GENOMICS **42,** 115–125 (1997)
ARTICLE NO. GE974724

Plectin Transcript Diversity: Identification and Tissue Distribution of Variants with Distinct First Coding Exons and Rodless Isoforms

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Received January 15, 1997; accepted March 10, 1997

Plectin is a widely expressed protein that is very large in size and that has all the attributes of a multifunctional crosslinking and organizing element of the cytoskeleton. It displays a multidomain structure, versatile binding activities, and subcellular localizations that enable it to strengthen cells against mechanical stress forces. Moreover, hereditary gene defects in plectin cause epidermolysis bullosa simplex (EBS)-MD, a severe skin blistering disease with muscular dystrophy. Here we report the analysis of the exon–intron organization of the rat plectin gene and the identification of several different isoforms on the transcriptional level. We show that of 35 coding exons identified, 4 serve as alternative first exons splicing into the same successive exon 2, which is the first of 7 exons encoding a highly conserved actin-binding domain. RNase protection mapping of transcripts containing 3 of the identified 4 alternate first exons revealed their coexpression in rat glioma C6 cells and in a series of different rat tissues that we examined. Significant variations in expression levels of first exons indicated the possibility of tissue-specific promoter usage. In addition, plectin splice variants lacking exon 31 (>3 kb), which encodes the entire rod domain of the molecule, were identified in a variety of rat tissues. This study provides first insights into a complex plectin gene regulatory machinery with similarities to that of dystrophin. © 1997 Academic Press

INTRODUCTION

Plectin, a cytoskeleton-associated protein of exceptionally large size, is abundantly expressed in a wide variety of mammalian tissues and cell types. It shows codistribution with different types of intermediate filaments (IFs),[3] and it is prominently located at the plasma membrane attachment sites of IFs and of microfilaments, such as hemidesmosomes (Wiche *et al.*, 1984), Z-line structures and dense plaques of striated and smooth muscle (Wiche *et al.*, 1983), intercalated discs of cardiac muscle (Zernig and Wiche, 1985), and focal contacts (Seifert *et al.*, 1992). Furthermore, in several tissues, including brain (Errante *et al.*, 1994) and kidney (Yaoita *et al.*, 1996), plectin expression is prominent in cells forming tissue layers at the interface of tissue and fluid-filled cavities. These observations are consistent with a model in which the role of plectin is to strengthen cells against mechanical stress both along their surfaces and at their internal anchorage sites of cytoskeletal filaments. This concept is supported by recent reports demonstrating defective expression of plectin in epidermolysis bullosa simplex (EBS)-Ogna, an autosomal dominant severe skin blistering disease (Koss-Harnes *et al.*, 1997), and EBS-MD, an autosomal recessive disease, characterized by skin blistering combined with muscular dystrophy (Gache *et al.*, 1996; McLean *et al.*, 1996; Smith *et al.*, 1996).

Plectin has been cloned and sequenced from both rat (Wiche *et al.*, 1991) and human (Liu *et al.*, 1996). Secondary structure predictions based on the deduced amino acid sequences of cDNAs and genomic clones as well as electron microscopy of the protein (Foisner and Wiche, 1987) revealed a multidomain structure composed of a central ~200-nm-long α-helical coiled coil structure flanked by large globular domains. The structure of the carboxy-terminal domain is dominated by six highly homologous repeats that also occur in lesser numbers in desmoplakin (three repeats; Green *et al.*, 1990), bullous pemphigoid antigen (BPAG) 1 (two repeats; Sawamura *et al.*, 1991), and the recently identified envoplakin (one repeat; Ruhrberg *et al.*, 1996). Analysis of the human gene locus revealed a complex organization of 32 exons spanning over 31 kb of DNA located in the telomeric region (q24) of chromosome 8 (Liu *et al.*, 1996; as confirmed by MacLean *et al.*, 1996).

The sequence data reported in this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. U96274, U96275, and U96276.

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[3] Abbreviations used: ABD, actin binding domain; EBS, epidermolysis bullosa simplex; IF, intermediate filament.

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On the molecular level plectin has been shown to bind to a variety of cytoskeletal proteins, including cytoplasmic and nuclear IF subunit proteins (vimentin, glial fibrillary acidic protein, cytokeratins, neurofilament proteins, and lamin B), subplasma membrane proteins (fodrin and α-spectrin), and high-molecular-weight microtubule associated proteins MAP1 and MAP2 (Herrmann and Wiche, 1987; Foisner *et al.*, 1988, 1991). The expression of mutant forms of plectin in cell lines transiently transfected with cDNA constructs led to the conclusion that plectin's C-terminal globular domain is involved in the binding to IFs (Wiche *et al.*, 1993). Recently, the binding site of plectin to vimentin and to keratinocyte cytokeratins was mapped to a stretch of ~50 amino acid residues within repeat 5 of plectin's carboxy-terminal domain, and a basic amino acid residue cluster within a nuclear targeting sequence motif was identified as an essential element of this site (Nikolic *et al.*, 1996).

Considering plectin's versatile binding activities, prominence at distinct strategically important locations within the cytoarchitecture, such as cytoskeleton anchorage junctions, its complex exon–intron organization, and the differential staining of tissues and cells as revealed using a battery of mAbs to plectin (Foisner *et al.*, 1994), one would expect the existence of different plectin isoforms that would perform different cellular tasks and thus have different subcellular localizations. We report here the identification and tissue distribution of several such variants on the transcriptional level in rat. We also report the complete exon–intron organization of the rat gene and extend the analysis of the plectin gene structure previously reported for the human species with the identification of three additional 5′ exons, all of which serve as alternate first coding exons.

MATERIALS AND METHODS

Cosmid cloning of the rat plectin gene. A rat genomic library in SuperCos 1 (Strata gene) was screened by standard procedures (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989) using various cDNA fragments as hybridization probes. Sixteen positive clones were isolated, and the DNA inserts of five of the clones were analyzed by restriction mapping and Southern blot analysis. The relevant restriction DNA fragments were isolated and subcloned into pBluescript plasmid vectors (Stratagene). DNA sequence analysis of portions of each of the clones confirmed the presence of DNA sequence stretches corresponding to rat plectin cDNA.

Genomic organization. Intron–exon boundaries were determined by comparing genomic DNA and cDNA sequences. The size of the introns was either determined by direct sequencing or estimated by PCR amplification based on primers in the flanking regions.

DNA sequencing and analysis. Nucleotide sequences were determined by the Sanger dideoxynucleotide chain termination method using either the Sequenase (U. S. Biochemicals) or the DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). All sequence data were compiled and analyzed using LASERGENE (DNAStar) or DNA Strider (Marck, 1988). Database searches were performed using the BLAST program (Altschul *et al.*, 1990).

