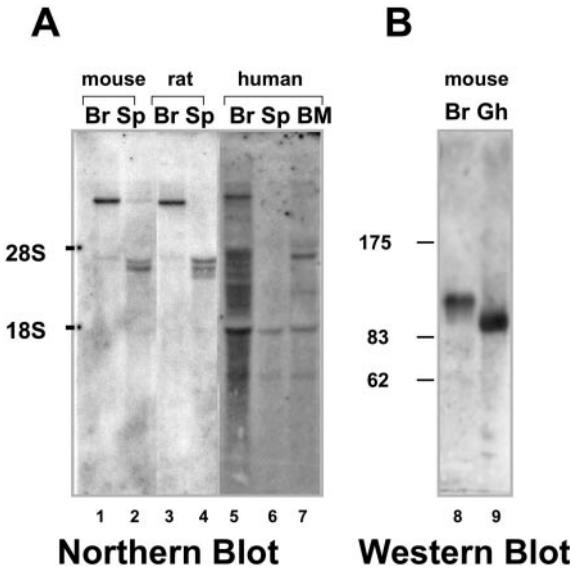

Figure 1. Northern and western blot analysis of brain and spleen β-adducin. (**A**) Northern blot analysis of mouse, rat and human RNAs. RNAs from mouse, rat and human brain and spleen were loaded in lanes 1–6 (25 μg). In lane 7, human bone marrow RNAs was loaded (25 μg). All RNAs were run in the same agarose formaldehyde gel, transferred to Hybond-N and hybridized with the mouse and human β-adducin probes (Lanes 1–4 and 5–7, respectively). The 28S and 18S rRNAs are indicated. (**B**) Brain and ghosts protein extracts (20 μg) were loaded in a 6% SDS–PAGE, blotted onto nitrocellulose, incubated with an anti-β-adducin antibody and visualized by chemioluminiscence. MWMs are indicated on the left. Br, brain; Sp, spleen; BM, bone marrow; Gh, ghosts.

human spleen RNA samples obtained from two different commercial suppliers (Figure 1A, lane 6), which might be due to the fast degradation of the RNA in this tissue before sample preparation. In fact, significant RNA degradation was also observed in human brain and bone marrow samples (Figure 1A, lanes 5–7) and some of the observed bands might be the result of alternative splicing and/or degradation products.

Therefore, the brain β-adducin mRNA contains about 5–6 kb of extra sequences in all three species and, as mentioned above, there is no report describing the molecular characterization of the brain β-adducin mRNA.

A brain-specific exon is present in the 5′-UTR of rats and mice

To identify the molecular basis of the size differences between the brain and spleen β-adducin mRNAs we performed RT–PCR analysis of brain and spleen RNA from mouse, rat and human tissues. The PCRs covered the complete 5′-UTR, coding regions and 3′-UTR of the reported β-adducin sequences in all three species, but produced in all cases the expected products and no extra band appeared that might explain the size difference between brain and spleen β-adducin mRNAs (data not shown).

To rule out that the amplification of a putative longer PCR product could be competed-out by the smaller expected product, we performed RNase protection analysis of rat brain and spleen total RNAs utilizing overlapping antisense riboprobes encompassing the entire reported ADD2 cDNA sequence. Again, no extra bands were observed when probes corresponding to the coding exons were used, indicating the absence of previously undetected exons inserted within the reported β-adducin open reading frame (ORF) (data not shown). In addition, this experiment demonstrated that the ORFs of the erythrocyte and the brain subunits of β-adducin were identical, providing supporting evidence that the size differences between brain and erythrocyte β-adducin observed in the western blot analysis are probably due to post-translational modifications.

Surprisingly, when we used a probe covering the 5′-UTR of the reported rat β-adducin sequence we detected the expected 168 bases protected fragment for the spleen RNA (Figure 2A and B, Lanes 2–3), but a smaller protected fragment of ∼100 bases was present in the brain RNA samples (Figure 2A and B, Lanes 4–5). This indicated the presence of different 5′-UTR in β-adducin mRNAs from brain and spleen tissues.

The 5′ RACE analysis of rat brain RNA led to the cloning and sequencing of a brain-specific exon. The longest clone contained a novel sequence of 258 bases (GenBank accession no. DQ231568, Figure 3). RNase protection analysis using the novel brain exon as probe produced the expected protected fragment of 198 bases in brain RNA samples (Figure 2A and C, Lanes 11–12) while a protected fragment of 104 bases was observed in spleen RNA samples (Figure 2A and C, Lanes 9–10). This result confirmed the exclusive presence of the novel exon in brain β-adducin mRNAs and that of the previously reported 54 bp exon in spleen β-adducin mRNAs.

To determine the transcription initiation site of rat brain and spleen β-adducin mRNAs we performed a primer extension experiment utilizing a primer complementary to the 3′ end of the 99 bases exon, the first exon common to brain and spleen mRNAs (Figure 2D and the scheme of Supplementary Figure 2A). Primer extension analysis of rat brain RNA showed two main bands of about 300 and 320 bases, ending very close to the 5′ end of the longest cDNA clone obtained by RACE–PCR of brain RNA (Figure 2D, Lanes 17–18). Northern blot analysis of RNA samples from mouse cerebellum, brain and spleen with a probe specific for the brain exon is shown in Figure 2E. A band of about 8–9 kb was observed in cerebellum and brain RNA samples (Lanes 19–20) while no band was observed for spleen RNA (Lane 21), confirming the tissue-specific expression of each exon as seen by the RNase protection experiment and suggesting a link between the use of the brain-specific promoter and the 8–9 kb long brain-specific mRNA. In the analysis of the spleen RNA two main bands of 163 and 168 bases were observed by primer extension, the bigger one coinciding with the reported transcription initiation site of the rat β-adducin gene and smaller the one only 5 bases downstream of that site (Figure 2D, Lanes 15–16, and Supplementary Figure 1B).

