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The Bromodomain-Containing Gene *BRD2* Is Regulated at Transcription, Splicing, and Translation Levels

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ABSTRACT

The human *BRD2* gene has been linked and associated with a form of common epilepsy and electroencephalographic abnormalities. Disruption of *Brd2* in the mouse revealed that it is essential for embryonic neural development and that viable *Brd2*$^{+/-}$ heterozygotes show both decreased GABAergic neuron counts and increased susceptibility to seizures. To understand the molecular mechanisms by which mis-expression of *BRD2* might contribute to epilepsy, we examined its regulation at multiple levels. We discovered that *BRD2* expresses distinct tissue-specific transcripts that originate from different promoters and have strikingly different lengths of 5′ untranslated regions (5′UTR). We also experimentally confirmed the presence of a highly conserved, alternatively spliced exon, inclusion of which would result in a premature termination of translation. Downstream of this alternative exon is a polymorphic microsatellite (GT-repeats). Manipulation of the number of the GT-repeats revealed that the length of the GT-repeats affects the ratio of the two alternative splicing products. In vitro translation and expression in cultured cells revealed that among the four different mRNAs (long and short 5′UTR combined with regular and alternative splicing), only the regularly spliced mRNA with the short 5′UTR yields full-length protein. In situ hybridization and immunohistochemical studies showed that although *Brd2* mRNA is expressed in both the hippocampus and cerebellum, Brd2 protein only can be detected in the cerebellar Purkinje cells and not in hippocampal cells. These multiple levels of regulation would likely affect the production of functional BRD2 protein during neural development and hence, its role in the etiology of seizure susceptibility. J. Cell. Biochem. 112: 2784–2793, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: BRD2; BROMODOMAIN; ALTERNATIVE SPLICING; TRANSLATION; EXPRESSION REGULATION

BRD2 (human *BRD2*, mouse *Brd2*) is the founding member of the BET family of double bromodomain-containing genes, which have two tandem bromodomains at the N-terminal region and an ET domain at the C-terminal side of the bromodomains. The BET family includes *Brd3*, *Brd4*, and *Brdt*, in addition to *Brd2* [Florence and Faller, 2001]. That these genes play critical functions is underscored by the observations that the members of this family that have been studied in the mouse model have been shown to be essential for development and differentiation. Specifically, the *Brd2* [Gyuris et al., 2009; Shang et al., 2009] and *Brd4* [Houzelstein et al., 2002] homozygous null states are embryonic lethal and male mice lacking the first bromodomain of the testis-specific gene *Brdt* are sterile, exhibiting profound defects in spermiogenesis [Shang et al., 2007].

The human *BRD2* gene has been shown to play a critical susceptibility role in a common form of epilepsy as well as other, more subtle, neural phenomena including abnormal electroenceph-alographic (EEGs) patterns [Greenberg et al., 1988; Durner et al.,

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1991] and photosensitivity [Tauer et al., 2005; Lorenz et al., 2006]. Using linkage [Greenberg et al., 2000] and association [Pal et al., 2003] analysis, *BRD2* was identified as *EJM1*, the locus on chromosome 6p21.3 linked to Juvenile Myoclonic Epilepsy (JME), but the mechanisms by which *BRD2* contributes to these abnormalities are unknown. Brd2 is expressed in embryonic and adult mouse brain and loss of function of *Brd2* (in *Brd2−/−* homozygous knockout mice) is embryonic lethal, with striking abnormalities in the developing nervous system [Gyuris et al., 2009; Shang et al., 2009]. Further, reduction of levels of Brd2 in *Brd2+/−* heterozygous mice leads to increased seizure susceptibility, spontaneous seizure, and epileptiform EEG patterns [Velisek et al., under revision]. These observations suggest a critical role for *Brd2* in brain function. The BET proteins, including Brd2, have been reported to play a role in a variety of cellular processes, including cellular proliferation, apoptosis, and transcription. However, which of these mechanisms are playing specific roles in neural development and functions remains to be determined.

In the present study, we demonstrate multiple levels of regulation, including transcription, alternative splicing and translation, which could contribute to altered levels of expression of Brd2 protein in the neural system. Critical to the potential regulation of *BRD2* function is our discovery of an alternatively spliced exon within a highly conserved intronic region in both the human and mouse genes. mRNA containing this alternative exon introduces a premature termination codon. Because of *BRD2*'s role as an epilepsy susceptibility gene, we examined its expression at the mRNA and protein levels in different regions of the brain. We also demonstrated that different *BRD2* promoters result in transcripts that differ in the lengths of their 5′ untranslated regions (5′UTR), which in turn affects the efficiency of their translation both in vitro and in cultured cells. Although *Brd2* transcripts are distributed in various regions of the mouse brain, the mRNAs are selectively translated in a region-specific and cell type-specific manner, suggesting that these multiple mechanisms for regulating its expression contribute to its role during brain development and in adult brain functioning as well.

MATERIALS AND METHODS

IDENTIFICATION OF DISTINCT BRD2 ALTERNATIVE TRANSCRIPTS

Through database searching we identified four different mRNA transcripts of the human *BRD2* gene: one long, regularly spliced (4.6 kb, without exon 2a), one short, regularly spliced (3.8 kb, without exon 2a), and one long, alternatively spliced (4.7 kb, with exon 2a), and one short, alternatively spliced (3.9 kb, with exon 2a) (Fig. 1 and text in Results Section).

cDNA CONSTRUCTS

cDNAs corresponding to the long transcripts of both the regularly spliced and alternatively spliced human *BRD2* mRNAs were obtained from ImaGenes (www.imagenes-bio.de). cDNAs with the short 5′UTR were derived by cutting the cDNAs with the longer 5′UTR at the unique *Nar*I site, which is located 20 bp downstream of the putative TATA-box in Exon 1. The four cDNAs were subcloned into a pcDNA3 vector, and a synthetic Flag-tag coding sequence was inserted into the unique *Nhe*I site of the cDNAs between the two bromodomains. The EGFP-BRD2 fusion construct was made by partially digesting the human *BRD2* cDNA of the regularly spliced form at a 5′ *Apa*I site. This site is 70 bp downstream of the putative translation start site ATG. The products were then ligated in frame into the *Apa*I site of pEGFP-C1 vector.