Construction and screening of a plectin-primed cDNA library. A cDNA library was constructed as described previously (Wiche *et al.*, 1991), with the exception that four antisense plectin-specific oligonucleotides (5′-GCCTTGATAAGGTGTTTATTGACCC; 5′-TTCTTGTGTGCCTCCTT; 5′-CAGGGTGGGCAGGGTTG; and 5′-GTCACCTCCTCCTGCAT), based on sequences in clones C2, C4, and C7 (Wiche *et al.*, 1991), were used instead of oligo(dT) to prime cDNA synthesis. In brief, these primers were annealed to mRNA isolated from cultured rat glioma C6 cells, and following reverse transcription and second-strand synthesis, the cDNAs were methylated at the *Eco*RI restriction sites, *Eco*RI linked, digested with *Eco*RI, and ligated into λgt11 (Amersham). The recombinant phages were packaged *in vitro* and plated onto *Escherichia coli* strain Y1088. The library was screened by standard procedures using the 5′ end *Eco*RI fragment of clone C7 and one oligonucleotide based on the 5′ end of the same cDNA (5′-TGATGGCCACAACCTCAT). λ-Phages hybridizing to both probes were plaque purified, and their DNA was isolated, subcloned into pBluescript (Stratagene), and sequenced.

Polymerase chain reaction (PCR). DNA prepared from the isolated genomic clones and from the extension cDNA library was used as template for PCR amplifications. Pairs of rat plectin-specific primers based on the cDNA sequence were used for genomic DNA amplifications, while combinations of plectin-specific primers with λ arm primers flanking the *Eco*RI cloning site of λ phage gt11 were used for cDNA amplifications. PCRs were carried out using either AmpliTaq polymerase, supplied by the Perkin–Elmer Cetus Corp. and used under conditions specified by the manufacturer, or a mixture of *Klen-Taq* (Ab Peptides) and *Pfu*I (Stratagene) polymerase under conditions described by Barnes (1994) for long and accurate amplifications.

For RT-PCR, 100 μg total RNA prepared from several adult rat tissues according to the method of Chomczynski and Sacchi (1987) was treated with DNase before reverse transcription and PCR amplification using *Taq* polymerase and buffer solutions from Promega. The following primers were used as specified in the text: 1a (5′-AGCTGGGCCGCCAACTTCGCTAC); 1b (5′-ACCTTGGGCTTCTTGGCGGGGGAG); 2a (5′-CTCCCCCGCCAAGAAGCCCAAGG); 2b (5′-CTCAAAGGTGTACTCGGGCTCGGCC); and 2c (5′-CGGCCTCCTCGGCCTGCAGTTTC).

RNase protection assays. Ten micrograms of total RNA prepared from various adult rat tissues and rat glioma C6 cells by the method of Chirgwing *et al.* (1979) was used for RNase protection analysis as previously described (Vitelli *et al.*, 1988), with the exception that hybridization was performed at 60°C. The probe used for transcriptional start mapping was amplified as a 370-bp fragment from a mouse genomic subclone using primer 1 (see above) and 5′ upstream primer 2 (5′-ACACTTCCGTCCTGCCTCACA), and it was subcloned into the pCRII vector (Invitrogen). Before transcription with Sp6 polymerase, the plasmid was linearized with *Sma*I to yield a 361-bp probe providing a maximum RNase protection of 281 bp. Probes specific for exons 1-2-3, 1a-2-3, and 1b-2-3 were amplified from rat cDNA clones encoding these exons using primer pairs 3 (5′-GGAGGAGCGGCAGGTCTATCG) and 4 (5′-ATGAGGTTGTGGCCATCA); 5 (5′-ATGTCTCAGCAACGGCTCC) and 4; and 6 (5′-ATGGAGCCGTCGGGCAGC) and 4, respectively. The resulting PCR products with sizes of 237 (E1-2-3), 230 (E1a-2-3), and 230 bp (E1b-2-3) were subcloned into pCRII (Invitrogen), linearized, and used to produce an antisense riboprobe by *in vitro* transcription with Sp6 polymerase. Similarly, a repeat 5-specific probe was amplified as a 164-bp fragment from a rat cDNA clone containing the repeat regions using primers 7 (5′-AAGGAAAAGAAGCGGGAG) and 8 (5′-GCACTCCTGCTCTGACAACT). The S16-specific probe was a gift from M. Busslinger.

RESULTS

Identification of Alternative First Coding Exons in a Plectin-Primed Rat cDNA Extension Library

When we first cloned and sequenced rat plectin cDNA, we found an open reading frame (ORF) of 12,420 bp within a mRNA of ~15 kb (Wiche *et al.*, 1991). In subsequent attempts to characterize the 5′ end of the molecule, a plectin-primed rat glioma C6 cell extension cDNA library was prepared in λ phage gt11. The exten-

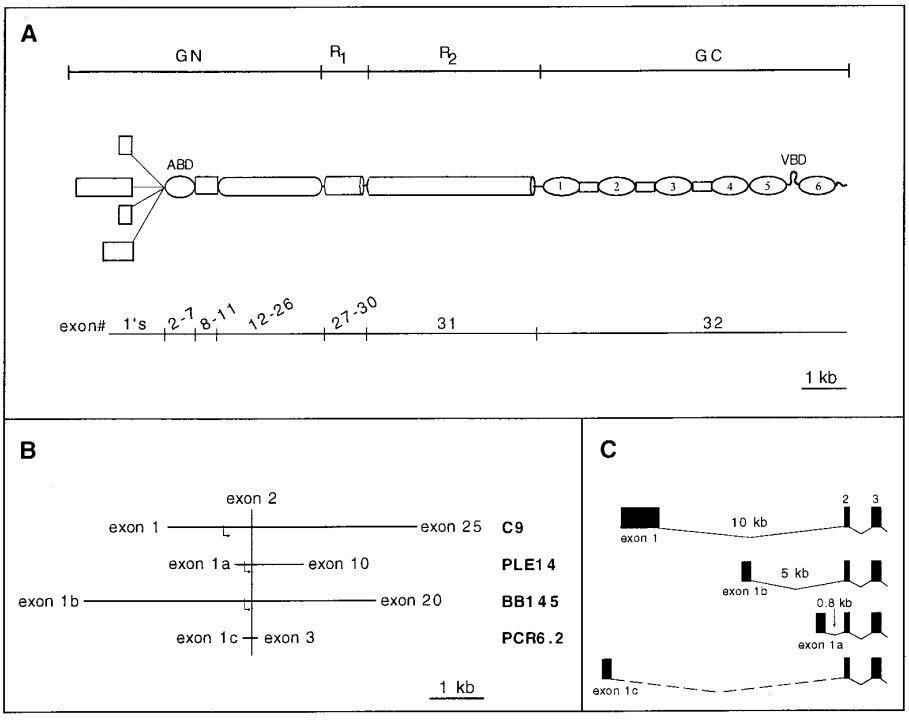

FIG. 1. Predicted domain and gene structure of plectin. (**A**) Schematic representation of the molecule with alternative starts. The domain designation given on top corresponds to that described previously (Wiche *et al.*, 1991), except for the extended size of the amino-terminal domain (GN), here based on the plectin isoform with the largest first coding exon (exon 1). ABD, actin binding domain comprising exons 2–7; VBD, vimentin binding domain in exon 32 (Nikolic *et al.*, 1996). Ellipsoids 1–6 correspond to the core of each of the six carboxy-terminal repeats, which consists of tandem repeats of a 19-amino-acid-residue motif. (**B**) Representative cDNA clones. Clones C9, BB145, and PLE14 were isolated from two different rat glioma C6 libraries (see text). Clone PCR 6.2 is a RACE-PCR clone. Exons covered by cDNAs are indicated. Positions of start codons in alternative first coding exons are marked by arrows. (**C**) Genomic organization of the 5′ end of the plectin gene. The four different transcripts identified at this locus are shown. Exons are indicated by black boxes; introns are indicated by lines. Approximations of intron sizes are given where known.