Structure of brain and spleen-specific promoters

The 5′ end of the human β-adducin mRNA has been determined by RACE analysis of RNA from a neuronal cell line and, using the amplified product as probe, the genomic region containing the putative human β-adducin promoter was cloned (1). However, comparison of the β-adducin exonic architecture and sequences among human, rat and mouse suggested a different genomic structure for the 5′ ends for the murine β-adducin gene. While there was an almost perfect matching

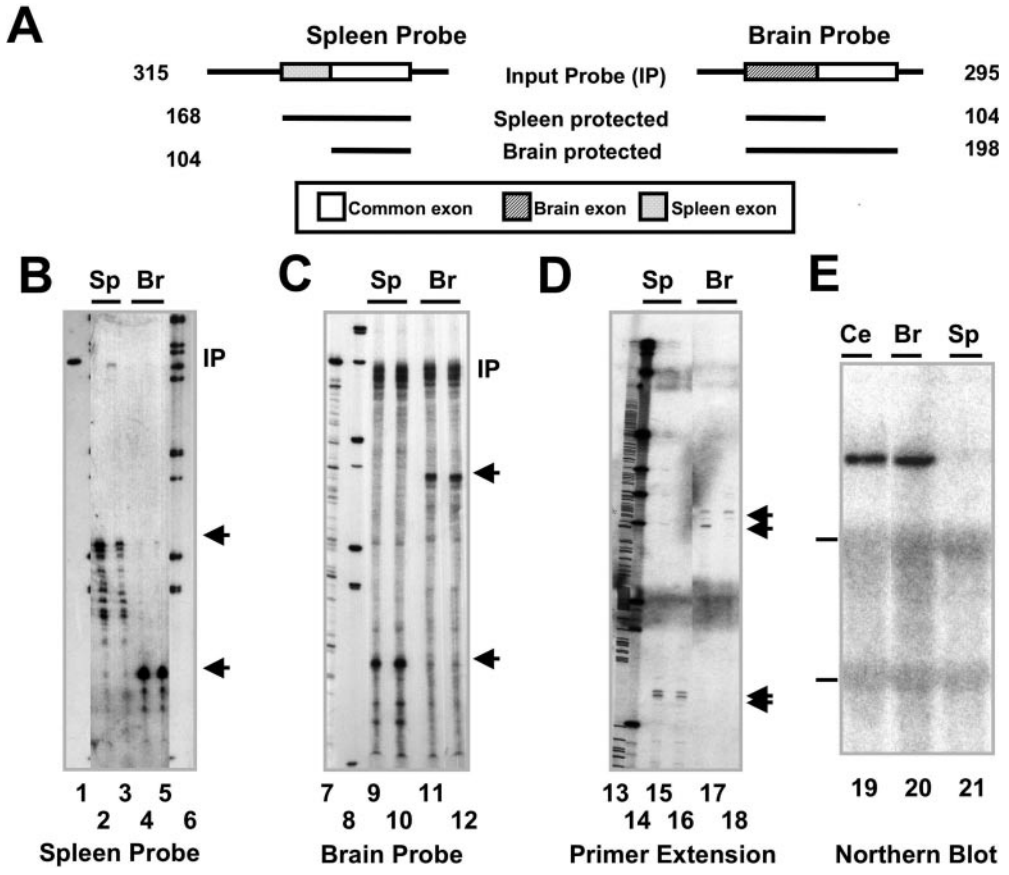

Figure 2. Detection of brain- and spleen-specific β-adducin exons in rats and mice. (**A**) Schematic representation of the riboprobes used in the RNase protection experiment shown in (B and C). The input probe and the size of the protected fragments for each probe and each tissue are indicated. (**B** and **C**) The spleen and brain probes shown in (A) were used in (B and C), respectively. Thirty micrograms of total spleen and brain RNA were annealed to the probe, digested with RNase, run in a polyacrylamide denaturing gel and autoradiographed. Lanes 1 and 7 correspond to the undigested probe (input probe), lanes 6 and 7 are radioactive molecular weight markers, lanes 2–3 and 9–10 spleen RNA, and lanes 4–5 and 11–12 brain RNAs. The arrows indicate the protected fragments. (**D**) Primer extension experiment with a primer annealing in the boundary between the constitutive 99 bp and the 217 bp exons (exons 2 and 3), that are present in both the brain and spleen forms of the β-adducin mRNA. The arrows indicate the primer extension products observed in brain and spleen RNAs. Lanes 15–16 and 17–18 correspond to spleen and brain RNAs, respectively. Lane 14 is a radioactive molecular weight marker, and lane 18 is a one-lane Sanger sequence using the same primer used in the experiment. (**E**) Northern blot experiment of cerebellum, brain and spleen mouse RNA (lanes 19–21, respectively) with a probe corresponding to the mouse brain-specific exon. The position of the 28S and 18S rRNA is indicated.

in the length and a high degree of homology of the protein coding exons among the three species, the conservation and length of the first exon highly differed (Supplementary Table 1). In fact, the human first exon was 312 bp long, while that of mice and rats had 219 bases. In addition, cloning and sequencing of the putative rat brain promoter region showed 90.8 and 94.1% of homology between mouse and rat sequences for the promoter region (up to position −300 bp) and for the brain-specific exon, respectively (Supplementary Figure 1A and Supplementary Table 1). On the contrary, the conservation between the human and rodent promoter region and first exons was only 47 and 53%, respectively.