NORTHERN BLOT HYBRIDIZATION AND RT-PCR ANALYSIS

For Northern blot hybridization analysis, total RNA was isolated from selected mouse tissues using Trizol Reagent (Invitrogen), electrophoresed, and transferred onto nitrocellulose membrane using standard procedures [Chomczynski and Sacchi, 1987]. The membrane was hybridized with a ^{32}P-labeled DNA probe corresponding to specific regions of the *Brd2* cDNA shown in Figure 1A. Briefly, specific regions of cDNA were PCR amplified. Single strand DNA probes were labeled using Taq polymerase in a thermocycler with only the reverse primer. Human brain RNAs were purchased from Stratagene (Stratagene.com). RT-PCR was performed using the ONE-STEP RT-PCR kit from Invitrogen. The primers used sequences in exon 2 and exon 3 (BRD2E2F 5′ACAAGGTAG-TGATGAAGGCTCTGTGGAA; BRD2E3R 5′CTTGTGGCATTGATG-CAACCTTC-TGTAGG).

IN VITRO TRANSLATION

Promega's TnT T7 Quick Coupled Transcription/Translation System and Amersham's [^{35}S]Methionine (1,000 Ci/mmol) were used in these experiments and the manufacturers' protocols were followed. Briefly, 20 μl TnT Quick Master Mix, 1 μl [^{35}S]Methionine (10 μCi), 1 μl (0.5 μg) plasmid DNA template were mixed in 25 μl volume and incubated at 30°C for 90 min. The translation products were separated on 10% SDS–PAGE gel and the gels were dried on a BioRad vacuum gel drier. The dried gels were exposed to X-ray film overnight at −80°C.

TRANSFECTION AND IMMUNOBLOTTING

The constructs described above were transfected into HEK293T cells using Lipofectamin (Invitrogen) in serum-free medium following the manufacturer's protocol. The medium was replaced with DMEM containing 10% fetal bovine serum 5 h later. The cells were harvested in SDS lysis buffer the next day and boiled for 5 min. For immunoblot analysis, the cellular lysates were separated by SDS–PAGE, and transferred to PVDF membranes. The blots were incubated with anti-Flag M2 antibody (Sigma, F3165) (1 μg/ml) or anti-Brd2 antibody (1:200), following routine protocols used in our laboratory [Rhee and Wolgemuth, 1997].

GENERATION OF *BRD2* MINI-GENES

A *BRD2* mini-gene construct was made by cloning a portion of a common allele of a human genomic DNA sample, from exon 2 to exon 3, into pcDNA3 (Invitrogen). This allele contains 13 GT repeats, 74 bases upstream of exon 3. Using this construct as a template, we generated subsequent mini-genes with 4, 3, 2, and 1 GT repeat number by GeneTailor™ Site-Directed Mutagenesis System (Invitrogen).