sion library was screened with a cDNA fragment derived from clone C7 (Wiche *et al.*, 1991) and with an oligonucleotide corresponding to the 5′ end of the same cDNA. Of the many clones isolated, several extended the previously reported ORF, but surprisingly the sequence homology between some of the clones diverged at their 5′ ends, and this divergence occurred always at the same site (later known to correspond to the 5′ end of exon 2) (Fig. 1; and see below). This suggested that the newly isolated cDNA clones represented differentially spliced transcripts, containing alternative 5′ coding exons. To verify this, several of these clones were analyzed in more detail together with previously uncharacterized clones isolated from the original rat glioma C6 cell cDNA library (Wiche *et al.*, 1991) and two cosmid clones isolated from a rat genomic library.

Clone C9 (Fig. 1B), with a cDNA insert of 4.5 kb, led to the identification of a new start ATG, 1632 bp upstream of the start site previously reported (Wiche *et al.*, 1991), preceded by 1 kb of untranslated sequence. This initiation codon and 10 bp of the adjacent untranslated sequence are conserved between human and rat, but the homology is lost further upstream (unpublished data). In contrast, downstream of the ATG, a single ORF of >14 kb is maintained in both cases, exhibiting over 90% sequence identity (Liu *et al.*, 1996). This lent support to our hypothesis that clone C9 indeed contained a first coding exon. The deduced amino acid sequence of full-length rat plectin, including the extension contributed by this clone, has been presented previously in the context of its comparison to human plectin (see Fig. 2; Liu *et al.*, 1996). Regarding the sequence divergence among the different cDNAs isolated, the entire unique coding sequence of clone C9 turned out to be contained within a single exon (see below), hereafter referred to as exon 1. A comparison of the exon 1 nucleotide and deduced protein sequences with database sequences revealed a region with substantial homology to the repeat domains found in ribosomal protein S10 (Fig. 3A). This region covers several of the putative basic repeats of the S10 protein possibly involved in its interaction with RNA or in nuclear targeting (Glück *et al.*, 1989).

A second clone, PLE14 (Fig. 1B), contained a 1.2-kb insert and extended the ORF of plectin upstream of its divergence point with clone C9 by 37 additional codons. This new ORF, designated exon 1a, started with an ATG that fully conformed to Kozak's (1987) consensus sequence for translation initiation and was preceded by two in-frame stop codons 42 and 138 bp upstream (Fig. 2). A third alternative first coding exon (exon 1b), was found within one of five *Eco*RI subclones derived from the 5-kb cDNA insert of lambda clone BB145 (Fig. 1B). The unique sequence contained in this subclone

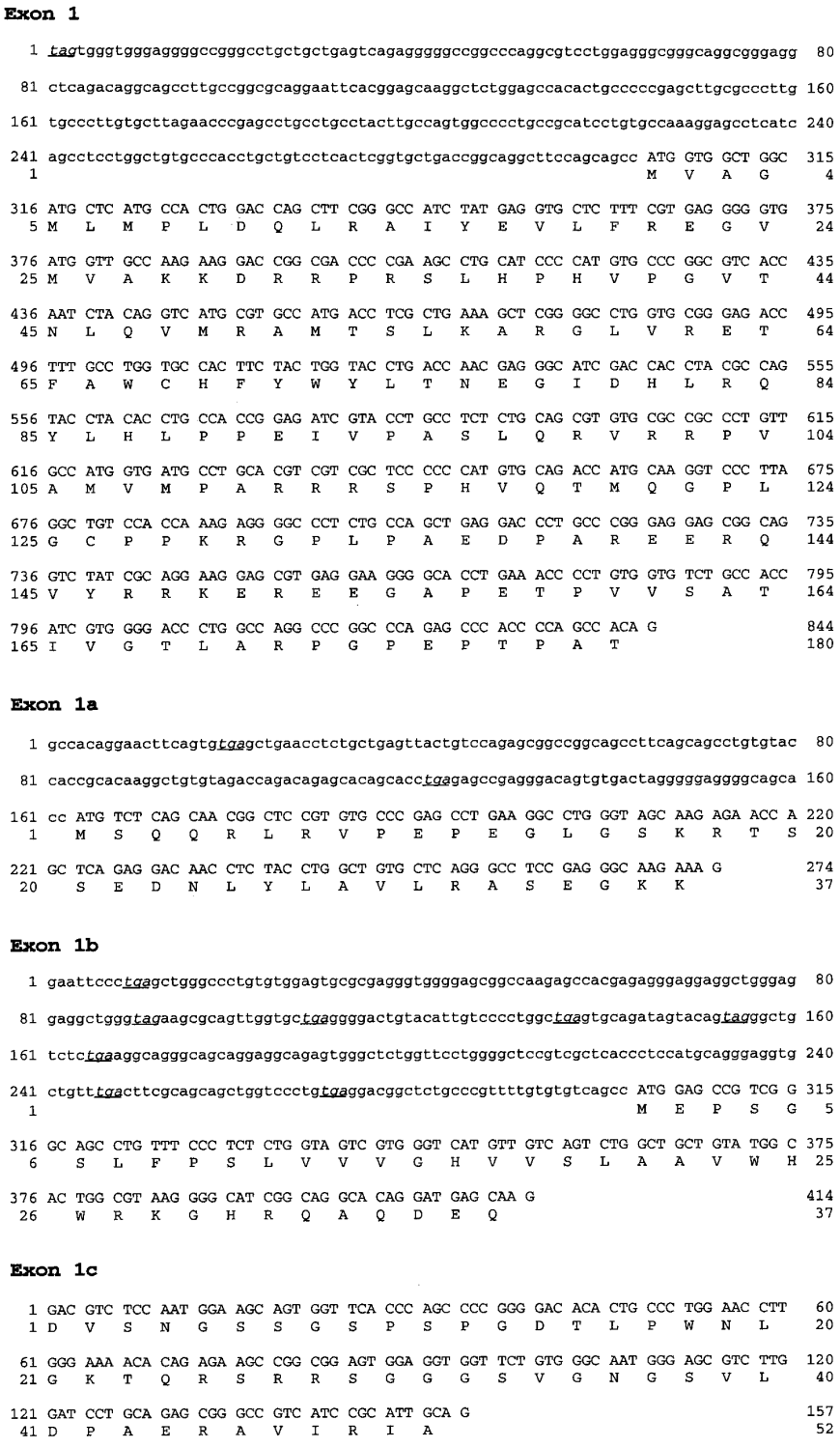

FIG. 2. cDNA and predicted amino acid sequences of four distinct first coding exons of plectin. The reported sequences stop at the splice junction to rat plectin exon 2. The in-frame stop codons in the 5′ untranslated sequences are marked.

extended the ORF of plectin by 38 codons 5′ of its divergence point with the other clones. Its start ATG, which again conformed to the consensus sequence of Kozak (1987), was preceded by 302 bp of untranslated sequence containing several in-frame stop codons (Fig. 2). Three of the other four *Eco*RI subclones derived from the same lambda clone could not be located in the cloned genomic region (see below), nor were other cDNA clones with similar sequences isolated, suggesting that they may have been the result of a cloning chimera between unrelated molecules. In a comparison of the cDNA and amino acid sequences of both exon 1a and exon 1b with other known sequences available from databanks, no significant homologies were found.

