Tissue-selective Expression of α-Dystrobrevin Is Determined by Multiple Promoters*

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α-Dystrobrevin, the mammalian orthologue of the Torpedo 87-kDa postsynaptic protein, is a dystrophin-associated and dystrophin-related protein. Knockout of the gene in the mouse results in muscular dystrophy. The control of the α-dystrobrevin gene in the various tissues is therefore of interest. Multiple dystrobrevin isoforms differing in their domain content are generated by alternative splicing of a single gene. The data presented here demonstrate that expression of α-dystrobrevin from three promoters, that are active in a tissue-selective manner, also plays a role in the function of the protein in different tissues. The most proximal promoter A is active in brain and to a lesser extent in lung, whereas the most distal promoter B, which possesses several Sp1 binding sites, is restricted to brain. Promoter C, which contains multiple consensus myogenic binding sites, is up-regulated during in vitro myoblast differentiation. Interestingly, the organization and the activity of the α-dystrobrevin promoters is reminiscent of those in the dystrophin gene. Taken together we suggest that the multipromoter system, distributed over a region of 270 kilobases at the 5′-end of the α-dystrobrevin gene, has been developed to allow the regulation of this gene in different cell types and/or different developmental stages.

Dystrobrevin, a dystrophin related protein, was originally identified from the Torpedo californica electric organ as an 87-kDa phosphoprotein associated with the cytoplasmic face of the postsynaptic membrane (1, 2). The protein is expressed in the electric organ, skeletal muscle, and brain, and it has been postulated to play a role in synaptic structure and function, because it copurifies with the acetylcholine receptors and rapsyn from the electric organ membranes. The protein is concentrated with the acetylcholine receptors at the synapse, but it is also found extrasynaptically at the sarcolemma of both Torpedo electric organ and vertebrate muscle. Furthermore, dystrobrevin is also found in association with dystrophin and the 58-kDa syntrophins in the Torpedo electric organ (3, 4).

By contrast to the single known Torpedo dystrobrevin molecule, cDNAs encoding several different isoforms differing in their domain content and tissue distribution have been identified in both human and mouse (5–7). The longest isoform, α-dystrobrevin-1 (94 kDa), has a tyrosine kinase substrate domain similar to the Torpedo protein (2, 5), in addition to a ZZ- (8) and two predicted α-helical-coiled coil domains (9), which it shares with α-dystrobrevin-2, while α-dystrobrevin-3 has simply the ZZ-domain. The genetic basis of this isoform diversity and additional alternative splicing was resolved by the determination of the genomic organization of the coding region of a single gene on mouse chromosome 18 (6). The conservation of the genomic organization between dystrophin and α-dystrobrevin is maintained across the homologous CRCT,† indicating that both genes evolved from an ancestral duplication event (10).

Considerable evidence supports an association of dystrophin and α-dystrobrevin. Dystrophin and α-dystrobrevin colocalize in skeletal muscle, copurify biochemically, and associate directly in vitro via the coiled-coil region of dystrophin (11–13). The expression pattern of the α-dystrobrevin gene also closely parallels that of dystrophin, where a set of diverse isoforms are generated in vivo by alternative splicing in brain and muscle. Dystrophin expression in muscle and brain results in three 14-kb transcripts (muscle-type, brain-type, and Purkinje cell-type), controlled by promoters at the 5′-end of the gene (14, 15). In addition, a whole family of smaller mRNAs are transcribed from promoters lying between exons within the rod domain of the gene. The first of these transcripts designated Dp71 (apolystrophin-1) and apodystrophin-3, encode the CRCT, or the first part of the CRCT, respectively, and are expressed predominantly in brain and non-muscle tissues from a housekeeping like promoter (16, 17). Other smaller transcripts are expressed in a tissue-specific manner, such as in peripheral nerve (Dp116, Ref. 18), the retina, brain, and cardiac muscle (Dp260, Ref. 19), and throughout the central nervous system (Dp140, Ref. 20).