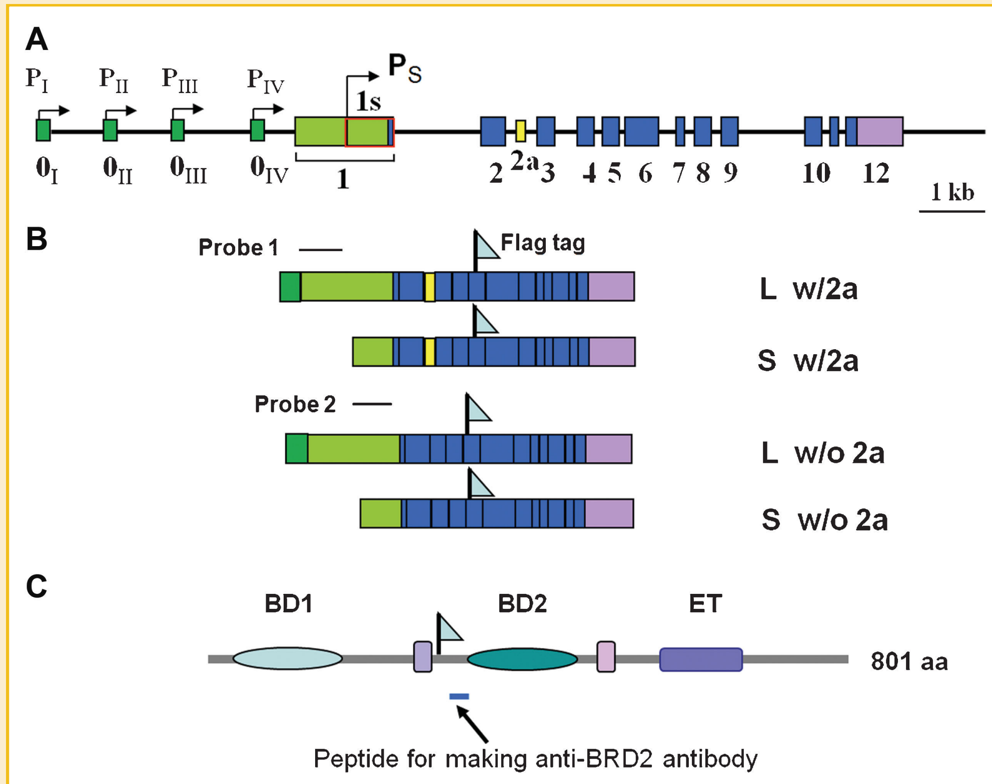

Fig. 1. Molecular organization of the *BRD2* gene and its multiple transcripts. A: A cartoon showing the presence of the exons, introns, and promoters of the human *BRD2* gene. P_I, P_{II}, P_{III}, P_{IV} represent multiple upstream transcription initiation sites; P_S represents the major transcription initiation site which produces the shorter transcript; 1 represents the first coding exon included in the long transcript; 1s represents the part of exon 1 included in the short transcript; 2a (yellow bar) represents the alternatively spliced exon; green bars represent the 5′ untranslated region; blue bars represent coding exons; purple bars represent the 3′ untranslated region. B: Cartoon depiction of the four corresponding mRNAs that result from the use of different transcription start sites and the alternatively spliced exon 2a (the color coding of various genomic regions is the same as that in A); the small flags indicate the Flag-tag sequences that were inserted in the expression constructs used in the transfection experiments; probes 1 and 2 indicate the region of sequence used as probes in the Northern blot hybridization analysis in Figure 2C,D. C: A cartoon of the full-length *BRD2* protein; the two ovals represent the two bromodomains; the blue rectangle represents the ET domain; the two small rectangles represent additional conserved regions; the region used for generating the antibody is indicated by the blue bar and arrow; the flag indicates the position of the inserted Flag tag.

ASSAY FOR DETECTING ALTERNATIVE SPLICING

We transfected these *BRD2* mini-genes into HEK293T cells using lipofectamine™ 2000 (Invitrogen) in serum-free medium following the manufacturer's protocol. The medium was replaced with DMEM containing 10% fetal bovine serum 6 h later. After 40 h, total RNA was harvested and purified using Trizol (Invitrogen). RNA was treated with RNase-free DNase I (Invitrogen), and 1 μg RNA was reverse-transcribed using random primers and SuperScript® III Reverse Transcriptase (Invitrogen). Resulting cDNA was used for PCR with specific primers, designed so as to only detect RNA from the transfected *BRD2* mini-genes, forward primer: 5′-CCAGGA-CGAGTTACCAACCAG-3′, and reverse primer: 5′-CAGGATCCC-TTGTTGTAAATG-3′. The PCR products were electrophoresed through 1.5% agarose gels.

IMMUNOHISTOCHEMISTRY AND IN SITU HYBRIDIZATION ANALYSIS

Adult C57Bl/6J mice (female, 6 months) were perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) and the brains were dissected and post-fixed overnight with 4% paraformaldehyde in PBS at 4°C. Fixed tissues were washed for 30 min each in PBS, saline, saline/50% ethanol, and then 70% ethanol and stored in 70% ethanol until paraffin embedding. After embedding in paraffin, 6 μm sections were cut and then the sections were de-paraffinized in Histoclear and rehydrated through an ethanol series. For immunostaining, the slides were boiled to enhance antigen retrieval and processed as described previously [Zhang et al., 1999]. The anti-Brd2 antibody (see below) was applied at a dilution of 1:200. The orange-brown signal was generated with the peroxidase/diaminobenzidine system (Vectastain ABC Kit, Vector Labs, Burlingame, CA). The blue nuclear counterstain was hematoxylin.

For in situ hybridization analysis, after rehydration, the slides were treated as described in Chapman and Wolgemuth [1992]. Digoxygenin-labeled anti-sense and sense RNA probes were transcribed from specific regions (as shown in Fig. 1A) of the cDNA subcloned into pCRII dual-promoter vector (Invitrogen) using Roche's Dig RNA labeling Kit (Roche) and either SP6 or T7 RNA polymerase.

GENERATION OF ANTI-BRD2 ANTIBODIES

Antibodies were generated using the 14 amino acid peptide RKDLPDSQQQHQSS, which is found between the two bromodomains of Brd2 protein (Fig. 1). A cysteine residue was added to the

N-terminal of the peptide to enable coupling of the peptide to agarose beads. The peptide was conjugated to KLH and used to immunize two rabbits by a commercial vender (Sigma-Genosys) (sigmaaldrich.com). The anti-Brd2 interbromo-peptide antibody was affinity-purified with the peptide coupled to Sulfolink Coupling Gel following the manufacturer's protocol (piercenet.com) and tested for its ability to recognize recombinant and endogenous Brd2 protein by immunoblotting (Fig. S1).

RESULTS

MULTIPLE TRANSCRIPTS OF THE *BRD2* GENE ARE TRANSCRIBED FROM DIFFERENT PROMOTERS

The mouse and human BRD2 proteins Exhibit 96% identity overall and 100% identity between their respective bromodomains and in the ET domains [Rhee et al., 1998]. The structural organization of the introns and exons of the human and mouse genes is also highly conserved (human *BRD2* is depicted in Fig. 1A). There are 12 coding exons, each of which contributes to the resulting BRD2 protein. We designated the exon containing the putative translation starting ATG as exon 1. There are multiple additional non-coding, alternative exons upstream of exon 1, all of which we designated exon 0 (see below).

Prior studies showed the presence of two different mRNA transcripts of *Brd2*, one long (4.6 kb) and one short (3.8 kb), transcripts [Rhee et al., 1998; Taniguchi et al., 1998], which we refer to as the "long" transcript and the "short" transcript in the present study. The molecular basis for the difference in the two transcripts was unknown. A search of cDNA sequences in the databases and aligning them to genomic sequences revealed that they are transcribed from different promoters. Specifically, the shorter transcript starts from the middle of exon 1 (P_S in Fig. 1), and the longer transcript starts from alternative exons further upstream (the exon 0 mentioned above), and is then spliced to exon 1.