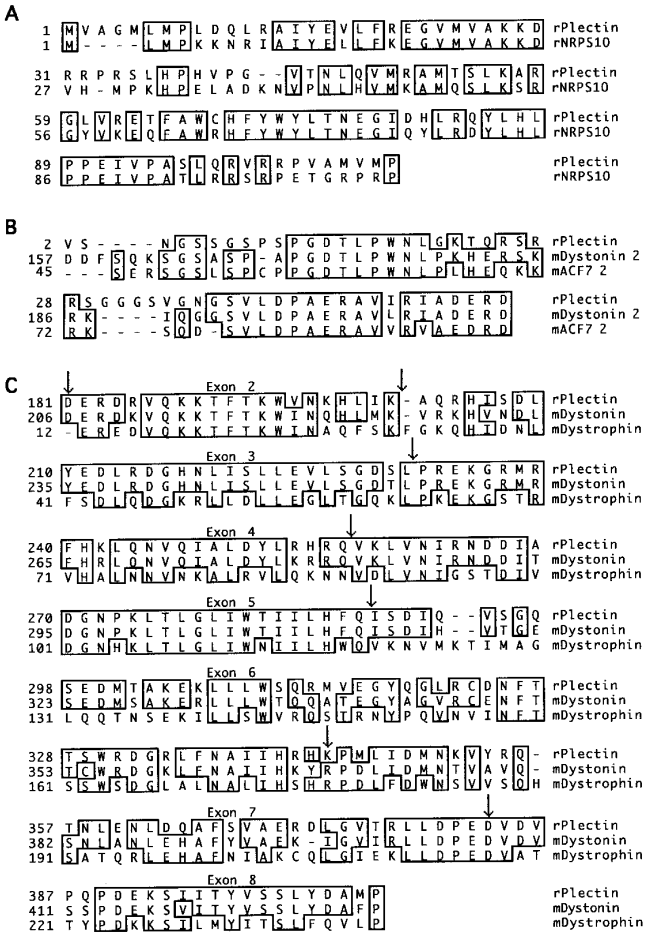

FIG. 3. Protein sequence comparison of the amino-terminus of rat plectin. Alignments shown are of rat plectin exon 1 with rat ribosomal protein S10 (X13549) in **A;** exon 1c with mouse dystonin, isoform 2 (U25158), and mouse ACF7, isoform 2 (U67204) in **B;** and exons 2–8 with the ABD of mouse dystonin (U25158) and mouse dystrophin (M68859) in **C.** Numbers on the left correspond to the amino acid positions in the different proteins. Boxes indicate identical amino acids, arrows indicate the exon boundaries, and dashes indicate gaps introduced to allow maximal alignment.

PCR amplification of cDNA extracted from the extension library with an antisense primer whose 3′ end was positioned 100 bp downstream of the divergence site (Fig. 1B) and a sense primer placed in the library vector (one for each of the lambda arms) led to the isolation of additional unique 5′ sequences with similar breakpoints at the 5′ exon 2 splice junction. One of them, designated exon 1c (Fig. 1B), was the rat homologue of the sole first coding exon of plectin reported for human keratinocytes (McLean *et al.,* 1996). Its expression, however, is not restricted to this cell type, since we have isolated it from a glioma cell line (this study) and from human placenta (unpublished data). The 3′ end of exon 1c (~150 bp) is nearly identical to the corresponding part of exon A found in one of the dystonin gene transcripts (isoform 2; Brown *et al.,* 1995a,b). Exon A of this dystonin transcript, like exon 1c and the other first coding exons of plectin so far identified, is spliced into the first exon encoding the ABD of dystonin. The same region of exon 1c is also homologous to mouse ACF7, isoform 2, a protein closely related to dystonin (Bernier *et al.,* 1996). The alignment of all three regions is shown in Fig. 3B. A second unique PCR product extending plectin's ORF upstream of exon 2 has not yet been confirmed by the isolation of a corresponding cDNA clone.

The region immediately downstream of the site where plectin 5′ sequence divergence occurred showed extensive homology to the ABD of known actin-binding proteins (Fig. 3C). This plectin domain, corresponding to exons 2–8 (see below), gives the highest homology score for dystonin (78% identity; 93% similarity), the neuronal isoform of BPAG1 (Brown *et al.,* 1995a), closely followed by protein ACF7 (Bernier *et al.,* 1996). The alignment of the corresponding sequences of plectin and dystonin is shown in Fig. 3C, together with the mouse dystrophin sequence, which was included for a comparison of exon–intron borders (see below). The ABD of plectin appeared to be functional, as demonstrated *in vitro* using a recombinant plectin mutant protein containing this domain (unpublished results). The homology between plectin, dystonin, and ACF7 extends beyond the ABD, spanning the whole region that is unique to the dystonin transcript upstream of exon 2 of BPAG1 (Fig. 1A; see also Fig. 1 in Bernier *et al.,* 1996).

RNase Protection Mapping of Transcripts Containing Alternate First Coding Exons in Glioma C6 Cells and Rat Skeletal Muscle

To investigate whether transcripts containing alternative first coding exons were expressed in glioma C6 cells at different levels, RNase protection assays were carried out using three different antisense riboprobes: one (E1-2-3) was specific for transcripts containing exons 1, 2, and 3; a second (E1a-2-3) corresponded to exons 1a, 2, and 3, and a third (E1b-2-3) corresponded to exons 1b, 2, and 3 (Fig. 4B). These probes were hybridized to RNA isolated from rat glioma C6 cells (Fig. 4A, lanes C6), and, for comparison, to RNA obtained from rat skeletal muscle (Fig. 4A, lanes M), a tissue known to be rich in plectin (Wiche *et al.,* 1983, 1991). In both cases, hybridization was carried out with and without an antisense probe specific to the carboxy-terminal repeat 5 domain (R5) of plectin (see Materials and Methods), on the assumption that the carboxy-terminal domain, which is encoded by the >7-kb exon 32 (see Fig. 1A), would be indicative of the total amount of plectin transcripts expressed from either source. Both glioma C6 cells and skeletal muscle showed the expected full-length protection of 237 bp for probe E1-2-3 and 230 bp for E1a-2-3 and E1b-2-3 (Fig. 4A). Additional transcripts containing only exons 1 and 2 (Fig. 4A; E1-2), or 1b and 2 (E1b-2), without exon 3, were detected in skeletal muscle, but not in glioma C6 cells. As determined by quantification of bands on a PhosphoImager, the relative abundance of transcripts E1-2-3 to E1-2 was ~1:3, and that of E1b-2-3 to E1b-2 was 1:6. This indicated that at least in skeletal muscle,