Promoter prediction analysis of the human, rat and mouse brain-specific promoters showed that the sequences 5′ to the mouse and rat brain-specific exons (mN1 and rN1 exons, respectively) contained a consensus Inr sequence (18) and several potential transcription binding sites (Supplementary Figure 1A). The transcription site determined in rat by the primer extension experiment exactly coincided with that reported for the mouse β-adducin mRNA (ENSEMBL, transcript ENSMUST00000077101, Supplementary Figure 1A). The *in silico* prediction showed that just a few transcription factor sites were common among all three species and in the human promoter numerous SP1 binding sites were predicted, a transcription factor found in many housekeeping genes that binds to GC rich regions, that were not so profuse in the rodent promoter. In fact, the GC value of the human promoter was 79.3% while in the rat and mouse brain rodent promoters it was of 55–60% supporting the observed differences in structure and suggesting a diverse transcriptional regulation of this β-adducin gene promoter.

Sequence comparison of the spleen-specific promoter region and first spleen-transcribed exon of rat and mouse β-adducin erythroid-specific exons (mE1 and rE1, respectively) with the recently reported human erythroid exon (hE1) (13) showed 84% homology between rat and mouse, while the homology between mE1 and rE1 sequences and the hE1 exon were only of 43% and 47%, respectively (Supplementary Figure 1B and Supplementary Table 1). Similarly to that observed for the human erythroid promoter (13), both the rat and mouse spleen promoters contained a conserved Inr sequence (18). In addition, other potential transcription sites

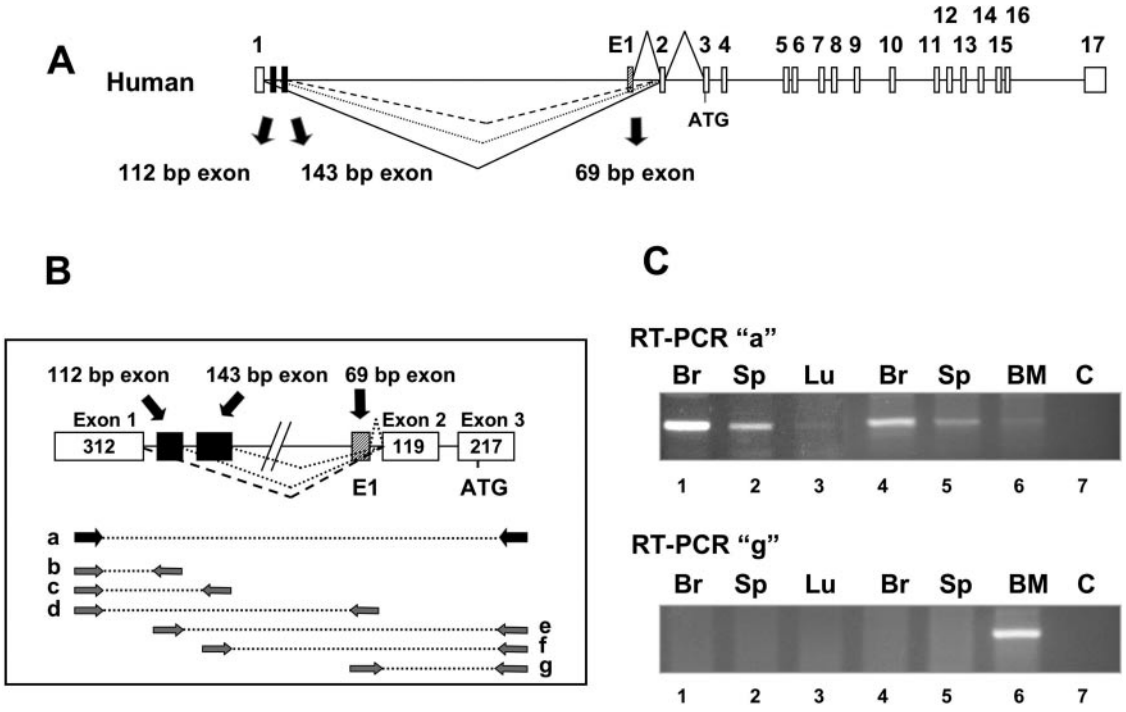

Figure 3. Analysis of new potential β-adducin exons in humans. (**A**) Schematic representation of the human β-adducin gene structure showing the position of the new potential exons composed of novel sequences flanked by known β-adducin exons. The open boxes indicate the exons 1–17; the closed boxes indicate the 112 bp and the 143 bp exons; and the dashed box indicates the erythroid-specific E1 exon. (**B**) Schematic representation shows the position of the oligonucleotides utilized in the RT–PCR to amplify the 112 bp exon (primer pairs 'b' and 'e'), the 143 bp exon (primer pairs 'c' and 'f') and the 69 bp exon (primer pairs 'd' and 'g'). As no product was obtained after the first round of amplification (primer pairs 'b', 'c', 'd', 'e' and 'f'), a nested PCR was performed with negative results (data not shown). (**C**) The primer pairs 'a' and 'g' showed in (B) that amplify a product from exon 1 to exon 3, and from exon E1 to exon 3, respectively, were utilized for an RT–PCR with samples from brain, spleen (two independent preparations, lanes 1–2 and 4–5), bone marrow and lung.

were found within the rat and mouse promoters and are shown in Supplementary Figure 1B. We observed that between rodents and humans most binding sites were not conserved. However, those of the hematopoietic GATA transcription factor family (19) predicted for the human erythroid E1 promoter (13) were present in conserved regions of the rodent promoters.

Tissue-specificity of the β-adducin pre-mRNA transcription

An additional functional difference between human and rodent promoters is their tissue-specificity. In fact, while the mouse and rat β-adducin pre-mRNAs are transcribed from a brain-specific promoter, the human one seems to be active not only in brain but also in spleen and bone marrow, and to a much lesser extent in lung.