In the middle of exon 1 there is a putative TATA box (TATATATAAA). Using the sequence of exon 1 to search the human EST database, we found that the majority of the cDNAs start immediately after the TATA box (Fig. 2A). Examination of the cDNA sequences that aligned with the second half of exon 1 confirmed that most of the alignments start from the 5′ ends of these cDNAs, suggesting that there is a transcription initiation site immediately after the TATA box. Since only the second half of exon 1 occurs in the short transcript, we designated it exon 1s. This observation is significant because in most database annotation, the longer transcript is used to represent the full length *BRD2* mRNA while the shorter transcript is mostly ignored. This is critical because, as we show in the following sections, it is in fact the shorter transcript that produces BRD2 protein, at least under all the conditions we tested.

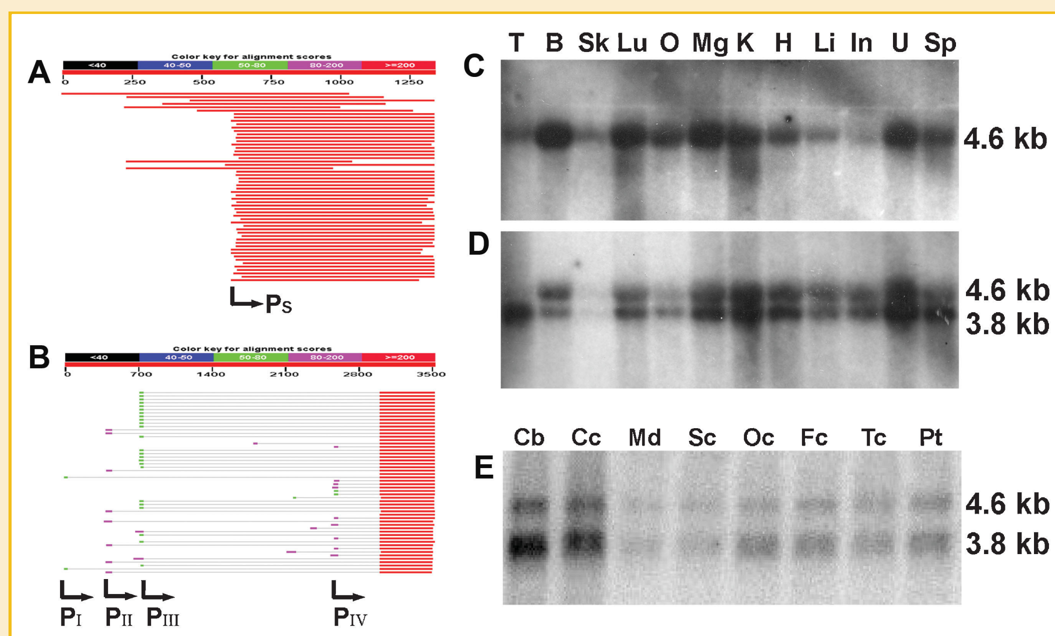

Fig. 2. Expression of the multiple transcripts in selected mouse tissues and in human brain. A: A cartoon depicting alignment of DNA sequences in the genomic region between the genomic DNA sequence of exon 1 and cDNA sequences in the EST database using NCBI's BLASTN, showing that most cDNA clones start from the middle of exon 1. B: A Cartoon depicting sequence alignment between the 3.5 kb genomic sequence upstream of the TATA Box in exon 1 and cDNA sequences in the EST database, indicating multiple transcription initiation sites of *BRD2* in a broad 3 kb region. The predicted promoters are labeled P_I to P_{IV}. C: Northern hybridization analysis of *Brd2* mRNAs in selected mouse tissues using probe 1 (indicated in Fig. 1) to detect the longer transcripts with long 5′ UTR. D: Northern hybridization analysis of *Brd2* mRNAs in selected mouse tissues using probe 2 (indicated in Fig. 1) to detect both the longer and shorter transcripts (with long and shorter 5′ UTR). T, testis; B, brain; Sk, skeletal muscle; Lu, lung; O, ovary; Mg, mammary gland; K, kidney; H, heart; Li, liver; In, intestine; U, uterus; Sp, spleen. E: Northern hybridization of human brain mRNA using human *BRD2* cDNA as a probe, showing the long and short transcripts. Cb, cerebellum; Cc, cerebral cortex; Md, medulla; Sc, spinal cord; Oc, occipital cortex; Fc, frontal cortex; Tc, temporal cortex; Pt, putamen. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Using the 3.5 kb sequence upstream of the TATA Box in exon 1 and NCBI's BLASTN program to examine the human EST database, we further found that the longer transcripts start, not from a single site, but rather from at least 4 sites (P_{I-IV}, Fig. 2B). Each of these upstream exons splice to exon 1 directly. We correspondingly designated these exons, from which the transcripts are generated, as exons 0_I, 0_{II}, 0_{III}, and 0_{IV}. There is no apparent TATA box associated with these upstream start sites. Such transcription start sites have been referred to as "dispersed promoters" [Sandelin et al., 2007; Juven-Gershon et al., 2008]. Database searches examining the tissue distribution of this variety of cDNAs showed that BRD2 transcripts from different tissues used different promoters. For example, site P_{III} in Figure 2B is preferentially used in brain and thymus tissues: 62% of the cDNA clones that align to P_{III} are from brain and 27% are from thymus. Among the cDNA clones aligned to the second most frequently used upstream initiation site (P_{IV} in Fig. 2B), 27% are of brain origin. P_{IV}-originating transcripts are also detected in placenta, uterus, neutrophils, dermal papilla cells, tongue, and teratocarcinoma.

We next used Northern blot hybridization analysis to confirm the tissue distribution of the long and short transcripts. We designed probes that would recognize either both the long and short transcripts of the mouse *Brd2* gene (Probe 2 in Fig. 1B), or only the longer transcript (Probe 1 in Fig. 1B). It was not possible to design a probe that recognized only the shorter transcript. Figure 2C,D shows that probe 1 recognized only the longer transcript while probe 2 recognized both the longer and the shorter species. The data also show that among the various mouse tissues examined, there were no tissues in which only the longer or only the shorter isoforms were expressed.