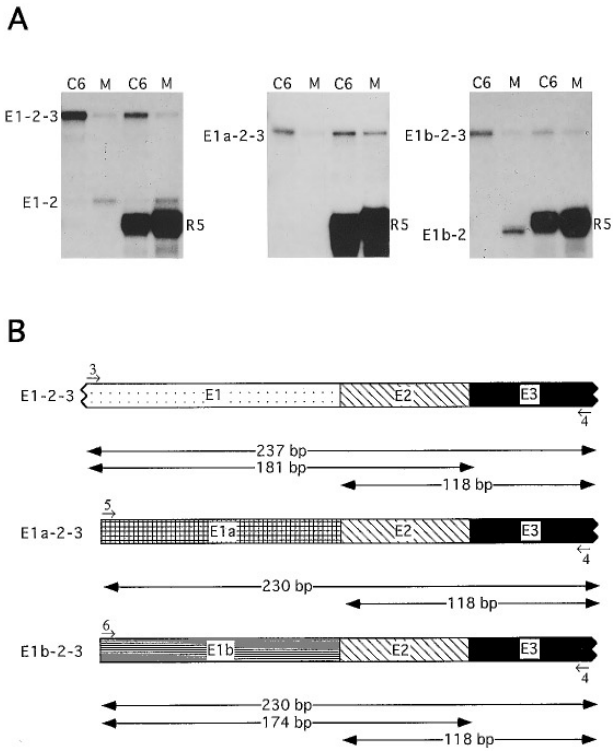

FIG. 4. Alternate expression of exons 1, 1a, and 1b in rat muscle and rat glioma C6 cells analyzed by RNase protection. Total RNA (10 μg) from rat skeletal muscle (**M**) or glioma C6 cells (**C6**) was mapped with RNA probes specific for transcripts containing (in succession) exons 1, 2, and 3 (E1-2-3), or exons 1a, 2, and 3 (E-1a-2-3), or exons 1b, 2, and 3 (E1b-2-3), and with an additional plectin repeat 5-specific probe (R5). (**A**) Autoradiography of RNase-protected bands (only relevant parts of electrophoresis gel are shown). Right-hand pair of gel lanes shown in each panel were comapped with the R5 probe. Note that E1-2-3 and E1b-2-3 autoradiographs were exposed for 16 h, while that of E1a-2-3 was exposed for 3 days. (**B**) Schematic representation of probes used. Numbered arrows indicate positions and orientation of primers used for cloning the probes. The expected lengths (bp) of antisense probes protected by plectin mRNAs containing different combinations of exons are indicated.

most transcriptional variants containing either exons 1 and 2, or 1b and 2, were not spliced to exon 3, but to other exons downstream of exon 3. No transcripts containing exons 1a and 2 without exon 3 were detected in C6 cells and skeletal muscle, even after 15 days of exposure time for autoradiographs.

The estimated ratios of C6 cell transcripts containing the different first coding exons spliced into exons 2 and 3 to total plectin transcripts (based on repeat 5 signals) varied from 1:8 (E1: R5) to 1:13 (E1b:R5) or 1:14 (E1a:R5). Corresponding ratios estimated for skeletal muscle were 1:17 (E1:R5), 1:7 (E1b:R5), and 1:100 (E1a:R5), based on the single signal obtained for exon 1a and both signals (with and without exon 3) obtained for exon 1 and exon 1b. Similarly, the relative levels of exon 1-, 1a-, and 1b-containing isoforms were 2:1:1.2 in glioma C6 cells and 6:1:14 in skeletal muscle. Since the combined levels of exon 1-, exon 1a-, and exon 1b-containing transcripts detected by these probes were significantly lower, both in glioma C6 cells and in muscle tissue, than those of total plectin (detected by the R5 probe), first coding exons other than those examined, such as exon 1c (see Fig. 2), or other yet unknown exons, are likely to make up for the missing fraction.

Tissue Distribution of Alternatively Spliced Plectin Transcripts in the Adult Rat

Probes similar to those shown in Fig. 4B were used to study the tissue-specific expression patterns of plectin transcripts containing alternate exons 1, 1a, and 1b in the adult rat by RNase protection mapping. Tissues were mapped concurrently with a ribosomal protein S16-specific probe as a control for RNA quantity and again with a R5 probe as an indicator of total plectin expression (Fig. 5A). All tissues tested (skeletal muscle, heart, brain, testis, lymph nodes, thymus, and kidney)

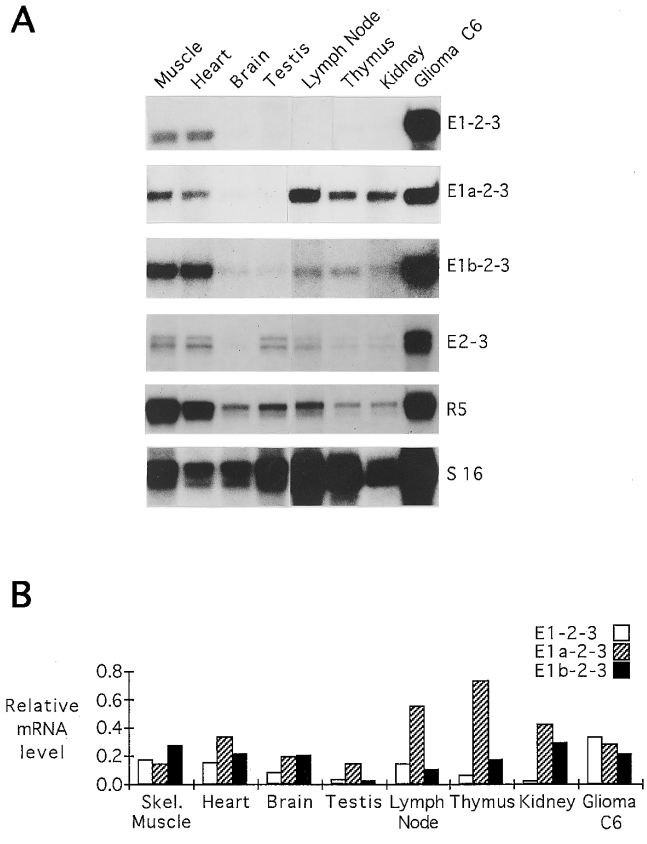

FIG. 5. Tissue distribution of alternatively spliced plectin transcripts in the adult rat using RNase protection analysis. Total RNA (10 μg) prepared from the tissues indicated was analyzed using exon 1-, exon 1a-, and exon 1b-specific RNA probes (see Fig. 6). Probes R5 and S16 were controls for plectin expression and RNA quantification, respectively. (**A**) Autoradiography of RNase-protected bands (only relevant parts of each gel are shown). R5 and E2-3 bands shown represent only one of the three autoradiographs (E-1-2-3). The exposure time for samples in the top four rows was 3 days, for the rest it was 16 h; an exposure time of >10 days was required to visualize weak bands (not shown). (**B**) Relative abundance of different 5′ plectin transcripts. Autoradiography signals were quantified on a PhosphorImager (Molecular Dynamics), and values were normalized against S16 for RNA loading. The relative abundance of exon 1 was calculated by dividing the E1-2-3 value by the sum of E1-2-3 and E2-3 values obtained from the same gel. Exons 1a and 1b values were obtained accordingly.