† The abbreviations used are: CRCT, cysteine-rich carboxyl-terminal domain; 5′-UTR, 5′-untranslated region; bp, base pair(s); kb, kilobases(s); YAC, yeast artificial chromosome; RT-PCR, reverse transcriptase-polymerase chain reaction; RACE, rapid amplification of cDNA ends; MED-1, multiple start site element; MEF-1, myocyte-specific enhancing factor 1; M-CAT, muscle-CAT heptamer CATTCT.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF092287, AF106836, AF106837, AF106838, AF106839, and AF106840.

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In view of the similarity between dystrophin and α-dystrobrevin, and the recent evidence that mice null for α-dystrobrevin suffer from muscular dystrophy and impaired aggregation of acetylcholine receptors (21), it was of interest to determine the regulation of this gene. Our preliminary evidence indicated a minimum of four 5′-UTR exons, suggesting that the regulation of the α-dystrobrevin gene might be as complex as dystrophin. While in the process of investigating whether particular α-dystrobrevin isoforms are associated with a specific 5′-UTR region, we identified additional 5′-UTR exon sequences, consistent with a regulation of expression by multiple promoters. Here we present evidence that the mouse 5′-region of the gene is composed of seven 5′-UTR exons covering 270 kb of genomic DNA and demonstrate that α-dystrobrevin is expressed from three promoters that are active in a tissue-selective manner. Our data suggest that this multipromoter system of the α-dystrobrevin gene has been developed to allow the regulation of this gene in different cell types and/or different developmental stages.

EXPERIMENTAL PROCEDURES

Isolation, Characterization, and Sequencing of Genomic Clones—To determine the order and estimate the distance between the 5′-UTR exons, a BAC (mouse 129/sv) genomic library was screened (Research Genetics, Huntsville, AL) by a PCR approach using primer sets specific for the 5′-UTR exons A (RnpAf, 5′-GGGGGAGAAAGAATCGTACTG-3′; RnpAr, 5′-CTCCTTTTATGATTCCAGC-3′) and the anchor-ligated DNA and demonstrating that α-dystrobrevin is composed of seven 5′-UTR exons, consisting with a regulation of expression by multiple promoters. Here we present evidence that the mouse 5′-region of the gene is composed of seven 5′-UTR exons covering 270 kb of genomic DNA and demonstrate that α-dystrobrevin is expressed from three promoters that are active in a tissue-selective manner. Our data suggest that this multipromoter system of the α-dystrobrevin gene has been developed to allow the regulation of this gene in different cell types and/or different developmental stages.

RESULTS

5′-UTR Exons and Alternative Splicing—We have described multiple transcripts for mouse α-dystrobrevin that encode three protein isoforms (5, 6, 11). Some of these transcripts differed in their 5′-UTRs, suggesting that they may be by alternative splicing of different 5′-exons, indicative of transcriptional regulation by multiple promoters. To determine the origin of these transcripts and the mechanism of α-dystrobrevin expression, we characterized genomic fragments covering the 5′-end of the gene. We have reported previously the 5′-intron boundaries of four 5′-UTR exons A–D, derived from different cDNA clones. Here, 5′-UTR sequences have been extended to their 5′-ends by sequencing of RACE products of the dystrobrevin mRNAs from mouse heart and brain. A PCR approach on a YAC vector library of the YAC clone ICRI8F02M0312Q positive for the dystrobrevin gene was used to clone additional 5′-UTR exons and determine their exon-intron boundaries.
boundaries (Fig. 1). Exon sizes and splice junctions sequences of all seven 5'-UTR exons are summarized in Table I. The splice junctions have the conserved GT and AG dinucleotides present at the 5' splice donor and 3' splice acceptor sites (25). The three most upstream exons, exons A, B, and C, are all of similar size and are larger than the internal exons D–G. Mapping of the 640-kb YAC clone ICRFy902M0312Q indicated that the maximum distance between the UTR exons is approximately 270 kb. To determine the order and estimate the distance between the UTR exons, a mouse BAC genomic library was screened using primer pairs for all 5'-UTR exons and the coding exon 3. This resulted in the isolation of three overlapping BACs, two of which (BAC 343 and BAC 547) had insert sizes of 170 and 140 kb and contained the first coding exon, UTR exons A and D. The third BAC, with an insert size of 160 kb, contained the other five UTR exons: exons C, G, E, F, and B. Fig. 2 shows the approximate positions of the seven UTR exons spanning a distance of approximately 270 kb relative to the 25 coding exons of the gene.