EXPRESSION OF *BRD2* IN THE BRAIN

Since *BRD2* has been correlated to human epilepsy, we are particularly interested in its expression during neural development and in the brain. In situ hybridization analysis of E12.5 mouse embryo histological sections [Rhee et al., 1998] showed robust expression of *Brd2* in several regions of the developing mouse brain and spinal cord, as did subsequent whole mount in situ hybridization analysis of E9.5 embryos [Shang et al., 2009]. These findings are consistent with the neural tube closure abnormalities seen in the absence of *Brd2* [Gyuris et al., 2009; Shang et al., 2009]. To examine the expression pattern of *BRD2* mRNA in human brain, we performed Northern blot hybridization analysis with RNAs from different regions of human brain. Both the long and short forms of *BRD2* mRNAs were detected in several dissected human brain regions including: cerebellum, cerebral cortex, medulla, spinal cord, occipital cortex, frontal cortex, temporal cortex, and putamen (Fig. 2E).

BRD2 mRNAs ARE ALTERNATIVELY SPLICED WITH A HIGHLY CONSERVED EXON 2A

When comparing the structure of the mouse and human *BRD2* genes, we noted striking homologies at the nucleotide level within non-coding regions of the genes. In particular, there is an about 180 nucleotide region of the second intron that is more conserved than sequences in the flanking coding exons (Fig. 3A). Not only is such striking sequence conservation not seen in other introns, this conservation extends across the evolutionary scale to monotremes (Fig. 3A), and strongly suggests that these sequences are functionally important [Veldhoen et al., 1999]. Indeed, previous work [Taniguchi et al., 1998] and examination of cDNA sequences in

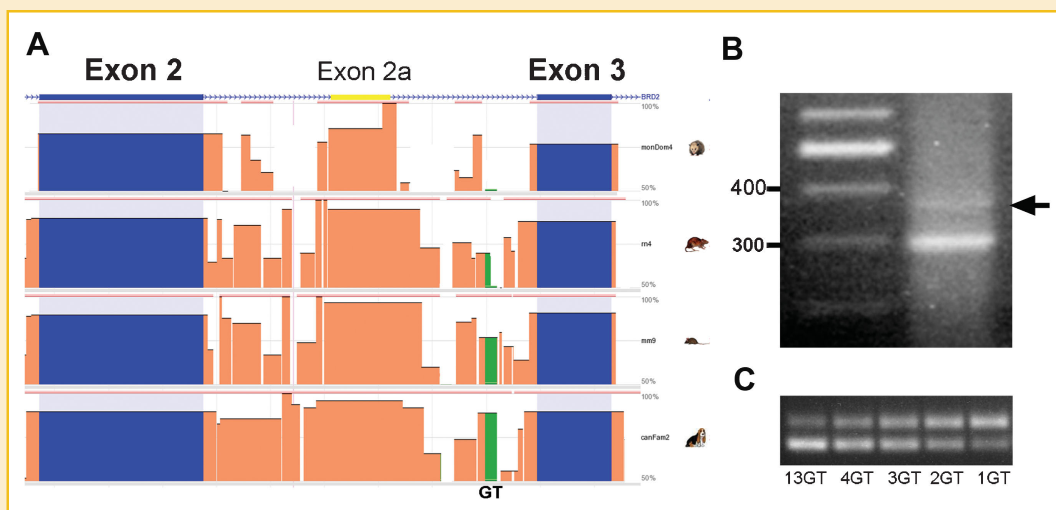

Fig. 3. The conserved region in intron 2 of *BRD2* contains an alternatively spliced exon. A: A cartoon portraying the ~180 bp region in intron 2 of *BRD2* that is highly conserved between human, dog, mouse, rat, and opossum, adapted from the ECR Browser. The yellow bar indicates the alternative exon 2a (92 bp) in intron 2. The green column near exon 3 is a microsatellite (GT repeats). A vertical axis cut-off of 75% identity is utilized to visualize only the significant alignments. Note that the level of conservation in intron 2 is higher than in the two adjacent coding exons. B: RT-PCR of human brain RNA using primers located in exon 2 and exon 3 of human *BRD2*. The upper weaker band contains a 92 bp conserved region in intron 2 (exon 2a), indicating the existence of mRNAs containing the alternatively spliced exon. C: RT-PCR of RNA from constructs containing different length of the GT repeats using primers across exon 2 to exon 3. It shows that the proportion of the alternatively spliced transcript (top band) increases relative to the regularly spliced transcript (lower band) with the shortening of the GT repeats. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

databases (Ensemble; ECR Browser) suggested to us that the conserved region of intron 2 might constitute an alternatively spliced exon. Using BLASTN and the third exon of the human *BRD2* gene to examine EST and cDNA databases to see if this 180 bp segment would harbor an alternatively-spliced exon, out of 100 hits, we identified 21 in which a part (92 bp) of this 180 bp genomic sequence appeared in alternatively spliced RNA transcripts. Importantly, inclusion of the 92 bp alternative exon 2a would introduce a premature translation termination codon in the middle of the first bromodomain and thus preclude the production of full length, functional Brd2 protein from transcripts containing exon 2a.

To determine if the alternative exon actually appears in RNA from brain, we performed RT-PCR using primers in exons 2 and 3 that would discriminate between the alternatively spliced forms. Figure 3B shows that, in addition to the 300 bp band corresponding to the major transcripts containing the constitutive exons 2 and 3, there is a minor band of ~390 bp. Sequencing of this minor PCR product confirmed that it contains the 92 bp-long alternatively spliced exon, which we designated as exon 2a. Thus, we confirmed that the highly conserved 180 bp-long region of intron 2 contains a 92 bp alternatively spliced exon. The resulting mRNAs are referred to as "regularly spliced" versus "alternatively spliced" transcripts.