showed varying levels of plectin repeat 5 expression, with thymus being the lowest and skeletal muscle being the highest (Fig. 5A). Transcripts containing alternative exons 1, 1a, and 1b were found in all tissues tested, although some tissues required longer exposure times to visualize bands compared to those shown in Fig. 5A. The relative representation of exon 1, exon 1a, and exon 1b in transcripts containing exons 2 and 3 in succession (Fig. 5A; probe E2-3) is plotted for all tissues tested in Fig. 5B. In skeletal muscle, where the level of plectin expression is highest, none of the first exons was clearly dominant. In fact, only 58% of transcripts containing exons 2 and 3 in succession were accounted for, again suggesting that first coding exons other than exons 1, 1a, and 1b were expressed in this tissue (see also Fig. 4).

A similar situation prevails in heart and brain, where again no striking differences in the expression patterns for these exons were observed. In testis only 20% of transcripts detected by the E2-3 probe are accounted for by transcripts containing exons 1, 1a, and 1b. In lymph node, thymus, and kidney, where the overall plectin expression was relatively low, the predominantly expressed first coding exon was exon 1a. A similar analysis of glioma C6 cell transcripts revealed a comparable representation of the three first coding exons examined.

Exon–Intron Organization of the Rat Plectin Gene

A rat genomic cosmid library was screened using cDNA fragments corresponding to the 5′ end of plectin coding regions as probes. Five of 16 clones isolated were characterized by restriction enzyme digestion and Southern analysis, and their insert DNAs were sequenced after subcloning into pBluescript (Stratagene) as a series of fragments generated by digestion with distinct restriction enzymes. The partially overlapping clones with inserts of ~32 kb contained sequences from both ends of the plectin ORF. The exon–intron organization of the plectin gene sequence was determined by primer walking along the genomic DNA using primers based on the available cDNA sequence. In addition, genomic DNA isolated from rat glioma C6 cells was subjected to PCR amplification with different sets of plectin-specific primers, and amplified products were sequenced after subcloning.

This analysis led to the identification of 35 exons, including the 4 first coding exons described above, one of which (exon 1) we had previously found also in the human gene (Liu *et al.*, 1996). Sequencing of the genomic region upstream of the putative first coding exons revealed that both genomic and cDNA sequences were identical and thus that no introns were interrupting the cloned untranslated regions. Locations of the alternative first exons were determined using PCR-amplified DNA fragments derived from the new exons 1a, 1b, and 1c as probes for Southern hybridization of restriction fragments obtained from rat plectin genomic clones and by sequencing the hybridizing fragments. Exon 1a was shown to be located between exon 1b and exon 2, and exon 1b was located between exon 1 and exon 1a (Fig. 1C). We could not localize exon 1c in the cloned genomic region, and thus, we believe it is located upstream of exon 1. McLean *et al.* (1996) have placed this exon at ~1.5 kb upstream of exon 2 (exon 3, by their numbering) in the human gene. However, we could not confirm these data, because neither in rat nor in human, where we have analyzed 14 kb of continuous sequence covering the region from exon 2 to upstream of exon 1, were we able to find sequences corresponding to exon 1c (unpublished results). All four alternate first coding exons of plectin characterized in rat have also been found in human, both on the genomic level and on the cDNA level (unpublished results). Thus the total number of exons encoding plectin identified in rat and human is 35, 3 more than previously reported for human plectin (Liu *et al.*, 1996; McLean *et al.*, 1996).

The positions of splice junctions identified in rat exactly matched those in human, but slight differences were found in the sizes of introns (Table 1). As in human, the most striking feature of the rat gene structure was that two of the major domains of the molecule, the R2 rod domain and the carboxy-terminal globular domain, were encoded by single very large exons of >3 and >6 kb, respectively (Fig. 1A).

The exon–intron organization of the plectin gene in the region of the ABD, including the type of codon splitting at junctions, was identical to that of dystrophin (Fig. 3C). This confirmed the idea that the ABD was acquired as a functional unit during evolution.

A Differentially Spliced Transcript Encoding a Rodless Plectin Is Found in Various Tissues of Rat

To confirm the existence of an alternatively spliced transcript corresponding to the previously isolated cDNA clone C4 (Wiche *et al.*, 1991), a series of RT-PCRs was performed using rat glioma C6 cells and various rat tissues, including skin, heart, brain, skeletal muscle, kidney, testis, lung, and liver. Plectin expression was first tested with primer set 1a/b (Fig. 6A), which spans an area upstream of the proposed 5′ splice site. The expected 300-bp band was amplified from all tissues tested (Fig. 6B), while a significantly larger band (600 bp) was amplified from control genomic DNA (data not shown). The expression of the rodless transcript was then tested with primer set 2a/b (Fig. 6A). Since signals on ethidium bromide gels were low in this case, the products were transferred to a nylon membrane and probed with a cDNA fragment that covered the area upstream and downstream of both splice sites. A positive signal corresponding to the spliced version lacking the rod (670 bp) was observed in all tissues except lung and kidney, although intensities were very low in some cases (Fig. 6B). For the unspliced transcript (4.1 kb), which should also have been amplified with this primer set, no signal was obtained, probably due to insufficient length of the reverse-transcribed cDNA template. To confirm, however, the expression