Comparison of the nucleotide sequences of the seven 5'-UTR exons to the sequences of 5'-RACE products from brain and heart revealed a complex pattern of alternative splicing. As indicated in Fig. 2B, exon A was spliced directly to exon 1, the first protein-coding exon, while both exon B and exon C were separated from exon 1 by a further exon, exon D. In a minority of RACE clones, one or the other of three additional 5'-UTR exons, exons E, F, and G, were spliced between exons B and D. All RACE clones were identical in sequence downstream of exon 1. By contrast, RACE clones positive for exon B and exon C ended at several different points, but in each case there was a cluster of clones. Thus for exon B clones terminated at nucleotide –95, –110, –121, –128, –134, –142, –148, –197, and –200, where the 3'-end of the exon is designated as –1. Similarly transcription initiation sites were identified at nucleotide positions –121, –146, –171, –185, and –209 for exon C. In all three populations of RACE clones we did not find any clones that were spliced within these regions. Moreover, there are no multiple AG sequences to serve as splice acceptor sites.

To confirm the location of the cap sites for the various α-dystrobrevin mRNAs, three genomic probes were constructed and used in the RNase protection assay. Probe A was a 299-bp PCR fragment of exon A and its 5'-flanking region. Using this probe, a major 243-bp fragment was protected by mRNA from mouse brain, heart, lung, and skeletal muscle. Additionally, a second product at 170 bp was also evident, albeit at very low intensity, indicating that this fragment might reflect a minor transcription start site (Fig. 3). By contrast probe B, a 268-bp PCR fragment of exon B and its 5'-flanking region, revealed multiple protected products ranging in size from 223 to 109 bp. None of these fragments were protected by mRNA from lung or muscle. Using a 258-bp PCR fragment of exon C and its 5'-flanking region revealed major protected products at 209, 195, 164, and 135 bp. Interestingly, the intensity of the 135-bp protected fragment was significantly higher in skeletal muscle than in all the other tissues. This protected fragment probably reflects a preferentially used cap site for α-dystrobrevin mRNAs in skeletal muscle. Weak muscle-specific products at 183 and 175 bp were also detected. In summary, multiple transcription start sites for all three promoters were detected.

Mapping of Transcriptional Initiation Sites—Twenty RACE clones for each type of end-terminal UTR exon (exons A, B, and C) were sequenced and provide an indication of the location of transcriptional start sites. The majority of the exon A-positive clones terminated 239 bp upstream of the 3'-end of exon A. By contrast, RACE clones positive for exon B and exon C ended at several different points, but in each case there was a cluster of clones. Thus for exon B clones terminated at nucleotide –95, –110, –121, –128, –134, –142, –148, –197, and –200, where the 3'-end of the exon is designated as –1. Similarly transcription initiation sites were identified at nucleotide positions –121, –146, –171, –185, and –209 for exon C. In all three populations of RACE clones we did not find any clones that were spliced within these regions. Moreover, there are no multiple AG sequences to serve as splice acceptor sites.

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Nucleotide Sequence of Regions Upstream of 5'-UTR Exons A, B, and C—To identify putative regulatory promoter elements that flank the most upstream 5'-UTR exons, we sequenced a 858-, 528-, and 994-bp upstream of exons A, B, and C, respectively (Fig. 4). In common with other genes with
multiple transcription start sites, no typical TATA or CAAT box was present in the most distal promoter B region. However, we found the motif GCTCCC downstream of the −100-bp multiple start site window, which is identical to a conserved downstream element defining a new subclass of RNA-polymerase II promoters (26). Computer-assisted analysis revealed several putative transcription factor binding sites including Sp1 and AP2. The promoter C sequence also contained no TATA box in non-GC-rich promoters (30). Several putative transcription factor sites, including AP1 and PuF, were found within the promoter A sequence.