THE LENGTH OF A MICROSATELLITE IN INTRON 2 OF *BRD2* AFFECTS THE ALTERNATIVE SPLICING

In the human *BRD2* gene, between the alternative exon 2a and exon 3 there is a microsatellite (GT-repeats) (Fig. 3A) that is highly polymorphic. Importantly, in the original linkage and association analysis, this highly polymorphic microsatellite was used as a marker and certain alleles were strongly associated with JME [Greenberg et al., 2000]. It has been reported that alternative splicing in other genes can be regulated by varying the lengths of nearby intronic microsatellites [Faustino and Cooper, 2003]. As this microsatellite is only 170 bp away from exon 2a of *BRD2*, we speculated that the length of this microsatellite may influence the inclusion of the alternative exon 2a. To test this hypothesis, we cloned the region from exons 2 to 3 from a human *BRD2* allele common in the population into an expression vector. That common allele contained 13 GT repeats (the prototype listed in Ensemble database). Using the construct as a template, we then generated constructs with shorter versions of GT repeat length using site-directed mutagenesis. Following transfection into HEK293T cells, total RNAs were isolated and analyzed by RT-PCR to examine the proportion of alternative to regular splicing products. As shown in Figure 3C, with the shortening of the GT repeats, the proportion of the alternative splicing product (the upper band) increases, from only a minor part in 13 GT to a major part in 1 GT (Fig. 3C).

THE FOUR TRANSCRIPTS OF *BRD2* EXHIBITED DIFFERENT TRANSLATIONAL PROPERTIES

The long and short transcripts, each combined with regular or alternative splicing, would result in four different mRNA isoforms. As mentioned earlier, we predicted that full length BRD2 protein would not be translated from both long and short transcripts that contain exon 2a, while the long and short transcripts that are regularly spliced, that is, not containing the alternative exon 2a, would yield full length protein. To determine if all four transcripts (long and short regularly spliced, and long and short alternatively-spliced) are indeed translated, we generated constructs containing human *BRD2* cDNAs that would yield the appropriate mRNAs. In vitro transcription and translation revealed that, as predicted, no detectable full length BRD2 protein was produced from either the long or short transcripts containing alternative exon 2a (Fig. 4A). What was surprising, however, was our observation that among the regularly spliced transcripts containing the full open reading frame (ORF), only the short transcript yielded full length BRD2 protein (Fig. 4A).

To determine if preferential translation of the shorter, regularly spliced *BRD2* transcript was also seen in vivo, each of the *BRD2* cDNA variants were engineered as Flag-tagged constructs, transfected into HEK293T cells, and examined for the production of the tagged BRD2 protein product. Immunoblot detection of the tagged BRD2 products using anti-flag antibody revealed that, as in the in vitro experiment, only the short, regularly spliced *BRD2* transcripts (exon 2a-exclusive) were translated (Fig. 4B).

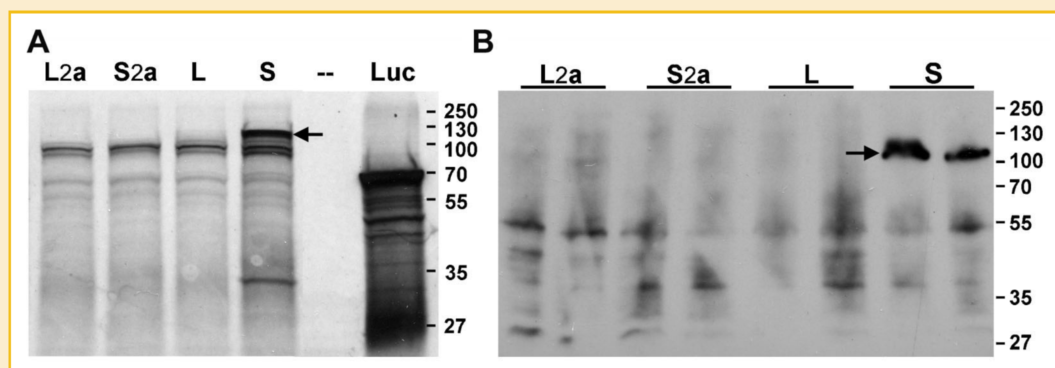

Fig. 4. Translation of the *BRD2* mRNAs. A: In vitro translation of *BRD2* mRNAs using ^{35}S methionine labeling. L, *BRD2* transcript with long 5′ UTR. S, *BRD2* transcript with short 5′ UTR. 2a, exon 2a containing-alternatively spliced. The arrow indicates the full-length *BRD2* protein. Luc, Luciferase positive control. (−), negative control. B: Expression of BRD2 protein from Flag-tagged *BRD2* cDNA constructs transfected into HEK293T cells as detected by immunoblot analysis with anti-Flag antibody.

TRANSLATION OF *BRD2* mRNA IS DIFFERENTIALLY REGULATED IN DIFFERENT REGIONS OF THE BRAIN

Since a major goal of our studies is to determine BRD2's potential function in brain, we next examined whether the different transcripts play a role in regulating BRD2 production in brain tissue. That is, we wished to know if there is a tissue specific differential distribution of the productive versus non-productive transcripts at the cellular level in vivo. If so, then a corresponding in vivo regulation at the level of translation would exist as well. To test this possibility, we first performed in situ hybridization analysis using the two probes we developed, one that recognizes only transcripts with the long 5′UTR (probe 1) and one that recognizes both transcripts (long and short) (probe 2). As noted above, because the short transcript is totally included in the longer one, we cannot generate a probe to detect the short transcript specifically. Thus, the in situ hybridization analysis could only reveal the general expression pattern of *Brd2* mRNAs. Nonetheless, we were able to conclude that there is not a region/cell type in the brain regions we examined that expresses only the short transcript; that is, the expression pattern detected by probe 1 (long transcript only) was similar to the pattern detected by probe 2 (both long and short). Attempts to examine the relative distribution of the alternatively spliced transcripts in histological sections of the brain were not successful, due to the short length of the specific region that can be used as probe.