TABLE 1

Exon–Intron Organization of the Rat Plectin Gene

Exon No.	Exon size (bp)	Exon–intron junctions		Intron size (bp)
		3′ acceptor site	5′ donor site	
01	541		. . .AGCCACAGgtcagg. . .	~5000
01b	112		. . .TGAGCAAGgtaagc. . .	~4000
01a	112		. . .CAAGAAAGgtagct. . .	~800
02	62	. . .ctccagATGAACGA. . .	. . .TTATCAAGgttagt. . .	309
03	90	. . .ctgcagGCTCAAAG. . .	. . .ACAGCCTGgtatgt. . .	~2600
04	78	. . .tctcagCCCCGAGA. . .	. . .ATCGGCAGgtaaag. . .	106
05	93	. . .tctcagGTGAAGTT. . .	. . .ACTTCCAGgtaggt. . .	~600
06	168	. . .tggcagATCTCAGA. . .	. . .AGGCACAAgtatgt. . .	640
07	115	. . .ccgcagGCCCATGC. . .	. . .CCCAGAAGgtatga. . .	71
08	107	. . .ctgcagATGTGGAT. . .	. . .GGGCCAATgtaagt. . .	93
09	120	. . .tcacagGAGCTGCA. . .	. . .AGATTGAGgtaggc. . .	72
10	96	. . .ctacagATCCTATG. . .	. . .CTTTGGAGgtaaag. . .	248
11	128	. . .tcgcagGGAGCAGT. . .	. . .TTTGAGAGgtgggc. . .	267
12	94	. . .cctcagGCTGGAGT. . .	. . .TGCAGTCGgtgagg. . .	704
13	155	. . .ttgcagGATATTCG. . .	. . .TACCGGAGgtaggt. . .	84
14	310	. . .ccttagGGTGTATC. . .	. . .ATGATGAGgtgagc. . .	83
15	78	. . .ctgcagGCCAGCTC. . .	. . .AGCTGCTGgtgagt. . .	73
16	162	. . .ttaaagAACTCCTC. . .	. . .GTTACTCGgtaagg. . .	173
17	105	. . .tgtcagGCCCTGAT. . .	. . .CAGTGGAGgtgggg. . .	110
18	96	. . .ctgcagTCCTTCCA. . .	. . .ACTTCCAGgtaagg. . .	~220
19	126	. . .tcctagTTCTTCTC. . .	. . .ATGCCCAGgtaagg. . .	573
20	153	. . .caacagGATGAGAA. . .	. . .AGGTGGAGgtaagg. . .	94
21	155	. . .tcccagGTGACTGT. . .	. . .GTTGCTAGgtgggt. . .	92
22	127	. . .tgccagGCTGGAGG. . .	. . .TAGTCACGgtagga. . .	103
23	184	. . .ccccagTTCCGCAC. . .	. . .GGAGCAGGgtatgg. . .	80
24	158	. . .ccccagGTGAGCAG. . .	. . .CACAACAGgtaagc. . .	91
25	179	. . .ctacagAAAGCACA. . .	. . .TTGGAGAAgtgagt. . .	1121
26	139	. . .acctagACTCAAGA. . .	. . .CACTAAAGgtacct. . .	87
27	357	. . .caacagAAGCTGCG. . .	. . .AGGAGAAGgtaggc. . .	120
28	84	. . .ctgtagGCCCTGCT. . .	. . .CAATCAAGgtaggc. . .	168
29	105	. . .ctatagGACTATGA. . .	. . .TCCAGGAGgtaggt. . .	91
30	99	. . .cctcagTACGTGGA. . .	. . .AGGAAGAGgtacgc. . .	~1000
31	3381	. . .gcacagCGGCTGGC. . .	. . .CTGAGGAGgtacca. . .	135
32	7386	. . .caacagATGCAGAC. . .	. . .TCAATAAA	

Note. The 14 bp surrounding the splice sites are shown for each exon: intron sequences in lowercase, exons in upper case. Exon sizes were determined by direct nucleotide sequencing or estimated from the sizes of the amplified PCR products. Sizes of exons 1, 1b, and 1a refer to coding sequences only. The polyadenylation consensus sequence (AATAAA) at the 3′ end of exon 32 is underlined.

of the unspliced transcript, another primer pair (2a/c) spanning the area over the 5′ splice site was used for amplification. The expected 750-bp band corresponding to the unspliced locus was observed again in all tissues tested (Fig. 6B).

DISCUSSION

In this study we have identified several splice variants of rat plectin on the transcriptional level. Four distinct plectin transcripts that could be generated by alternative splicing or differential promoter usage were characterized. In three of these cases the alternate exons preceding exon 2, exons 1, 1a, and 1b, could clearly be defined as first coding exons of plectin according to their nucleotide sequences, which indicated exon-based in-frame stop codons upstream of consensus start ATGs. The fourth alternate exon identified (exon 1c) is likely to represent another first coding exon, because our partial sequence analysis indicated that it represented the rat counterpart of the first coding exon reported for human plectin by McLean *et al.* (1996). However, considering that the exon 1c sequence analyzed shows high homology to the unique 5′ exons A of dystonin and ACF7, isoforms 2, which seem to be preceded by other coding exons (Brown *et al.*, 1995a,b; Bernier *et al.*, 1996), the possibility that exon 1c represents a unique but not a first coding exon of rat plectin cannot be ruled out. There might even be more than the four alternate first coding exons of plectin characterized in this study, as we have preliminary evidence pointing to the existence of a fifth transcript of rat plectin containing alternate coding sequences upstream of exon 2 (unpublished data).

The expression of plectin variants distinguished by different amino-termini may determine distinct functions of the various molecules encoded. One possibility is that alternate amino-terminal domains bind to distinct interaction partners located within different compartments of the cytoplasm, enabling the targeting of

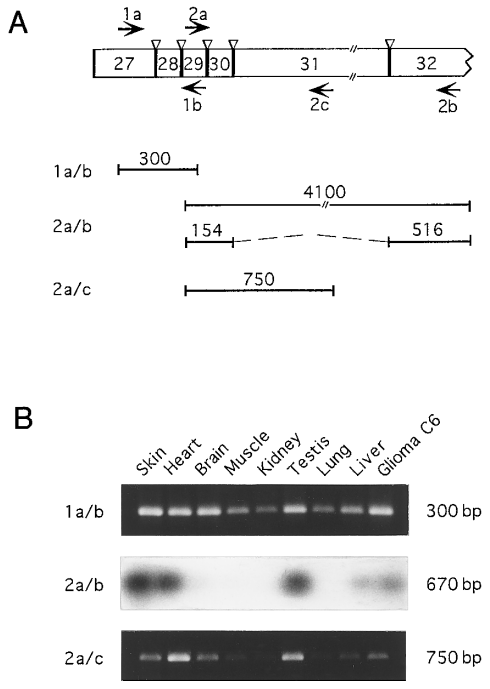

FIG. 6. Identification of a novel plectin transcript lacking the rod domain (exon 31) in various rat tissues by RT-PCR. (**A**) Schematic representation of the alternatively spliced locus, depicting exons (numbered open boxes), the location and orientation of primers used (arrows), and the expected sizes (in bp) of amplified products. Open triangles indicate intron positions. (**B**) Detection of RT-PCR products on ethidium bromide-stained gels (primer pairs 1a/b and 2a/c) or autoradiographs (primer pair 2a/b) of Southern blots of the gel hybridized with a cDNA probe encoding sequences up- and downstream of exon 31. Sizes (bp) of amplified products are indicated. Only relevant portions of gels are shown. Note that longer exposure of the autoradiograph was needed to visualize weak bands in brain and muscle (not shown).

variants to distinct subcellular sites. However, except for the putative RNA-binding domain identified in exon 1, no other potentially significant domains have yet been found in any of the alternate exons identified. Another intriguing scenario would be that variants with different first coding exons have their own independent promoters that may regulate their expression by specifically responding to cell-type- or developmental stage-specific transcription factors. A similar situation has been described for dystrophin, for which at least five distinct promoters drive the cell-type-specific expression of the gene product (Ahn and Kunkel, 1993). This concept would be consistent with our observation that distinct plectin transcripts are expressed at significantly different levels in various tissues. The expression of different first coding exons may also be related to distinct splice patterns of subsequent exons, resulting in the generation of plectin isoforms with potentially different functions, such as the rodless version described in this study. Future research will be directed toward establishing the precise role of alternate first coding exons of plectin molecules.