We analyzed the pattern of expression of the brain and spleen-specific exons in kidney, liver, brain, lung, heart, placenta, bone marrow and spleen of embryos, newborn and adult mice of different ages. We observed that the mouse brain-specific promoter was active in brain of 13.5 day-embryo, postnatal 1- and 14-day-old animals, and 3-month-old mice (Supplementary Figure 2B, primers pair 'a'). On the contrary, no expression was observed in other tissues. The spleen promoter was active in liver embryo and placenta, and had a low level of expression in other embryonic tissues (Supplementary Figure 2B, primers pair 'c'). In 14-day-old mice and adults, the expression in the liver and other tissues seemed to be completely shutdown and was active only in the spleen and bone marrow.

To determine the expression pattern of the human exon 1 we used the same RNA samples with the pair of primers 'a' shown in Figure 3B and Supplementary Table 3, that amplify the fragment from human exon 1 to exon 3. A specific PCR product was observed for brain, spleen, bone marrow and lung as shown in Figure 3C (primers 'a'), although the amount of RT–PCR product observed in lung was very low. This experiment strongly suggests differences in activity and tissue-specificity between the rodent and human promoter. Those variations might be related to the structural and base composition changes observed among the species, as described above and shown in Supplementary Figures 1 and 2.

The brain-specific β-adducin mRNA has an unusually long 3′-UTR

We showed above the existence of a novel brain-specific first exon and its putative promoter for rats and mice. However, despite that this exon was longer than that used in spleen and other organs, it did not account for the observed size differences between brain and spleen mRNAs (Figure 1 and Supplementary Table 1). Therefore, we focused the search in the 3′-UTR region to determine the molecular basis of the brain 8–9 kb β-adducin mRNA. Database search (UCSC Genome Browser http://genome.ucsc.edu/) showed the presence of ESTs transcripts in mouse, rat and humans in the region downstream to the known β-adducin 3′-UTR, that in all three cases have not been assigned as part of the β-adducin mRNA. A scheme showing the alignment of the genomic