Promoter Activities in the 5′-Flanking Regions of Upstream Exons—Genomic fragments containing the putative promoter regions A, B, and C (Fig. 4) were cloned upstream of the luciferase gene. These constructs were then transiently transfected into the mouse fibroblast NIH3T3 and mouse myogenic H2K-tsA58 cell lines. All constructs directed luciferase expression in NIH3T3 cells (Fig. 5A). The level of luciferase activity produced by the constructs containing sequences of the 5′-flanking regions of exon B and exon C was substantially higher in forward (Bf and Cf) than in reverse orientation (Br and Cr). The promoter strength of the construct Bf was comparable with the activity of the herpes simplex virus thymidine kinase promoter used as a control. The construct Cf was about 3-fold stronger than this promoter. The 5′-flanking region of exon A

Table I
Exon-intron boundaries of the α-dystrobrevin 5′-UTR exons

| Exon Name | 3′-Splice acceptor site | Exon/intron boundaries | 5′-Splice donor site |
|-----------|-------------------------|------------------------|---------------------|
| UTRA      | AGCTCTTTT               | −243  242              | −2 AAAGACAggttaaggtc. . . |
| UTRB      | GCTTCAGG                | −352  23               | −129 ATGGAAGgtggtgtg. . . |
| UTRC      | GTTAAAGA                | −338  209              | −129 GGCTCAGAggtgaag. . . |
| UTRD      | . . . cattacaagATAATAG  | −128  127              | −2 AATTITAGgtgagtgc. . . |
| UTRF      | . . . tcttcaagGCCCGAAGA | −128  128              | −129 CCTGGCAGttgtacag. . . |
| UTRG      | . . . tgggtcaagGTACTTC  | −93    | −129 CACTCCAGgtcagtgag. . . |

Fig. 2. Genomic organization and alternative splicing of α-dystrobrevin 5′-UTR exons. A, long range restriction map of the 5′-end of the α-dystrobrevin gene. The three promoters (arrow) and the approximate position of exons A–G in the first 270 kb of the α-dystrobrevin gene are shown relative to the 25 coding exons 1–25. Data on placement of exons 1–25 are derived from previous studies (6). Restriction sites for Mlu1, NotI, SalI, and XhoI are indicated by M, N, S, and X, respectively. Exons are shown as open boxes. The positions of the mBAC clones (mBAC 467, mBAC 547, and mBAC 343) are shown above the gene. B, schematic illustration of the predominant splicing patterns in brain and muscle. The previously described m24, m21, and m32 transcripts (5) are generated by alternative usage of exons A, B, C, and D (open boxes) with lines indicating regions that are spliced out of the primary transcripts. In a minority of m32 transcripts one or the other of three internal exons, exons F, E, and G (black boxes), were found to be spliced between exon B and D and therefore generate further complexity in the 5′-untranslated region.
showed orientation-independent expression in this cell line and was excluded from further analysis.

To investigate the effect of myoblast differentiation on α-dystrobrevin expression, we transfected the aforementioned constructs into H2K-tsA58 myoblasts and analyzed luciferase activity in total cell extracts prepared from H2K-tsA58 muscle cells at different time points during myoblast fusion. The construct Cf exhibited higher luciferase activity in myotubes than in undifferentiated myoblasts, with a 200-fold increase in differentiated H2K-tsA58 myotubes over that obtained with the promoter construct Bf (Fig. 5A), which showed similar low levels of activity in myoblasts and myotubes. Luciferase activities in extracts of cells transfected with a construct containing the same region in reverse orientation (Cr and Br) and a promoterless pGL-3 basic vector are shown as negative controls.