The new 5′ plectin coding sequences characterized in this study contain regions of high homology to sequence domains previously implicated in protein function. The basic amino acid repeats contained in the homologous sequence domain of ribosomal protein S10 shared with exon 1 of plectin have been implicated in ribonucleotide binding, although their precise function is unknown (Glück *et al.*, 1989). Provided this domain has retained a similar function after becoming an integral part of plectin isoforms containing this exon, it would be interesting to test the hypothesis that plectin networks comprising this isoform serve as docking sites for structures containing RNA, such as ribosomes or mRNA particles. Plectin structures may thus provide the structural framework for site-specific synthesis of proteins required, for instance, for the polarization of cells during morphogenesis and differentiation.

A second region potentially of great importance for plectin's proposed function as a versatile cytoskeletal cross-linking element is the amino-terminal ABD. The existence of plectin gene products containing this domain is in line with our previous studies showing colocalization of plectin structures with actin filaments in cultured cells and tissues (Wiche *et al.*, 1983; Seifert *et al.*, 1992; Foisner *et al.*, 1995). It remains to be shown, however, whether all plectin isoforms contain this domain or whether variants without ABD exist. Such a situation would be similar to that reported for BPAG1, whose epidermal isoform, BPAG1e, in contrast to its neuronal isoforms, lacks a functional ABD (Yang *et al.*, 1996).

As expected, the exon–intron structure of the rat gene was found to be identical to that of the human gene (Liu *et al.*, 1996). Moreover, the extensive similarity in gene organization of plectin and BPAG1, demonstrated by the remarkable conservation of exon–intron boundaries in the amino-terminal domains preceding their respective rod sections (plectin: exons 12–30; BPAG1: exons 2-20) (Liu *et al.*, 1996), seems to hold also for dystonin, the neuronal isoform of BPAG1 (Brown *et al.*, 1995a,b). Like the various plectin isoforms with alternative first exons, the two isoforms of dystonin are generated by splicing one (isoform 1) or possibly more (isoform 2) different exons to the first exon encoding the ABD of the molecule (exon 2). Although the exon–intron structure of the ABD of dystonin has not yet been reported, we predict highly conserved exon–intron boundaries of plectin and dystonin over the bulk of their amino-terminal domains, based on the overall similarity of the plectin and dystonin gene structure and the conservation of this locus in general, as revealed in a comparison of the corresponding regions of plectin and dystrophin (see above). Interestingly, according to the intron phase at the exon–intron junctions in both human and rat plectin (and dystrophin), any of the alternative first coding exons of plectin could be spliced either to exon 2 or to exon 8, generating plectin isoforms with or without an ABD.

The significance and function of rodless plectin molecules encoded by transcripts lacking the >3-kb-long exon 31 remain unclear. The RT-PCR experiments reported here indicate that such transcripts are widespread among different tissues, although they are con-

siderably less abundant than the rod-containing isoforms (data to be published elsewhere). The α-helical domain encoded by exon 31 forms coiled coils by alignment of two α-helices in parallel, creating a stable dimeric rod structure flanked by globular domains endowed with functional sites, such as the recently identified IF binding site in the carboxy-terminal repeat 5 domain (Nikolic *et al.*, 1996). Because of the dimeric nature of coiled-coil structures, any functional sites encoded in the flanking domains occur at least in duplicate, which is likely to augment their effectiveness. Moreover, rod (exon 31)-mediated coiled-coil formation between plectin isoforms differing in their amino- or carboxy-termini may contribute to the functional diversity of plectin molecules. As none of these aspects is applicable to rodless plectin, such versions may represent less versatile forms of the molecule.

RNase protection mapping of transcripts containing alternate first coding exons (1, 1a, and 1b) in rat glioma C6 cells and a series of different rat tissues afforded several new insights. First, similar to glioma C6 cells, where they were initially detected, transcripts representative of all three isoforms were found in every tissue examined, although some tissues showed extremely low-level expression of certain transcripts, such as testis (exon 1 and exon 1b) and kidney (exon 1). Second, the intensity of 5′ transcript signals combined was generally considerably lower than that of 3′ (repeat 5) transcripts. This may indicate the existence of alternate first exons other than those tested for, particularly in those cases where the combined relative levels of alternate first coding exons examined were relatively low, such as in testis (20%). However, part of the deficit may have been due also to gross or differential losses of transcripts with intact 5′ ends during their isolation from the various tissues, a likely scenario considering the large size of the transcripts. Thus, regarding the percentage of usage of the first exons in the various tissues examined, the estimates based upon their abundance compared to transcripts containing the immediately following exons 2 and 3 (Fig. 5A; probe E2-3) were probably more accurate than those based upon 3′ transcripts. However, any isoforms (yet unknown) containing first exons spliced to exons other than exon 2 would still not be accounted for. A more precise evaluation of first exon usage will be possible once the structures of all isoforms containing these exons have been analyzed in full. Finally, clearly predominant isoforms were found in a only few cases, such as exon 1a in lymph node and thymus. Since these were tissues expressing low overall levels of plectin, high-level expression of plectin may correlate with a greater variety of transcripts.

Given the remarkable similarity of plectin and dystrophin 5′ gene regions, in particular with respect to several alternate first coding exons being spliced into a common second exon, we anticipate that, as found for dystrophin, the different alternate first exons of plectin will be found to have their own, probably tissue-specific, promoters. In this study we have not addressed this issue, yet the data available are consistent with this concept, showing that transcripts containing alternate first coding exons are differentially expressed in different tissues. Furthermore, as demonstrated by RNase protection assays in one case examined (exon 1), there seem to exist multiple transcriptional start sites of transcripts containing this exon, and they vary considerably between different tissues (unpublished data). Whether a similar complexity applies to the initiation sites of transcripts containing first coding exons other than exon 1 remains to be established. Studies addressing this question and other questions relevant to plectin gene expression are in progress.

ACKNOWLEDGMENTS

Special thanks go to a number of former co-workers who contributed to this work while they were in our laboratory: Dr. Thomas Frischmuth characterized some of the cDNA clones extending the previously published rat sequence; Elisabeth Einzenberger isolated the genomic clones and together with Zarko Hrzenjak characterized some of the exon–intron borders. We also thank Dr. John Dubendorff for his contributions to the characterization of exon 1a and for critically reading the manuscript. Dr. Christian Stratowa (Ernst Boehringer Institut, Vienna) is thanked for his supervision of the cDNA library preparation, and Dr. Meinrad Busslinger (IMP, Vienna) is thanked for providing some of the reagents used for RNase protection assays. This study was supported by grants from the Austrian Science Research Fund.

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