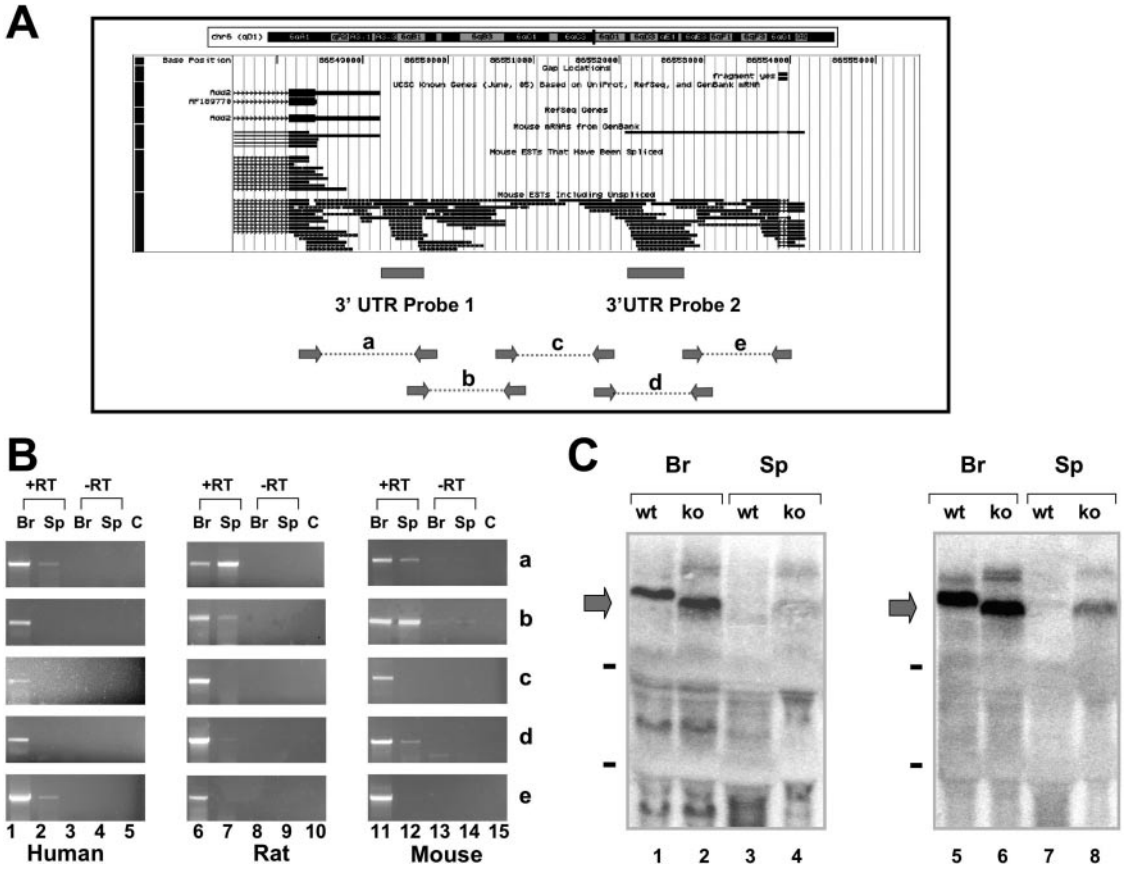

Figure 4. The brain β-adducin mRNA has an unusually long 3′-UTR. (**A**) Schematic representation corresponding to the mouse genomic region downstream of the reported polyadenylation site (UCSC browser). The ESTs corresponding to that region are indicated. The position of the PCR primers used the RT–PCRs shown in (B) are schematically indicated. The probes used in the northern blot experiment of (C) are shown. (**B**) RT–PCRs of brain and spleen RNA, from human (lanes 1–5), rat (lanes 6–10) and mouse (lines 11–15) tissues were performed with different set of primers of the region downstream of the reported β-adducin polyadenylation site. Lanes 3–4, 8–9 and 13–14 were performed with the same RNAs of lanes 1–2, 6–7 and 11–12, respectively, but without the reverse transcriptase reaction. Lanes 5, 10 and 15 correspond to the PCR without the addition of cDNA. (**C**) Northern blot experiment of brain and spleen total RNA (20 μg per lane) from control and β-adducin KO mice (control mice: lanes 1 and 5, brain; lanes 3 and 7, spleen; β-adducin KO mice: lanes 2 and 6, brain; lanes 4 and 8, spleen). Two identical membranes were hybridized with 3′-UTR probes 1 and 2 (Lanes 1–4 and 5–6, respectively). An arrow indicates the detected bands. The size of the band observed in the β-adducin KO brain sample has a lower molecular weight due to the targeted deletion. The position of the 28S and 18S rRNA is indicated.

region downstream of the reported β-adducin gene and the available mouse ESTs is shown in Figure 4A. Human and rat EST distribution covered a similar region to that schematically shown in Figure 4A for mice (data not shown).

To determine whether those ESTs could belong to the β-adducin brain mRNA, a series of overlapping RT–PCR analysis was performed using RNA from brain and spleen, prepared from rat, mouse and human tissues. As schematically shown in Figure 4A, the first pair of primers (called 'a' in Figure 4A and Supplementary Table 3) should amplify a portion of the known β-adducin 3′-UTR and up to 800–1100 bases, depending on the species, of the sequence downstream of the reported 3′-UTR of β-adducin. The other pairs of primers ('b', 'c', 'd' and 'e', Figure 4A and Supplementary Table 3) were complementary to sequences downstream to the known β-adducin 3′-UTR, and were designed in order that each PCR product overlapped the flanking ones covering the entire region up to the most downstream EST shown in Figure 4A.

The RT–PCR analysis showed specific PCR products in brain samples for all primer pairs used (Figure 4B, Lanes 1, 6 and 11). In addition, an RT–PCR using a reverse primer located 80 bases after the last reported EST and the forward one used in pair 'e' produced no product, suggesting that all the β-adducin brain mRNA ends at the same site (Figure 4A and data not shown). These results strongly suggested that the 8–9 kb mRNA transcript observed in brain could be generated by alternative usage of polyadenylation sites. To confirm this hypothesis we performed northern blot analysis of mouse brain and spleen RNAs from control and β-adducin KO mice using two different probes of 600 and 800 bp that mapped downstream of the β-adducin stop codon present in exon 16, in the novel 3′-UTR of brain β-adducin mRNA (starting ∼1400 and 3500 bases downstream of the stop codon, respectively; see scheme of Figure 4A). Both probes showed the presence of a band of similar size to that detected for the mouse brain β-adducin mRNA (Figure 4C, Lanes 1 and 5), confirming that the difference in size of the brain β-adducin mRNA is due to the presence of an extremely long 3′-UTR. In addition, taking advantage of the β-adducin KO mice previously generated in our laboratory (6), we run in the same gel RNA samples prepared from the brain of KO mice and we also observed

a high molecular weight band having a small reduction in size indicating, unambiguously, that the detected band was indeed the product of the β-adducin gene and not the result of unspecific hybridization (Figure 4C, Lanes 2 and 6). The targeted deletion in the β-adducin locus corresponds to a reduction of 744 bases in the β-adducin97 mRNA (exons 9 to 13) (6). A faint band of similar size to that observed in brain RNA samples of KO mice was also observed for spleen RNA samples of KO mice (Figure 4C, Lanes 4 and 8). These bands could be the result of altered polyadenylation regulation in spleen of the β-adducin deficient mice due to the loss of important regulatory sequences by the targeted genomic deletion. In fact, no band is observed in the spleen RNA samples of control mice (Figure 4, Lanes 3 and 7).

In addition, northern blot analysis using an Add63-specific probe showed that the ADD63 family of transcripts also has a long 3′-UTR in brain (Supplementary Data and Supplementary Figure 3). Moreover, hybridization of a parallel membrane with an Add97-specific probe suggested that the relative abundance of the Add63 transcripts is considerably less than the Add97 forms.

A highly-conserved brain-specific polyadenylation site is used in mice, rats and humans

According to the scheme shown in Figure 4A, EST sequences were aligned to genomic sequences of the region downstream of the known β-adducin poly(A) site reported in the last exon and were examined for the presence of poly(A) tails after the alignment. Several EST-containing poly(A) tails were found at chromosomal positions 86 554 176, 120 280 485 and 70 795 572 for mouse chromosome 6q, rat chromosome 4q and human chromosome 2p, respectively (see below and Supplementary Tables 1 and 2), thus generating extremely long brain-specific last exons of 6034, 5592 and 6951 bases and a 3′-UTR of 5831, 5289 and 6642 bases for mouse, rat and human, respectively (Figure 5A and Supplementary Table 1). We will call these distal brain-specific polyadenylation sites mA_4, rA_4 and hA_4 for mice, rats and humans, respectively (Figure 5A and C).

The DNA sequences of the last exon up to the detected poly(A) sites, including the adjacent downstream regions, were retrieved for all three species. Sequence comparison among mouse, rat and human genomic sequences showed a very high degree of conservation of the 3′ end of the 8–9 kb brain β-adducin transcript, specially in the region of the polyadenylation site used in brain. In fact, within the 200 bp region flanking the distal brain polyadenylation signal we observed a 99.1% homology between mouse and rat sequences, and a 75% homology among all three species (Figure 5C).