While our in situ hybridization results did not show differential expression at the mRNA level, immunostaining revealed striking differences in the translation of Brd2 protein in specific regions of the brain. For example, although the regional and cellular distribution of *Brd2* mRNAs was similar in the two brain structures that we selected for further analysis (hippocampus and cerebellum), the protein expression was quite different. In the Purkinje cell layer in the cerebellum, both *Brd2* mRNA and protein were readily detected (Fig. 5A,C). However, in the hippocampus, while *Brd2* mRNA was expressed quite robustly, no protein could be detected (Fig. 5B,D). The observation that *Brd2* mRNA was transcribed but not translated could result from an absence of the short, regularly spliced transcript in the hippocampal cells. That is, these cells would be expressing only the non-translated isoforms, long transcripts with or without exon 2a and/or the short isoform with exon 2a. Alternatively, it is also possible that the translation of even the normally productive shorter transcript lacking exon 2a is suppressed in hippocampus.

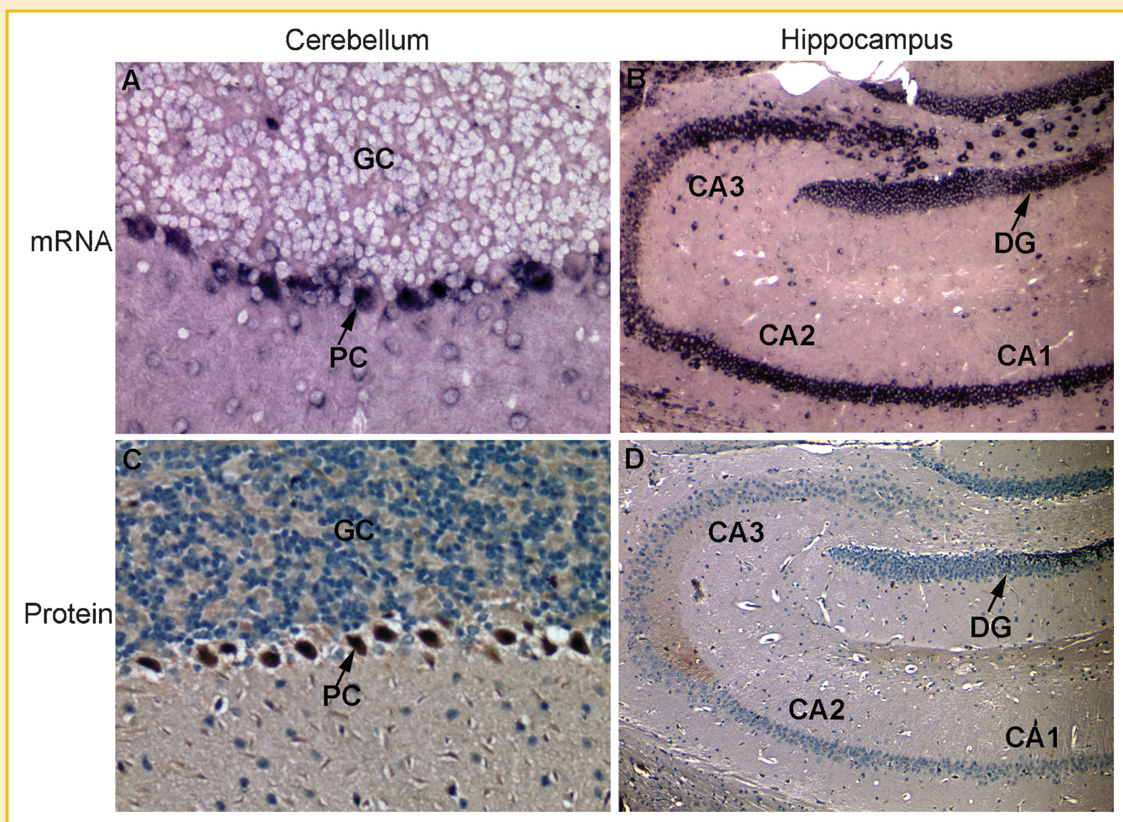

Fig. 5. Expression of *Brd2* mRNAs and protein in selected regions of the adult mouse brain. A: In situ hybridization in cerebellum. B: In situ hybridization in hippocampus; the pictures shown in panels A and B were obtained with probe 2 (indicated in Fig. 1) which recognizes both the long and short transcripts. C: Immunostaining in cerebellum. D: Immunostaining in hippocampus. PC, Purkinje cells; GC, granule cells; DG, dentate gyrus. CA1, CA2, CA3, sub-regions of hippocampus.

DISCUSSION

The human *BRD2* gene has been linked and associated with a common form of epilepsy, JME [Greenberg et al., 2000; Pal et al., 2003; Cavalleri et al., 2007], and also with photoconvulsive response on EEG [Tauer et al., 2005; Lorenz et al., 2006], but the mechanisms by which *BRD2* leads to epilepsy-related phenomena are unknown. As no mutations have been identified in the coding regions of the human *BRD2* gene in patients, we suspect that abnormal regulation of *BRD2* gene expression may be the critical step in susceptibility. The fact that *Brd2* haplo-insufficient mice are more susceptible to seizures [Velicek et al., under revision] argues that proper levels of Brd2 expression are critical and suggests that regulatory disruption of *BRD2* expression is the ultimate disease mechanism in humans. Thus, we considered potential mechanisms that could contribute to an alteration in the amount of *BRD2* mRNA and protein, including transcription, alternative splicing, and translation.

It has been suggested that >50% of human genes exhibit alternative splicing and that about 30–50% of human genes and about 50% of mouse genes have multiple alternative promoters [Sandelin et al., 2007; Chen and Manley, 2009]. For some genes, alternative promoters are used to generate proteins with different N-termini. For example, *Neuregulin 1* (*NRG1*) uses at least nine different alternative promoters to transcribe corresponding mRNAs that produce distinct NRG1 protein isoforms [Tan et al., 2007]. Interestingly, the presence of particular isoforms of NRG1 has been linked to schizophrenia [Tan et al., 2007].