In order to determine the endogenous transcription pattern of α-dystrobrevin in the H2K-tsA58 cell line, total RNA was isolated from myoblast cultures at various stages of differentiation. First, the differential usage of 5'-UTR sequences was investigated by RT-PCR using UTR exon-specific forward primers and a common forward primer and isoform-specific reverse primers. Southern blot analysis of the PCR products using a radiolabeled m32 cDNA probe (5) revealed single bands for all three dystrobrevin isoforms in proliferating myoblasts (Fig. 6B). After switching to differentiation medium, the level of α-dystrobrevin-3 (Fig. 6B, α-db-3) increases and then stabilizes as myoblast fusion proceeds. In the case of α-dystrobrevin-1 (Fig. 6B, α-db-1) and -2 (Fig. 6B, α-db-2), an additional upper band appears at increased levels, whereas the lower band seen in proliferating myoblasts remains at almost the same level. This amplification product probably reflects an alternatively spliced form of α-dystrobrevin-1 and -2 containing the vr3 sequence (5).

Western blot analysis of protein extracts prepared from H2K cells at different time points during myoblast fusion using an antibody against α-dystrobrevin-1 detects two proteins of similar relative mobility (Fig. 6C). Consistent with our detection of α-dystrobrevin-1 mRNA (Fig. 6B, α-db-1), the protein is found at low levels in undifferentiated H2K-tsA58 myoblasts. After switching to differentiation medium, α-dystrobrevin-1 becomes more abundant and remains expressed at constant levels as myoblast fusion proceeds. At this time, the muscle-specific splice variant of α-dystrobrevin-1 containing the vr3 sequence is also detected (5). Similar results were obtained for α-dystrobrevin-2 from expression studies in the myogenic C2C12 cell line (11). Taken together these results indicate that in the H2K-tsA58 cell line the transcriptional activation of the mouse α-dystrobrevin gene occurs upon differentiation of myoblasts into multinucleated myotubes. Promoter C, which contains multiple consensus myogenic binding sites, is active, whereas promoters A and B are not. However it is formally possible that a fourth as yet unidentified promoter is active as well.

**Tissue-selective Expression of α-Dystrobrevin mRNAs Containing Different 5'-UTR Exons**—To examine the expression...
To determine whether mRNA species encoding the three major α-dystrobrevin isoforms are associated with a particular promoter, we hybridized 5′-UTR exon-specific probes to multiple tissue Northern blots. The hybridization patterns are summarized in Fig. 8. Hybridization with a common probe spanning exons 1–6 illustrates the five predominant transcripts encoding the three major isoforms, which are estimated to be 7.5 and 4.0 kb (α-dystrobrevin-1), 5.0 and 3.6 kb (α-dystrobrevin-2), and 1.7 kb (α-dystrobrevin-3). All three UTR exon probes hybridized to the dystrobrevin-1 and dystrobrevin-2 transcripts in brain. Both exon A and exon C sequences were found in the full-length dystrobrevin-1 transcript expressed in lung and in a 3.8-kb transcript that has not been assigned to any of the three isoforms (6). The 1.7-kb transcript corresponding to the recently described muscle expressed α-dystrobrevin-3 isoform (11) was detected by the exon C probe in muscle, but also hybridized, albeit weakly, with the exon A probe in brain and possibly lung. These results indicate that the promoters are active in a tissue-selective rather than a tissue-specific manner and that the formation of individual dystrobrevin variants is independent of...
promoter activity and is probably the result of a post-transcriptionally regulated process.