Analysis of the sequences lead us to the identification in all three species of all elements required for polyadenylation in mammals: a polyadenylation signal, a putative poorly conserved downstream elements (DSE) downstream of the poly(A) site and a putative U-rich region (upstream element, USE) located upstream of the polyadenylation motif (Figure 5B and C). The polyadenylation motif AGTAAA was found as the brain poly(A) signal in all three species. A scheme of the last exon showing the relative position of all the polyadenylation sites found in the last β-adducin exon of all three species is shown in Figure 5A.

In addition, several ESTs containing poly(A) tails were found for all three species, confirming the use of these distal polyadenylation sites (see the scheme of Figure 4A, Supplementary Data and Supplementary Table 2). Other ESTs that mapped nearby upstream to those mentioned above did not contain the characteristic poly(A) tail. The observation that no ESTs mapping downstream of the proposed polyadenylation site were present (Figure 4A) together with the absence of an RT–PCR product with primers at either side of the mA_4 poly(A) site support the hypothesis that the brain β-adducin pre-mRNA is completely polyadenylated at the proposed A_4 site.

Several proximal polyadenylation sites are present in mice, rats and humans

Based on the available deposited sequences and ESTs, we were able to identify two proximal polyadenylation regions in rodents and three in humans that might generate the spleen β-adducin mRNAs. The first one was located between bases 1000 and 1150 relative to the start of the last exon (named mA_1, rA_1 and hA_1, for mice, rats and humans, respectively, see scheme of Figure 5A), should generate a 3′-UTR of about 750–800 bases and showed a high conservation among the three species (Figure 5A and Supplementary Figure 4). The second region was between the bases 1480–1640 (having more than one cleavage site) and coincided with the polyadenylation region observed in humans at position 1600 relative to the start of the last exon (named $mA_{2\text{-}3}$, $rA_{2\text{-}3}$, and hA_2 for mice, rats and humans, respectively). The putative human polyadenylation signal of hA_2 region was not conserved in rodents as they bear a deletion of the region including that element. However, ESTs with a variable position of the poly(A) tail were found in the $mA_{2\text{-}3}$ and $rA_{2\text{-}3}$ regions (Supplementary Table 2) but no consensus polyadenylation signal was identified in this rodent region (Supplementary Figure 4B, underlined region). Another polyadenylation site (hA_3) mapping at position 2532 of the last human exon was detected and its functionality is supported by the presence of numerous EST containing a poly(A) tail (Figure 5A and Supplementary Table 2).

Additional supporting evidence derives from the observation that more than one band is present in the 3.5–4.5 kb region in the northern blot shown in Figure 1A. In fact, we observe two bands in rat and mouse spleen samples that might correspond to the mRNA forms utilizing the A_1 and $A_{2\text{-}3}$ polyadenylation sites. In the human brain, we see three bands that might be the results of the use of the hA_1, hA_2 and hA_3 polyadenylation sites (depicted in Figure 5A). In the bone marrow sample we mainly detected the higher molecular weight band, which might correspond to the use of the A_3 polyadenylation site. The human bands were of higher molecular weight than the rodent ones, which is on line with the expected position of the different polyadenylation sites and, in consequence, the length of the human transcripts (Figures 1A and 5A). Alternatively, these bands might be the consequence of the generation by alternative splicing of the β-Add97 and β-Add63 families of transcripts. However, this possibility is less probable as the Add63 levels are much lower than those detected for the Add97 family of transcripts (Supplementary Figure 3) and we have observed no other major band in the RNase protection experiment of the