We have confirmed that the *BRD2* gene contains multiple promoters in a large region (over 3.5 kb). Importantly, we discovered that there is a downstream promoter in the middle of exon 1, and that mRNAs transcribed from this promoter are actually the class that produces protein in the assays we employed. Consistent with our findings, newly released maps of histone H3K4me3 in human brain and multiple cell lines on the UCSC website [Cheung et al., 2010; Ernst et al., 2011], identified by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq), showed that a broad region from upstream of the first promoter to intron 2, is covered with promoter marker H3K4me3.

While the different promoters we identified for *BRD2* did not change the ORF of the resulting distinct transcripts, they generated transcripts with different 5′ UTRs. The longer transcript contains an extremely long 5′UTR of 1.7 kb, which could affect their stability or translation efficiency. For example, the *Drosophila insulin-like receptor* gene (*dINR*) has an unusually long 5′UTR with an internal ribosome entry site that makes the translation of the mRNA cap-independent [Marr et al., 2007]. Although our experiments suggested that the longer *BRD2* transcript was poorly translated, if at all, we have not excluded the possibility that it is translated in certain cell types, and/or under certain situations. It is also possible that the transcription of the longer transcript "interferes with", that is, regulates, the transcription of the productive shorter transcript [Mazo et al., 2007], as has been suggested for the human *dihydrofolate reductase* (*DHFR*) gene [Martianov et al., 2007]. Expression of the longer untranslated *DHFR* RNA reduces binding of TATA binding protein (TBP) and TFIIB in vitro, and of TBP, TFIIB, and Pol II in vivo, hence interfering with transcription of the shorter transcript.

We also found that *BRD2* exhibits another regulatory control mechanism, differential splicing. There are at least two ways in which the alternative splicing could regulate expression of BRD2 protein. First, the inclusion of exon 2a introduces a frame shift and thus a premature termination codon in the transcript. However, immediately after the stop codon there are several in-frame ATGs in exon 3 that could potentially serve as translation start sites. If this alternatively spliced mRNA did indeed initiate translation at these sites, it would be predicted to yield a truncated protein containing the second bromodomain, the ET domain, and the C-terminal region but lacking the first bromodomain. As yet, we have no evidence for any protein arising from the alternatively spliced mRNAs. Interestingly, deletion of the first bromodomain of another BET family member, *Brdt*, results in a truncated protein containing only the second bromodomain, the ET domain, and the C-terminal region [Shang et al., 2007]. The expression of this truncated Brdt protein still resulted in a dramatic phenotype: disruption of spermiogenesis and complete male sterility in mice.

Second, exon 2a is a potential target of the brain-specific RNA splicing factor Nova-1 as it harbors 4 copies of the "YCAY" motif, the Nova-1 binding site, in its sequence [Buckanovich and Darnell, 1997; Dredge et al., 2005]. This number of YCAY motifs is significantly greater than one would expect from a random distribution of one "YCAY" in stretch of DNA of this length [Ule et al., 2003]. The fact that the alternatively spliced exon is the most evolutionarily conserved part of *BRD2* argues strongly for its functionality.

The alternatively spliced exon 2a is unique to mammals and its sequence is extremely conserved among mammalian species. In our original linkage and association studies of JME with *BRD2*, some alleles of a microsatellite polymorphism (length of GT or CA repeats) in intron 2 were strongly associated with JME [Greenberg et al., 2000]. This polymorphic GT repeat region, 170 bp downstream of the conserved alternatively spliced exon 2a, raises the question as to whether it could be involved in regulating the splicing event. In support of this possibility, there is a growing body of literature that shows that dinucleotide repeats can act as splicing enhancers and silencers [for review see Faustino and Cooper, 2003]. Our preliminary studies showed that changing the length of the GT repeat has a profound effect on the splicing event: shortening of the GT repeat length increased the ratio of alternatively spliced relative to the regularly spliced product. The increase in the levels of the non-productive alternatively spliced transcript would be expected to be reflected in a corresponding decrease in the amount of BRD2 protein. Thus, polymorphisms of GT repeat length in the microsatellite in JME patients could result in alterations in the amount of BRD2 protein produced which in turn affect neural development and function.

Changes in the lengths of microsatellites have been implicated in several diseases [Thomas et al., 1995; Pagani et al., 2000; Stangl et al., 2000; Gabellini, 2001]. Repeat regions influence splicing by causing either the skipping or inclusion of exonic sequences, decreasing or increasing the levels of the various splice variants. Slight alterations in the number of repeats can have profound effects on splicing by changing the recognition sites for the splicing machinery. For example, a change from 10 to 16 dinucleotide CA

repeats in *NCXI*, a Na^+/Ca^{2+} cation exchanger, greatly enhances a splicing decision that includes a particular exon, while removing the repeat abolishes the inclusion [Gabellini, 2001]. Also, a significant increase in exon skipping occurred in splicing of cystic fibrosis transmembrane regulator mRNA when the length of a CA repeat in an intronic region was altered from 11 to 13 repeats in a specific allele [Pagani et al., 2000]. The balance of the two alternative splicing events could thus reflect the function of *BRD2*.

In conclusion, we have demonstrated a unique structural organization of the mouse and human *BRD2* genes which results in complex modes of transcriptional and post-transcriptional regulation of expression of the protein products. We further showed that Brd2 expression differs in different regions of the brain and elucidated possible mechanisms by which the choice of alternative promoters and alternative splicing events could result in differential regulation of *BRD2*'s function. The alternative splicing in particular may further link polymorphisms in the surrounding region of the alternative exon in individuals affected with JME to the disease mechanism.

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