**DISCUSSION**

In the present study we have identified multiple promoters that control the expression of the \( \alpha - \)dystrobrevin gene. The exon-intron arrangement of the unusually long 5′-end of the gene explains the origin of the different mRNA species that we have characterized (Ref. 5 and Fig. 2B). It is clear from the structure of the gene that both the alternative use of three promoters and the differential splicing of the resulting transcripts is involved in the generation of the multiple \( \alpha - \)dystrobrevin mRNAs. The most distal brain promoter is separated by 120 kb of genomic sequence from the muscle promoter. A third promoter located 70 kb further downstream is separated by another 80 kb of genomic sequence from exon 1, which contains the translational start site. Remarkably, the region containing the seven small UTR exons A-G spans 270 kb of genomic DNA. Considering that the \( \alpha - \)dystrobrevin gene is organized into 25 coding exons contained within a genomic interval of 170 kb, the total size of the gene can now be estimated as at least 440 kb (6).

The \( \alpha - \)dystrobrevin gene exhibits a structural arrangement similar to a number of other genes in which multiple promoters generate tissue-specific mRNAs that differ only at their 5′-untranslated region. Four short 5′-noncoding exons of the rat gene for brain-derived neurotrophic factor can be spliced to a common coding exon and are each regulated by separate promoters (31). These promoters confer tissue-specific, axotomy- and neuronal activity-induced expression in transgenic mice (32). Our results show that all three promoters of the \( \alpha - \)dystrobrevin gene are active in brain. It is therefore possible that the \( \alpha - \)dystrobrevin transcripts detected in the brain are transcribed in a region or cell specific manner from these promoters. Interestingly, the arrangement of the control region of the \( \alpha - \)dystrobrevin gene is very similar to that described for the dystrophin locus, where tissue-specific promoters regulate expression of full-length dystrophin isoforms (14, 33). One of these promoters, which is located upstream of the muscle promoter, regulates the expression of dystrophin in the cortex and hippocampus (34), while a third promoter, active in brain Purkinje cells, has been identified between the muscle promoter and the second exon of dystrophin (15). However, whereas the dystrophin isoforms have different first coding exons, the dystrobrevin transcripts described utilize the same first coding exon and
In cases in which a promoter switch does not affect the coding region, as in the α-dystrobrevin gene, the translation efficiency of the mRNAs can be affected (35). We found that the long 5' UTRs of the α-dystrobrevin mRNAs contain small partially overlapping upstream open reading frames that precede the major translation initiation site, which are highly conserved between human and mouse (data not shown). It has been suggested that such an arrangement might be particularly suitable for translational regulation (36). The mouse gene for the retinoic acid receptor-β2 has a similar complex 5' UTR exon organization. Mutation in the small open reading frames of the 5' UTR affected expression of the downstream major open reading frame, resulting in an altered regulation of gene expression in vivo (37).

The regions upstream of the 5' UTR exons A and B of the α-dystrobrevin gene exhibited none of the consensus features that define proximal promoter regions of tissue-specific promoters. The presence of multiple transcriptional start sites could be expected, since these regions lack a canonical TATA box-like element conferring a unique transcription start site (38). This situation is reminiscent of housekeeping genes (30). Despite the fact that the discovery of TATA-less promoters is steadily increasing, there is little information how this multiple selection process occurs. Recently, a protein binding sequence GCTCCC (MED-1; multiple start element downstream) was found to be positionally conserved in a number of promoters that initiate at multiple unclustered start sites (~100-bp window) and was shown to be involved in the regulation of this...
multiple initiation process (26). We found an identical sequence stretch immediately downstream of the multiple cap sites in exon B, and therefore the exon B promoter might be a member of this new subclass of TATA-less promoters.

Our RT-PCR studies clearly indicate that in H2K-tsA58 myoblast the transcriptional activation of the α-dystrobrevin gene occurs upon differentiation of myoblasts into multinucleated myotubes. Functional promoter studies show that the 1169 bp promoter C fragment is able to direct luciferase expression during myoblast differentiation. The proximal part of this region is composed of cis-acting sequences that have been implicated in muscle specific expression. Four copies of the CANNTG motif present in the MyoD target sequence and characteristic of many muscle-specific regulatory regions (27, 39) and a “M-CAT” similarity and a TGCCTGG sequence, both of characteristic of many muscle-specific regulatory regions (27, 39)

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