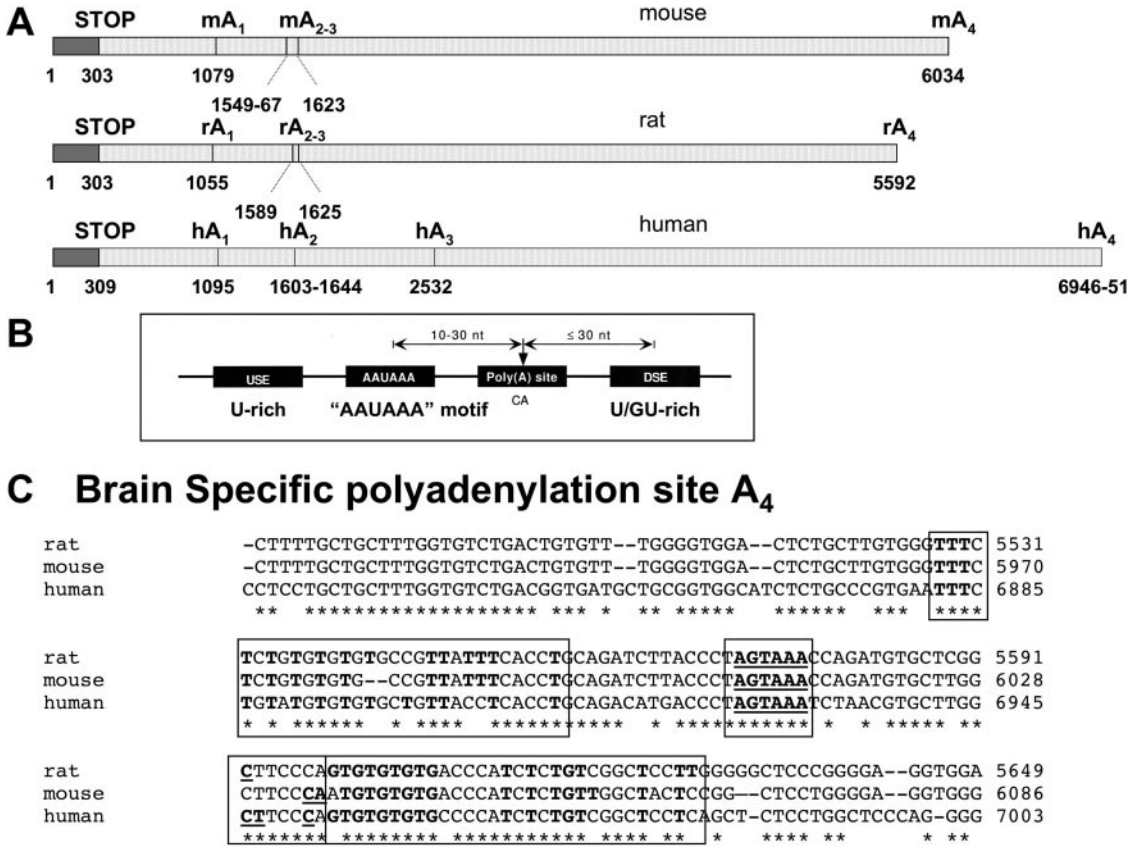

Figure 5. Brain-specific alternative polyadenylation sites. (**A**) A schematic representation of the last exon of the β-adducin gene is shown. The ORF (gray box) and the stop codon are indicated. The positions of the polyadenylation sites used in brain are indicated. The 3′-UTR (5731, 5289 and 6642 bases for mice, rats and humans, respectively) are indicated as a dotted box. (**B**) Schematic representation showing the position of the elements found in the region of the polyadenylation sites of genes. USE, upstream element; DSE, downstream element. (**C**) Sequence comparison among rats, mice and humans of the brain-specific polyadenylation sites. The AGTAAA motifs and the polyadenylation sites are underlined. The U-rich and U/UG-rich elements are indicated. Numbers are relative to the first base of the last exon of each species.

complete β-adducin ORF that might have been produced by alternative splicing.

DISCUSSION

This report describes the complete primary structure of the β-adducin mRNA of brain in humans, rats and mice. We show that the brain mRNA form contains an unusually long 3′-UTR in all three species. Moreover, we present evidence that there is a brain-specific promoter in rodents that was apparently not conserved in humans. Consequently, the architecture of the ADD2 gene is extremely complex with alternative use of tissue-specific promoters and polyadenylation sites, in addition to the intricate alternative splicing pattern of the pre-mRNA already described (2). Moreover, considering the use of the brain-specific mN1 and rN1 exons, the maximum predicted length of the primary transcript of the β-adducin gene in brain was of 96 144 bases, 111 835 bases and 111 412 bases for mice, rats and humans, respectively. We also provide evidence demonstrating the identity between the ORFs of the brain and erythroid β-adducin subunits, suggesting that the observed molecular weight differences in the proteins are the result of post-translational modifications.

Genes encoding intracellular proteins tend to have alternative poly(A) sites (20) and many genes coding for proteins of the membrane skeleton fall within this group having both alternative promoters and polyadenylation sites. For example, the ANK-1 gene has different polyadenylation sites and two promoters that direct its expression to erythroid cells or muscle cells (21,22), protein 4.1 has three promoters that generate distinct N-termini (23) and human band 7.2 gene also utilizes alternative polyadenylation signals (24). In the case of ADD2, we show here that there are two main distinct promoters in rodents, one specific for hematopoietic tissues such as spleen and bone marrow, and the other one specific for neuronal tissues such as brain and cerebellum.

The observations that the homology between the human promoter and rodent brain-specific promoter was only of 48% and that the structure and base composition were highly different among species (80 and 55% of CG content for humans and rodents, respectively) suggested important differences in the regulation of the brain-specific β-adducin transcript in humans and rodents. This hypothesis is supported by the expression data of the human β-adducin gene promoter (Figure 3). The presence of a human brain-specific promoter was previously suggested [M. Bulger, unpublished data; (13)] but we detected transcription from this promoter not only in brain but also in hematopoietic tissues such as spleen and bone

marrow, and at lower levels in lung (Figure 3). However, our observations were performed by RT–PCR and need further analysis for their confirmation. The ubiquitous transcription from the human promoter might be the consequence of the presence of numerous binding sites of the transcription factor SP1, which is required for ubiquitous transcription of multiple housekeeping genes and play an important regulatory role in cellular processes during development and differentiation (25). On the contrary, the rodent brain-specific promoters showed a very tight expression pattern, as we were able to detect its activity only in mouse brain of 13.5 day embryo, postnatal 1- and 14-day-old babies, and adults, but not in other tissues, and no band corresponding to the product of this promoter was detected in the rat spleen sample of the RNase protection experiment. Therefore, we propose the designation of the rodent brain-specific promoters and first exons as mN1 and rN1, standing for neuronal mouse and rat first exons, respectively, but we suggest that the human promoter and first exon are not exclusive to brain (13).

Similarly to that observed in humans, [Figure 3C and (13)], the activity of the mouse and rat spleen-specific promoter was mainly detected in erythropoietic tissues such as liver embryos and placenta, and was also active in other embryonic tissues, although at a lower level of expression (Supplementary Figure 2B, primers pair 'c'). In 14-day-old mice, the expression in the liver and other tissues seemed to be completely shutdown and was active only in the spleen and bone marrow.

Promoter comparison of the rodent spleen-specific promoter with the human E1 promoter (13) showed a 43 and 47% homology with rats and mice, respectively, while the rat and mouse sequences showed a higher homology (91%). Prediction of the regulatory sites present in the rodent promoters showed conservation of some of the sites but most of the human-predicted ones were not conserved. However, the binding sites of the hematopoietic GATA transcription factor family (19) predicted for the human erythroid E1 promoter (13) were present in conserved regions of the rodent promoters suggesting that these erythroid promoters might be regulated, at least in part, by mechanisms common to all three species. Nevertheless, functional analyses are needed to more precisely determine the *in vivo* regulation of both the brain- and spleen-specific ADD2 promoters.

Despite the fact that the 8–9 kb ADD2 mRNA was detected in brain about 15 years ago (7,12) and at least three attempts that were made to completely characterize the ADD2 gene structure (1,2,13), the molecular basis of this long brain-specific form remained unknown. After ruling out the presence of undetected internal exons or a long 5′-UTR in the brain isoform, we focused our attention to the 3′-UTR. We detected the presence of transcripts in the region downstream of the reported ADD2 gene, and we demonstrated that they belong to the brain-specific β-adducin mRNA, generating an 8262, 7817 and 9286 bases long mRNA for mice, rats and humans, respectively. On the contrary, the longest form containing all internal exons of the erythroid-specific transcript was 3664, 3574 and 4631 bases long for mice, rats and humans, respectively, when the A_3 polyadenylation site was used. The functionality of all of the proposed polyadenylation sites is supported by the presence of several ESTs and mRNAs containing poly(A) tails.

We present evidence that the β-adducin pre-mRNA is processed at many alternative polyadenylation sites, with the more proximal ones mainly used in erythroid tissues and the distal site in brain. In fact, in addition to the northern blot data of brain RNA (Figure 4), almost all of the EST and RNAs found in the databases that corresponded to the distal A_4 polyadenylation site were derived from brain or brain-related tissues (Supplementary Table 2). On the contrary, libraries originated from a variety of tissues including spleen and liver embryo were the source of the RNA sequences containing the other polyadenylation sites.

The UTRs of mRNAs regulate the expression of genes by modifying the mRNA stability due to the presence of elements affecting the degradation rate, the efficiency of transcription, the translation efficiency by the presence of binding sites for specific proteins and/or antisense RNAs, and the differential concentration of the corresponding proteins in different subcellular regions (26). It is striking the high degree of conservation of the brain-specific polyadenylation region (A_4) in all three species studied. We observed a 99.1% homology between mouse and rat sequences, and a 75% homology among all three species, while the complete coding region of β-adducin has 95.8% homology between rat and mouse sequences, and 86.3% among all three species (6) and the complete last exon showed 81 and 57% homology between mice and rats, and rodents and humans, respectively (Supplementary Table 1). Therefore, the highly-conserved polyadenylation configuration of mouse, rat and human β-adducin genes highlights the importance of producing tissue-specific mRNA products and suggests that the brain-specific 3′-UTR could be involved in the regulation of the function of the β-adducin gene by one or more of the mechanisms mentioned above. The AU-rich elements (AREs) are associated to the regulation of mRNA stability and translation (17) and one ARE is found 149 bases upstream of the brain-specific polyadenylation signal in a highly-conserved region of all three species, suggesting that it might play a regulatory role in the turnover and/or translation of the β-adducin mRNA in brain. However, the regulatory role of the initiation step of translation by elements at the 3′ end of eukaryotic mRNAs is still controversial (27,28).

Polyadenylation of the pre-mRNA is a process common to the majority of genes transcribed by RNA polymerase II. Three major steps in polyadenylation are (i) recognition of the authentic poly(A) site; (ii) cleavage of the pre-mRNA; and (iii) addition of up to 250 adenosine residues (29). The recognition step is determined by at least two sequences: (i) the polyadenylation signal (AAUAAA or similar) and (ii) the U-rich or GU-rich elements found downstream of the cleavage site. These two sequences are recognized by the cleavage and polyadenylation factor (CPSF) and the cleavage stimulation factor (CstF), respectively. CstF stimulates binding of CPSF to non-AAUAAA sites conferring specificity to polyadenylation site choice (30). In this respect, the poly(A) sites in mice, rats and humans meet all these basic sequence requirements.

The most frequent poly(A) signal is AATAAA that is found in 50–60% of genes (20,31,32), and polyadenylation signals differing with the canonical sequence are usually associated with alternative or tissue-specific polyadenylation (29). Although the consensus sequence for GU- and U-rich elements have been determined (29), some DSE contain no match to either the U- or GU-rich consensus motifs and large deletions are required to abolish its function. The USEs are often U-rich, supporting the idea that those sites are poorly defined and

possible redundant (29). The putative poly(A) signal for the brain mRNA form was AGTAAA was fully conserved in all three species and is found in about 3% of genes (20,31,32). In the brain-specific A_4 region, one polyadenylation site was observed in rodents and two in humans (separated by only 5 bases), while multiple sites were found in each of the A_1, A_2 and A_3 regions in all three species. The reasons for the use of a single poly(A) site in the A_4 region could reside in presence of a well-defined and highly-conserved GU-rich element downstream of the brain-specific A_4 poly(A) site. On the contrary, the GU-rich element was poorly defined in the proximal polyadenylation regions (A_1, A_2 and A_3 regions). The associated elements (U- and GU-rich elements) found in distal brain poly(A) region might be recognized by brain-specific CstF and/or other tissue-specific factors favoring the binding of CPSF, as observed with the tissue-specific tCstF-64 factor found in germ cells (33), and prevailing over the use of the proximal poly(A) sites. However, it is not clear how a distal poly(A) site might influence the use of the upstream proximal poly(A) site (30), but given the coupling between transcription and pre-mRNA processing, it is conceivable that part of the decision may occur at the promoter (29). Recent studies have shown the coupling between the transcriptional processes and pre-mRNA processing including pre-mRNA splicing and polyadenylation (34–36). An intriguing possibility is that the transcriptional complexes formed at the brain-specific promoter could direct the polyadenylation machinery to the distal polyadenylation sites, as already observed in the coupling of transcription to pre-mRNA splicing (37,38).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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